Association between IL-23R rs11209026 Gene polymorphism and soluble interleukin 23 receptor (Δ9) in Iraqi patients with and without lupus nephritis: A promising therapeutic target

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Abstract---Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the uncontrolled formation of antibodies, overproduction of pro-inflammatory cytokines with a genetic background that is poorly understood. This case-control study comprised 80 SLE patients, SLE patients who met the American College of Rheumatology (ACR) criteria (40 SLE and 40 LN groups), and 40 healthy controls. All participants’ serum IL-23R levels were determined using an enzyme-linked immunosorbent assay (ELISA). 40 patients (20 SLE and 20 LN groups) and 12 age- and sex-matched healthy controls were genotyped for IL-23R gene polymorphisms (rs11209026 G>A) by direct DNA sequencing. As for sIL-23R levels, the results showed non-significant differences between the groups of the patients (0.18(0.11-7.50) vs 0.17(0.12-3.73) (P= 0.751) in SLE and LN, respectively) and the control group (0.17 (0.12-2.69) (P=0.713) vs (P=0.934) in SLE and LN, respectively). Analysis of the rs11209026 sequencing results showed that most patients and the control group share the same genotype, which is GG, and that the A allele is underrepresented in the studied groups, as the AA genotype did not appear in the results. Our results suggest that rs11209026 affects the production of the soluble form of IL-23R. Because of this, we think that this pathway protects against the development of complex events of this disease which caused by IL-23 activation and that soluble receptors can be used in gene therapy to reduce immune inflammation.
Introduction

Systemic lupus erythematosus (SLE) is a multisystem and multifactorial autoimmune connective tissue disease with various clinical aspects and obscure pathogenesis (Paradowska-Gorycka et al., 2016). Kidney involvement, also known as lupus nephritis (LN), is one of the most severe forms of organ involvement in systemic lupus erythematosus (SLE), as evidenced by the associated morbidities and negative effects on patient and kidney survival (Mohan & Putterman, 2015). The genetic involvement in SLE is supported by the family aggregation of SLE. Despite the well-known family clustering of SLE, single nucleotide variations (SNVs) relatively infrequently connect with SLE in a Mendelian fashion, with the majority of genetic predisposition following complicated inheritance patterns (Stetson et al., 2008).

IL-23R is considered the primary initiator of the biological functions of IL-23. IL-23 initiates its functions by binding to IL-12R1 and IL-23R membrane receptor complex, which is made up of two type I membrane proteins (Wojno et al., 2019). On chromosome 1 (1p31.3), the IL-23 receptor (IL-23R) gene is located. Many single nucleotide polymorphisms may be found in the promoter region of the human IL-23R gene (Raymond et al., 2015). The rs11209026 SNP, encoding either R or Q at residue 381, is found in exon 9 at the 39th position. Exon sequences include cis-acting regions that control the utilization of adjacent splice sites and so may govern mRNA processing in addition to encoding information for amino acids (Woodley & Valcárcel, 2002). Serine/arginine-rich (SR) proteins, a class of important splicing factors, often bind to such cis-acting regions. Exon-bound SR proteins are involved in the control of alternative splicing as well as promoting splicing of nearby introns (Lin & Fu, 2007).

Human IL-23Ra (HuIL23Ra) mRNA is 2.8 kb long and has 11 exons in its natural form. This mRNA is translated into a 629 amino acid type I transmembrane (TM) protein. A signal peptide, an N-terminal fibronectin-III-like domain, and a 252-residue cytoplasmic region with three possible tyrosine phosphorylation sites are all present in this protein (Parham et al., 2002). The soluble form of IL-23R that lacks TM and intracellular domains is one of the classes of putative translation products of alternative IL-23Ra mRNA sequences after extensive alternative splicing (Raymond & Gallagher, 2010).

A number of previous studies have found that the SNP rs11209026 of IL-23R gene is associated with strong protection against a range of autoimmune diseases, but the molecular basis for these findings is still unclear in patients with SLE or the development of LN, and the role of sIL-23R in SLE patients remains unknown, so the current study sought to investigate for the first time the relationship between rs11209026 and the soluble form of IL-23R, as well as the latter's levels and effect on SLE Iraqi patients with and without LN.
Materials and methods

Study population

During November and December 2020, 120 persons from the Rheumatology Unit of Baghdad Teaching Hospital and the Kidney Diseases and Transplant Center in Medical City, Baghdad, Iraq participated in this case-control study. The study's participants were divided into three groups: 40 SLE patients (Female: Male 40(100%): 0(0%)), 40 LN patients (Female: Male 37(92.5%): 3(7.5%)), and 40 healthy individuals (Female: Male 35(87.5%): 5(12.5%)) matched patients with average age ± SE (29.60 ± 1.49, 29.98 ± 1.30 and 29.45 ± 1.23 years, respectively). All of the patients had active/ongoing disease. After a full clinical laboratory evaluation and information was gathered from them, none of the healthy were found to have any medical diseases other than renal disorders, infections that may affect blood components and interleukin levels, or cancer, and none of them had a family history of SLE. The research was conducted with the consent of the University of Anbar's ethical approval committee. Furthermore, the General Departments of Medicine at City Hospital and Baghdad Teaching Hospital have granted their consent, and all subjects have supplied written informed consent. SLE disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). There were twenty-four symptoms linked to lupus discovered, and each one was given a weighted score (Ahmed et al., 2017).

Collection of blood samples

Eight milliliters of venous blood were collected from each volunteer. The obtained blood sample was divided immediately into three aliquots. The first aliquot (3ml) was used for clinical tests. The second aliquot (3ml) was transferred to a Serum Separating Tube (SST) left to clot at room temperature (20-25 ºC), then centrifuged at 3000 rpm for 10 minutes to obtain serum and stored at (-20 ºC) until assayed for immunological tests. The third aliquot (1ml) was transferred to a sterile EDTA tube and stored at (-20 ºC) until assayed for IL-23R genotyping.

Serum IL-23R determination

The serum levels of IL-23R were measured using a commercially available quantitative sandwich ELISA technique (Al-shkairate establishment for medical supply, Amman, Jordan) according to the manufacturer's instructions. Optical densities at 450 nm were measured immediately after the stop solution was applied. For IL-23R, the detection range was 0.156-10 ng/ml.

Genotyping for IL-23R gene

DNA Extraction

The Quick-gDNATM Blood MiniPrep equipment (Zymo/USA) was used to extract genomic DNA from blood samples using breakthrough Fast-Spin column technology and according to the manufacturer's instructions. In a summary, 800 µl of Genomic Lysis Buffer was mixed with 200 µl of blood and incubated for 5-10 minutes at room temperature. A new Collection Tube was filled with 400 µl of
DNA Pre-Wash Buffer. The tubes were then centrifuged at 10,000 \( \times \) g for one minute. Washing was carried out in two phases with 500 \( \mu l \) g-DNA Wash Buffer, followed by final elution in 100 \( \mu l \) DNA Elution Buffer by centrifugation at maximum speed for 30 seconds at room temperature.

**Detection of IL-23R gene polymorphisms**

PCR amplification was carried out using AURA TM PCR Cabinet (EuroClone, Italy) with ALHP system which includes i-Taq\textsuperscript{TM} DNA Polymerase and all PCR reaction components (dNTP mixture, reaction buffer, etc.) in each PCR tube of the Maxime PCR PreMix Kit (i-Taq\textsuperscript{TM}) (Intron, Korea). The PCR product was utilized to assess genetic polymorphisms in the IL-23R gene at one position: IL-23R rs11209026 by Sanger Method using conventional PCR. In a total volume of 25 \( \mu l \), all polymerase chain reactions were carried out. Each reaction combination for traditional PCR reactions includes 5 \( \mu l \) of Taq PCR PreMix (Intron / Korea), 1 \( \mu l \) of forward primers, 1 \( \mu l \) of reverse primer, 1.5 \( \mu l \) of genomic DNA, and 16.5 \( \mu l \) of nuclease-free water. Table 1 shows all of the PCR conditions and shows the primer sequences for detecting the genotypes and sequencing.

Table 1: primer sequences, PCR conditions, and the Gene ID numbers for IL-23R

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Primer Sequences</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-23R G&gt;A</td>
<td>F:5'-CACCCCTTTCTCCTTTGAGACCT-3'</td>
<td>-An initial denaturation at 94(^\circ)C for 3 min.</td>
</tr>
<tr>
<td>(rs112090</td>
<td>R:5'-TGATTCTGGGCTGAGGACTT-3'</td>
<td>-Then, 35 cycles each cycle consisted of denaturation</td>
</tr>
<tr>
<td>26)</td>
<td></td>
<td>94(^\circ)C for 45s, annealing at 62(^\circ)C for</td>
</tr>
<tr>
<td>GENE ID:</td>
<td></td>
<td>45s and extension-1 at 72(^\circ)C for 45s.</td>
</tr>
<tr>
<td>149233</td>
<td></td>
<td>- A final extension at 72(^\circ)C for 7 min.</td>
</tr>
</tbody>
</table>

**Sequencing and Alignment of Sequences**

After amplification of the extracted DNA of the SNP yielded a band of predicted size, the PCR products with forward primer were delivered to Macrogen Corporation in Korea for Sanger sequencing using an ABI3730XL-automated DNA sequencer. The results were emailed and analyzed with geneious software and the Basic Local Alignment Search Tool (BLAST) on the National Center of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) to align them to the IL-23R gene sequences.

**Statistical analysis**

The data were checked for normality, homogeneity, and normal distribution using IBM SPSS version 26.0. For non-parametric data, the median, minimum, and maximum values were calculated. The Kruskal Wallis and Mann-Whitney U tests, as well as Pearson’s chi-square test, were used to compute the probability. For genotyping and allele frequencies, WinPepi version 11.65 (Abramson, 2011) calculated the odd ratio, 95 percent confidence interval, and Fisher’s exact probability. Use an online Hardy-Weinberg calculator (McMurran, 2010) for genotyping and allele frequency estimations.
Results

Clinical characteristics of participants
Table 2 shows the demographic characteristics as well as laboratory results. Serum creatinine and blood urea nitrogen were measured as indicators of renal function. C3 and C4 levels, anti-dsDNA, and SLEDAI (SLEDAI-2K) were all assessed in the patients.

Table 2. The study subjects' baseline clinical data and demographic information

<table>
<thead>
<tr>
<th>parameter</th>
<th>SLE Group (n=40)</th>
<th>LN Group (n=40)</th>
<th>SLE vs LN P value</th>
<th>Healthy Control (n=40)</th>
<th>SLE vs Controls P value</th>
<th>LN vs Controls P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Year)</td>
<td>29.60 ± 1.49</td>
<td>29.98 ± 1.30</td>
<td>&gt; 0.05</td>
<td>29.45 ± 1.23</td>
<td>&gt; 0.05</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>23.15 ± 1.05</td>
<td>39.78 ± 4.50</td>
<td>&lt; 0.05</td>
<td>27.53 ± 2.11</td>
<td>&gt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.66 ± 0.03</td>
<td>1.12 ± 0.14</td>
<td>&lt; 0.05</td>
<td>0.80 ± 0.06</td>
<td>&gt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>C3</td>
<td>93.58 ± 7.72</td>
<td>77.50 ± 7.93</td>
<td>&gt; 0.05</td>
<td>158.37 ± 8.75</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>C4</td>
<td>88.39 ± 6.79</td>
<td>80.18 ± 7.96</td>
<td>&gt; 0.05</td>
<td>152.38 ± 7.80</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>404.01 ± 32.17</td>
<td>506.58 ± 38.04</td>
<td>&gt; 0.05</td>
<td>222.19 ± 31.37</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>20.48 ± 1.66</td>
<td>22.70 ± 1.45</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SLE duration</td>
<td>57.40 ± 8.76</td>
<td>69.58 ± 10.30</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

SLE, Systemic lupus erythematosus; LN, lupus nephritis; BUN, blood urea nitrogen; C3,4 complement 3,4; anti-dsDNA, anti-double strand DNA; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index. The quantity data are given as mean± SE. P < .05 was considered significant.

IL23R serum levels

As for the serum levels of IL-23R, the results showed non-significant differences between the groups of the patients (0.18(0.11-7.50) vs 0.17(0.12-3.73) (P=0.751) in SLE and LN, respectively) and the control group (0.17 (0.12-2.69) (P=0.713) vs (P=0.934) in SLE and LN, respectively; table 3, Fig 1).
Table 3: Comparison between SLE, LN groups, and controls in terms of the serum level of IL-23R

<table>
<thead>
<tr>
<th>Groups</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>0.18</td>
<td>0.11</td>
<td>7.50</td>
<td>0.751</td>
</tr>
<tr>
<td>LN</td>
<td>0.17</td>
<td>0.12</td>
<td>3.73</td>
<td>0.713</td>
</tr>
<tr>
<td>Control</td>
<td>0.17</td>
<td>0.12</td>
<td>2.69</td>
<td>0.934</td>
</tr>
</tbody>
</table>

**IL-23R level (pg/ml)**

*Fig 1: Comparison between the studied groups in terms of IL-23R level. The quantity data are given as median.*

**The genotype and allele frequencies of the IL-23R gene**

In SLE patients and control groups, the genetic polymorphisms of the IL-23R gene were identified at one position; IL-23R rs11209026, which was presented with two genotypes (GG and GA) for rs11209026.

**IL-23R rs11209026SNP**

IL-23R rs11209026SNP gene was detected using a conventional PCR approach, and the band visualized by gel electrophoresis was 494 bp in size, as shown in fig 2.
Fig 2: Gel electrophoresis of 494bp IL-23R rs11209026 of control and SLE groups on agarose gel (2%) stained with red safe stain, electrophoresed in 70 volt for 1hr, M: molecular marker (100 bp DNA ladder), lanes from 1-12 control and lane from 13-30 SLE patients.

Most patients and control groups share the same genotype, GG (wild type), as shown in table 4, fig 3.

Table 4: Genotypes and alleles frequency of IL-23R rs11209026 SNP in SLE and LN patients and control groups

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>Total No.</th>
<th>Genotyping frequency</th>
<th>Alleles’ frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GG %</td>
<td>GA %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The Submission Numbers for rs11209026 sequences in NCBI

OM066349.1; OM066350.1; OM066351.1

Discussion

To our knowledge, after reviewing the literature, this is the first study to investigate the effect of the soluble form of IL-23R on patients with SLE, as well as to investigate whether there is a relationship between the rs11209026 genetic polymorphisms and the soluble form of IL-23R in SLE patients with and without LN in the Iraqi population.

A number of laboratory analyses were conducted to assess the patients’ condition and the development of LN’s condition, especially since the study was conducted during follow-up. The results followed the well-known deterioration of the
indicators of kidney function in LN patients, as well as the decrease in the level of complement proteins C3 and C4, which coincided with a high anti-dsDNA level, which is an indicator of LN activity and poor outcome.

As mentioned earlier, all study patients are active or ongoing disease, and yet the present study showed non-significant differences in IL-23R levels in patients and the control group. The soluble form of IL-23R acts as a specific inhibitor of human IL-23 (Raymond & Gallagher, 2010), which is a major player in autoimmune diseases because it is responsible for the stability and production of the final phenotype of TH17 (Quiniou et al., 2014), which is the third arm of immunity, especially since SLE is a Th17-mediated disease. sIL-23R affects Th17 differentiation and stability because it causes the failure of STAT3 signaling, thus preventing excessive immune inflammation in the body and reducing Th17-dependent disease damage (Raymond et al., 2015). Recently, Liu et al. (Liu et al., 2020) demonstrated sIL-23/sIL-23R as a new serological marker for predicting disease progression as well as using sIL-23R (D9) to predict inflammatory diseases. In our previous work, as well as a number of studies (Larosa et al., 2019; Vukelic et al., 2020, 2019) we demonstrated an elevated IL-23 level in patients with SLE, especially those who develop lupus nephritis. Failure to obtain IL-23R/IL-23 balance in the circulation indicates that immune cells may overexpress membranous IL-23R and secrete a low-soluble form, which promotes the development of inflammatory processes in patients. From the above, we conclude that the excessive elevation of IL-23 in patients while the soluble form of IL-23R remains at low levels is a sign of patients' poor response to the treatments used. It can thus be speculated that patients do not have cofactors to convert the surface-associated form of IL-23R to the soluble form, and if these factors are present, they will help reduce activation of Th17 cells and limit consequent damage.

The results of the study showed the prevalence of the G allele in the studied groups, as the GG genotype was common in patients and healthy subjects. We do not believe that our results are without statistical power because the A allele is an under-represented allele in the autoimmune disease population and the AA genotype is very rare because, according to some studies results, it causes a poor complete response to IL-23 both in patients and healthy subjects, which is considered the main factor for activating Th17 (Di Meglio et al., 2011; Duerr et al., 2006; Rahman et al., 2009). Th17 involved in autoimmunity, in contrast to its protective role in the host’s immunity against bacteria and fungi (Miossec et al., 2009), but as it is known that what happens in autoimmune diseases is the excessive production of IL-23 and the additional stimulation of these cells (Tang et al., 2012).

Our results are supported by some studies that have looked at the variant’s relationship with autoimmune diseases in general and lupus erythematosus in particular. Sanchez et al. (Sanchez et al., 2007) obtained no evidence for an association of the rs11209026 gene variant with SLE in a Spanish population. Likewise in a recent study, Rezaei et al. (Rezaei et al., 2020) found no significant differences in the frequency of IL-23R rs11209026, rs1343151, and rs10489629 variations between the juvenile systemic lupus erythematosus and control groups. Ferguson et al. (Ferguson et al., 2010) discovered that the frequency of a
functional single nucleotide polymorphism (SNP) in the IL-23 receptor gene (IL-23R; rs11209026, 1142 G wild-type A reduced function, Arg381Gln, R381Q) is significantly higher in healthy controls than in patients, implying that the rare allele may protect against immune-mediated chronic inflammation. R381Q is a causal variation because the SNP rs11209026 encodes an amino acid change (Arg381Gln) in the protein product that has functional effects. As a consequence, based on the findings of our study, we anticipate it will protect against lupus erythematosus. In our results, we did not determine the AA genotype, so we believe that the A allele may be a protective factor for the disease in the Iraqi population. This is consistent with some previous studies that investigated the relationship of the allele with autoimmune diseases. Inflammatory disease protection is conferred by A allele of rs11209026 SNP (Raymond et al., 2015). Mosayebian et al. (Mosayebian et al., 2015) explained that the protective effect of the A allele of the IL-23R R381Q gene variant against autoimmune illness may be due to an impairment of TH17 cell effector activities, such as IL-17A production, rather than TH17 cell differentiation. This SNP’s allele A is markedly underrepresented in patients with Crohn’s disease, ulcerative colitis (Duerr et al., 2006), and a variety of chronic autoimmune inflammatory illnesses (Cargill et al., 2007; Elder, 2009; Huber et al., 2008; Rahman et al., 2008, 2009), indicating that it mediates a protective effect.

The results of our study suggest that IL-23R381Q has an effect on the production of the soluble form of IL-23R in SLE patients with and without LN. Previous studies have shown that the A allele affects the production of sIL-23R (Δ9) because it changes the way IL-23R a-chain mRNA is spliced by decreasing the binding of the SF2 splicing enhancer by disrupting its binding site, which prevents mistakes in the mRNA splicing process. This means that exon 9 is skipped, which increases the expression of the soluble form of IL-23R mRNA that acts as an antagonist of IL-23 and reduces the ability to develop a Th17 phenotype. whereas the G allele causes the expression of membrane IL-23R because it binds the SF2 binding enhancer, which results in no skipping of exon 9 and results in expression of membrane IL-23R and not the soluble form (Pidasheva et al., 2011; Raymond et al., 2015) (Figure 4). And since the GG genotype was common in patients and healthy subjects, we did not get clear differences in the soluble form of IL-23R and a slight increase in the SLE group, we expect that the A allele has an effect on this.
Fig 4: The effects of A and G alleles on both membranous and soluble IL-23R. A. Exon 9 splicing and skipping model, which results in the synthesis of Δ9. *AON displacement of SF2 in a model, resulting in Δ9 expression (Raymond et al., 2015).

What we have explained above makes us ask the question whether the therapeutic tools based on exon skipping will succeed in reducing the inflammatory damage caused by Th17 cells stimulation in patients with SLE, after we demonstrated, in line with the results of previous studies, this molecular pathway in patients. One of the therapies that mimics the role of the A allele is antisense oligonucleotides (ASOs), but Stebbins et al. (Stebbins et al., 2019) demonstrated that these treatments pose a greater risk of immunogenicity in patients with SLE, as nucleosomes released from apoptosis result in an accumulation of DNA and histones in the circulation, which increases the appearance of antibodies for double-stranded DNA. This raises other questions about whether genetic engineering experiments will succeed in reducing the immune reaction, especially after studies on autoimmunity have proven the importance of balancing lymphocyte activation and regulating the immune response. Therefore, the approach to gene therapy should be to enhance the regulatory and immune pathways and reduce pro-inflammatory factors. The IL-23-controlled Th17 lymphoid differentiation pathway is a major target of these approaches (Edo & Espinosa-Parrilla, 2017). Or will targeting the SF2 binding enhancer be possible in the event that the A allele is under-represented in the disease population? As well as mechanisms that target the stalk region of IL-23R or factors that increase the production of the soluble form of IL-23R (Hummel et al., 2017), we must work in future studies to find promising solutions to the disease.

**Conclusion**

The results of the study concluded that IL-23R381Q affects the soluble form of IL-23R (Δ9) in SLE patients with and without LN. Thus, this molecular pathway has become clear in autoimmune diseases in general and in SLE and the development of lupus nephritis in particular, so future work should be done on gene therapy
based on soluble receptors. It is an innovative and promising solution to reduce disease damage caused by immune inflammatory activation and improve the wellness and life expectancy of patients.

**Abbreviations**

ACR American College of Rheumatology, anti-dsDNA anti-double strand DNA, ASOs antisense oligonucleotides, bp base pair, BUN blood urea nitrogen, C3 complement 3, C4 complement 4, EDTA Ethylenediaminetetraacetic acid, ELISA enzyme-linked immunosorbent assay, HuIL23Ra Human IL-23Ra, Kb kilo base, LN lupus nephritis, mRNA messenger RNA, nm nanometer, R381Q Arginine 381 Glutamine, sIL-23R serum interleukin receptor, SLE Systemic lupus erythematosus, SLEDAI Systemic Lupus Erythematosus Disease Activity Index, SNP single nucleotide polymorphism, SNVs single nucleotide variations, SR Serine/arginine, SST Serum Separating Tube, STAT3 Signal transducer and activator of transcription 3, Th17 T helper 17, TM transmembrane.

**Conflict of interest**
The authors declare that they have no conflict of interest.

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**References**


