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ORIGINAL ARTICLE

The association of single nucleotide polymorphism of interleukin-21 gene and serum interleukin-21 levels with systemic lupus erythematosus

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KEYWORDS

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Abstract *Background:* Systemic lupus erythematosus (SLE) is a common autoimmune disorder which commonly results from the combined effects of a large number of genes. Variations in the DNA sequence in the Interleukin-21 (IL-21) gene may lead to altered IL-21 production and/or activity which can affect an individual's susceptibility to SLE. IL-21 is a novel class I cytokine produced by activated $CD4^+$ T cells, natural killer T cells and T helper (Th) cells. There is increasing evidence that IL-21 contributes to the pathogenesis of SLE due to its biological activity.

Aim of the study: To investigate the association between single nucleotide polymorphism (SNP) of IL-21 rs2221903 gene and serum IL-21 levels with SLE and to detect the possible association between IL-21 serum levels and the pathogenesis of the disease.

Subjects and methods: This study was conducted on 30 SLE patients and 20 age and sex matched healthy controls. Serum IL-21 levels were measured using enzyme-linked immunosorbent assay (ELISA) technique and SNP of IL-21 rs2221903 gene was detected by genotyping assay, using real time polymerase chain reaction (RT-PCR).

Results: Serum Il-21 levels were significantly higher in patients compared with controls ($p < 0.001$). Patients with high activity index of SLE had significantly higher levels of serum IL-21 (p value < 0.001). A statistically significant association was found between the T allele of SNP rs2221903 and SLE, whereas; no association between SNP of IL-21 rs2221903 genotypes and SLE or serum IL-21 levels could be detected.

Conclusion: IL-21 plays an important role in the immune-pathogenesis of SLE and could be used as a possible target for novel immunotherapy. The T allele of SNP rs2221903 suggests that the IL-21 gene may contribute to an inherited predisposition to SLE.

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1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic, systemic, autoimmune disease. It is characterized by a diverse array of autoantibody production, complement activation and immune-complex deposition, which causes tissue and organ damage. The etiology and pathogenetic mechanisms of SLE have not been clearly elucidated [1]. Epidemiological studies suggest strong contribution of genetic factors in the development of SLE [2]. SLE commonly results from the combined effects of a large number of genes. Each allele contributes only mildly and the accumulation of several genes is presumed necessary to significantly increase the risk of SLE. The combinations of risk alleles that lead to predisposition and the mechanisms through which they contribute to autoimmunity are poorly understood. In fact, most single nucleotide polymorphisms (SNPs) associated with SLE fall within non coding DNA regions (introns) and represent markers of co-segregated alleles. Most of them are associated with genes presumed to be involved in the immune response [3].

In humans, the Interleukin-21 (IL-21) gene is located on chromosome 4q26-q27, which consists of 5 exons spanning approximately 8.44 kb of genomic DNA [4]. Variations in the DNA sequence in the IL-21 gene may lead to altered IL-21 production and/or activity which can affect an individual's susceptibility to SLE. Several polymorphisms in the IL-21 gene have been identified [5–9]. Many of them may be associated with different autoimmune and inflammatory diseases, such as Graves' disease, rheumatoid arthritis and inflammatory bowel disease suggesting a common genetic background for these diseases [10–13,5].

IL-21 is a novel four helix bundle class I cytokine produced endogenously by activated $CD4^+$ T cells, natural killer T cells and T helper (Th) cells [14–16]. IL-21 is an important factor for the activation, proliferation, differentiation, antibody production, or death of murine B cells, with the outcome being dependent on the context of co-stimulation [17]. Also, IL-21 is an important regulator of T cell expansion, differentiation and effector function and can act in concert with other type I cytokines to further enhance the growth of $CD4^+$ T cells. It also regulates human $CD8^+$ T cells activation, survival, and memory formation. IL-21 is also important for the expansion and maintenance of the Th17 lineage that produces an array of pro-inflammatory molecules including IL-1, IL-6, IL-17, IL-21, IL-22, and tumor necrosis factor-α (TNFα) [18].

Recently, there is increasing evidence that IL-21 contributes to the pathogenesis of chronic inflammatory and autoimmune diseases due to its biological activity. Animal studies of SLE have indicated that IL-21 is important in the pathogenesis of murine lupus. SLE patients have higher serum levels of IL-21 than healthy controls. Moreover, association of IL-21 and IL-21R polymorphisms with susceptibility to SLE has been reported [5,19].

2. Aim of the work

The present study aimed to find if there is an association between SNP of IL-21 rs2221903 gene and SLE and whether it can serve as a novel genetic marker of susceptibility to the disease, and to detect the possible association of IL-21 serum levels and the pathogenesis of the disease.

3. Subjects and methods

3.1. Patients and controls

This observational cross sectional study was conducted on thirty patients (27 females and 3 males) diagnosed with systemic lupus erythematosus (Group 1) based on Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE [20], and twenty apparently healthy subjects matched for age and sex (Group 2) with no history of autoimmune diseases (17 females and 3 males). Patients were recruited from Rheumatology Department, Ain Shams University Hospitals between June and November 2014. Patients and control individuals participated in this study after informed consent was obtained. The work has been carried out after approval of Ain Shams University Ethics Committee and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

All the patients were subjected to full history taking, thorough clinical examination, and laboratory investigations which included complete blood count (CBC), erythrocyte sedimentation rate (ESR), antinuclear antibody (ANA), and anti-double stranded DNA. An assessment of disease activity in patients with SLE was done by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI); twenty-four features that were attributed to lupus were listed, with a weighted score given to any present feature. The more serious manifestations (such as renal, neurologic, and vasculitis) were weighted more than others (such as cutaneous manifestations) [21].

3.2. Blood sampling

Five milliliters of venous blood were taken from all study participants under complete aseptic conditions. Each sample was divided into 2 tubes. Three milliliters were added to EDTA containing sterile tubes and stored at −80 °C till real time polymerase chain reaction (RT-PCR) was done. Two milliliters were added to sterile empty tubes and were allowed to clot for 30 min then centrifuged for 15 min at $1000\times g$. Serum was then aliquoted and stored at −20 °C until used in enzyme-linked immunosorbent assay (ELISA).

3.3. Serum IL-21 determination

The serum levels of IL-21 were measured using a commercially available quantitative sandwich ELISA technique (IBL international GMBH, Hamburg, Germany) following the manufacturer's instructions. Optical densities at 450 nm were read within 10 min of adding stop solution. The detection limit of the assay was 20 pg/mL.

3.4. Genotyping for IL-21 gene

We used TaqMan single nucleotide polymorphism (SNP) genotyping assay, using RT-PCR.

3.4.1. DNA extraction

It was done using PureLink® Genomic DNA Mini Kit (Invitrogen, USA), for purification of DNA from blood according

to the manufacturer's instructions. In brief, 20 µl Proteinase K and 20 µl RNase A were added to 200 µl blood sample in sterile microcentrifuge tube, then incubated at room temperature for 2 min. 200 µl buffer was added and incubated for 10 min, followed by 200 µl ethanol. 640 µl lysate was added to spin column and centrifuged at 10,000×*g* for 1 min at room temperature. Washing was done in 2 steps using 500 µl washing buffer, final elution was then done in 200 µl elution buffer by centrifugation at maximum speed for 1 minute at room temperature.

3.4.2. PCR amplification

PCR amplification was carried out using a thermal cycler (*Applied Biosystems, USA*) with *SNP Genotyping Assay*. The assay contained sequence-specific forward and reverse primers to amplify the polymorphic sequence and two TaqMan® minor grove binding (MGB) probes (One probe labeled with VIC® dye which detected the C allele sequence and one probe labeled with FAM™ dye which detected the T allele sequence). Context sequence [VIC/FAM] ACAGACAATGGGGTTTTG TTTTCTT**[C/T]** TGTTCTGCAAGCAG AGAGCTGTGT. PCR was performed in 25 µl of reaction mixture containing 20 ng of each DNA sample, TaqMan Universal PCR Master Mix (*Applied Biosystems, USA*) and SNP genotype assay (*Applied Biosystems, USA*). PCR was done as follows; enzyme activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

3.4.3. Post-PCR plate read and analysis (Allelic Discrimination Plate Reading and Analysis)

After PCR amplification, an endpoint plate read was performed using an Applied Biosystems RT-PCR System. The Sequence Detection System (SDS) Software used the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicated which alleles were in each sample. The blue fluorescent FAM dye indicated the **T** allele, while the green fluorescent VIC dye indicated the **C** allele. ROX is a reference dye with red fluorescence. Identification of genotypes was based on the relative fluorescence from each well on a 48-well plate (Figs. 1–3).

Fig. 1 is an example of a heterozygous **C/T** genotype. The multi-component plot shows both green and blue curves which corresponds to the **C** and **T** alleles respectively.

Fig. 2a is an example of a homozygous **T/T** genotype. The multi-component plot shows one blue curve which corresponds to **T** allele. While Fig. 2b is an example of a homozygous **C/C** genotype, the multi-component plot shows one green curve which corresponds to **C** allele.

3.5. Statistical analysis

Continuous data e.g. age were presented as range, mean and standard deviation. Qualitative data e.g. sex were presented as numbers and percentages. Student's t test was used to compare continuous data between two independent groups and a One Way ANOVA test was used to compare continuous data between more than two groups. Chi square test was used to compare qualitative data between different groups. The significance level was set at $P \leqslant 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

4. Results

4.1. Clinical characteristics of the study participants

The age of Group 1 (patient group) ranged from 21 to 42 years with a mean of 32.7 ± 5.58 years, regarding sex, 27 (90%) patients were females and 3 (10%) patients were males. The age of Group 2 (control group) ranged from 23 to 41 years with a mean of 32.65 ± 5.70 years, 17 (85%) were females and 3 (15%) were males. There was no statistically significant difference between the 2 groups (Table 1). Clinical manifestations in patients are presented in Fig. 3.

4.2. IL-21 serum levels

The serum IL-21 levels were significantly higher in the patient group (group 1) than those in the control group (group 2) (mean = 310.35 ± 129.61) vs (mean 223.67 ± 30.53) (Table 2). Patients with high activity index of SLE had significantly higher levels of serum IL-21 than those with lower index score (p value < 0.001) (Fig. 4). A statistically significant association between nephritis and serum IL-21 levels (p value = 0.01) was detected, while no association was found between serum IL-21 levels and other clinical findings Table 3.

4.3. The genotype and allele frequencies of IL-21 gene

4.3.1. Genotype assessment

The homozygous **T/T** was the predominant genotype in the patient group (73%); whereas, both homozygous T/T and heterozygous C/T types were equally represented in the control group (45% each). No statistical significant difference was detected between the 2 groups regarding genotype distribution (Table 4). No correlation was found between SNP in IL-21 rs2221903 and serum IL-21 levels p value = 0.15 (Fig. 5).

4.3.2. Allele assessment

The frequency of (**T**) allele was much higher than (**C**) allele in both SLE patients and controls. The frequency of **T** allele was (85%) n = 51 in SLE patients while the **C** allele was (15%) n = 9. The frequency of the T allele was significantly higher in the patient group than in the control group (p = 0.038) (Fig. 6).

5. Discussion

To our knowledge, this is the first study to determine whether the IL-21 gene polymorphism and its soluble levels are associated with SLE in Egypt, other studies were done on other ethnic populations such as Europeans, African-Americans, and Asians.

In this study, serum levels of IL-21 were significantly elevated in SLE patients in comparison to healthy controls. The mechanisms leading to elevated IL-21 are unknown but this is believed to lead to increased production of auto-antibodies [22,23].

Our results come in agreement with the results obtained by other studies who found that the plasma soluble IL-21 levels were significantly higher in SLE patients compared to controls [9,24,25]. In addition, Wang et al. found that concentrations of

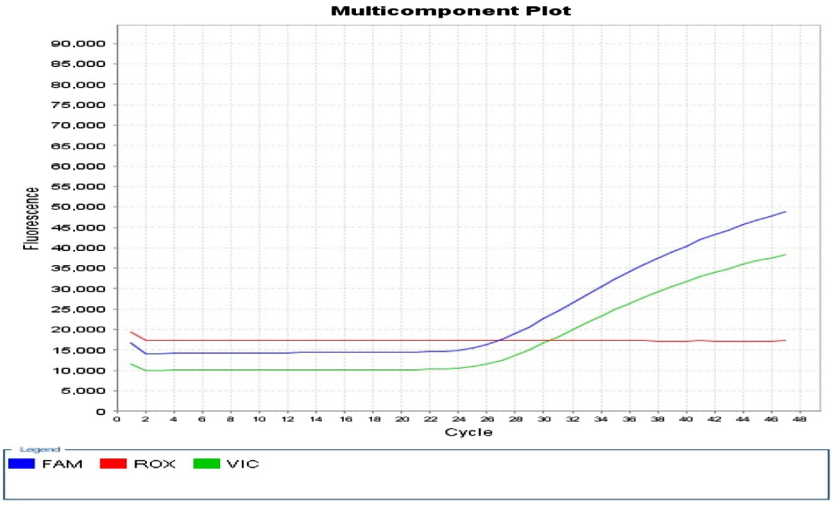

Figure 1 Plotted fluorescence signals indicating both C and T alleles in a heterozygous C/T genotype.

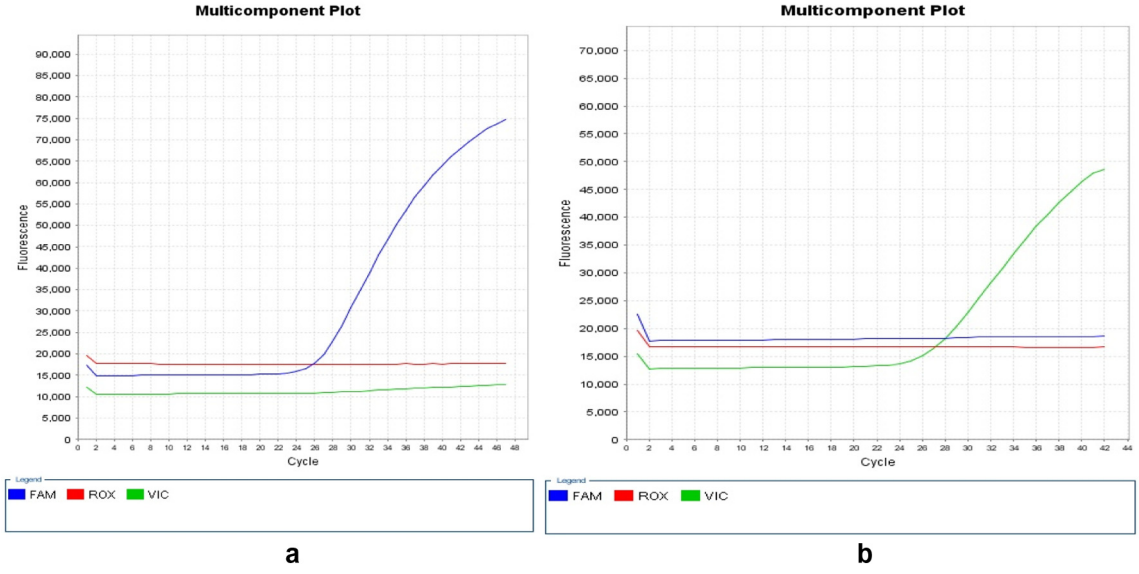

Figure 2 Plotted fluorescence signals indicating homozygous T/T genotype and homozygous C/C genotype in 2 different samples.

serum IL-21 were gradually reduced, after initiation of treatment particularly at week 12. They suggested that increased levels of serum IL-21 may be associated with the early process of SLE in Chinese patients [24]. Lee et al., also found that the mRNA expression of IL-21 was significantly higher in PBMCs and $CD4^+$ T cells from SLE patients than in those from healthy control [25].

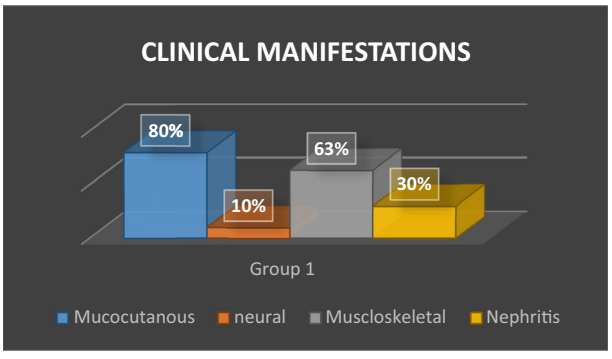

Figure 3 Clinical manifestations of SLE patients.

In contrast Pan et al. found that IL-21 levels were significantly decreased in the serum of patients with SLE compared with controls [26]. Nakou et al. found no difference in serum IL-21 levels between patients and controls. However, they found a 4-fold increase in mRNA and significantly higher intracellular levels of IL-21 in T-cells in active SLE patients compared to inactive SLE patients and healthy individuals [22]. The discrepancy with our results could be explained by several potential explanations; first, the treatment might influence the expression level of IL-21, second, it was also possible that there was retention of IL-21 within the tissues or organs in SLE patients, resulting in reduction in peripheral blood levels, so, further detection of IL-21 expression level in tissues or organs, such as renal tissue is essential [26].

Table 1 Demographic data of the studied groups.

	Patients ($N = 30$)		Control subjects ($N = 20$)		Student t test	P value
	Mean ± SD	Range	Mean ± SD	Range		
Age (years)	32.7 ± 5.58	21–42	32.6 ± 5.70	23–41	−0.05	0.96 NS
			No (%)	No (%)	Chi Square test	P value
Sex	Female		27 (90.0%)	17 (85.0%)	0.28 (Fisher Exact)	0.67 NS
	Male		3 (10.0%)	3 (15.0%)		

Table 2 IL-21 serum levels in SLE patients and controls.

	IL-21 serum levels			Student t test	p Value
	Mean ± SD	Mini (pg/ml)	Max(pg/ml)		
Patients ($N = 30$)	310.35 ± 129.61	220	790.3	−3.52	**0.001***
Control subjects ($N = 20$)	223.67 ± 30.53	160.6	281.2		

* p Value 0.001 highly significant.

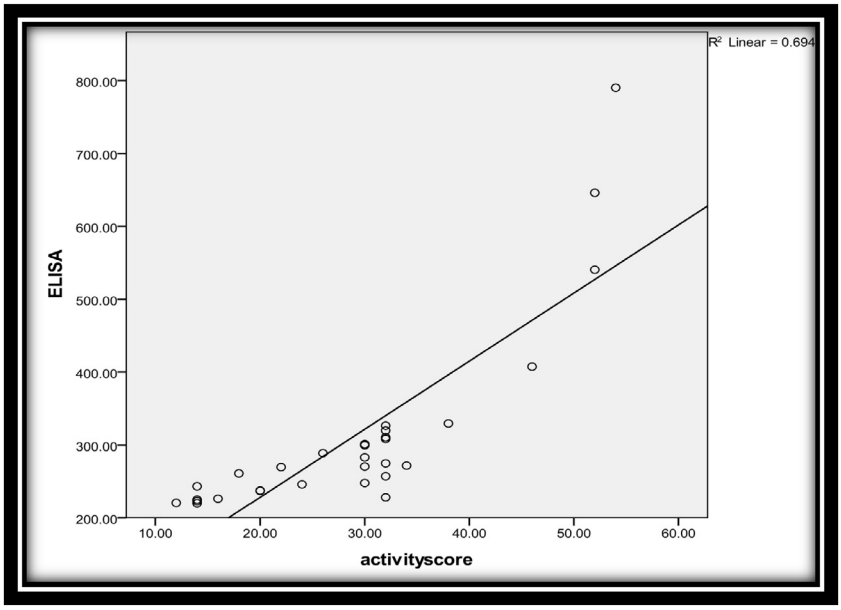

Figure 4 Relation between levels of serum IL-21 and activity index of SLE.

Table 3 Correlation between IL-21 serum levels and clinical findings of the patients.

		IL-21 serum levels	Student t test	P value
		Mean ± SD		
Muco-cutaneous	−ve	268.77 ± 8.15	−1.36	0.19
	+ve	334.42 ± 153.35		
Musculo-cutaneous	−ve	297.51 ± 104.27	−0.57	0.57
	+ve	325.02 ± 156.51		
Neurological defects	−ve	257.7 ± 31.45	−3.78	0.09
	+ve	429 ± 237.8		
Nephritis	−ve	253.52 ± 27.79	−3.05	**0.01***
	+ve	424.01 ± 175.97		

* p Value 0.01 significant.

Table 4 Genotype distribution of SNP in IL-21 rs2221903 among patient group and control group.

		Groups		Total ($N = 50$)	Chi Square test	P value
		Patients ($N = 30$) No (%)	Control subjects ($N = 20$) No (%)	No (%)		
SNP	T/T	22 (73.3)	9 (45)	31 (62)	4.20 (Fisher Exact)	0.12
	C/T	7 (23.3)	9 (45)	16 (32)		
	C/C	1 (3.3)	2 (10)	3 (6)		

In the current study, statistically significant association was found between serum levels of IL-21 and nephritis. On the other hand, no statistically significant association was found between serum IL-21 levels and other clinical findings. MRL-Faslpr mice with blockade of IL-21 by treating with anti IL-21R/Fc showed significantly reduced proteinuria, circulating autoantibodies and levels of glomerular IgG deposits in the kidney with no thickening in glomerular basement membranes by histological test [27]. However, Pan et al. found no significant difference regarding serum IL-21 level between SLE patients with nephritis and those without nephritis [26].

In our study, we found that SLE patients with a higher activity index score had significantly higher levels of serum IL-21 than those with a lower index score; these results verify the active role of IL-21 in immune-pathogenesis of SLE and its contribution to severity of clinical findings. In concordance to our results Nakou et al. and Wang et al. found that the levels of serum IL-21 were correlated positively with the values of

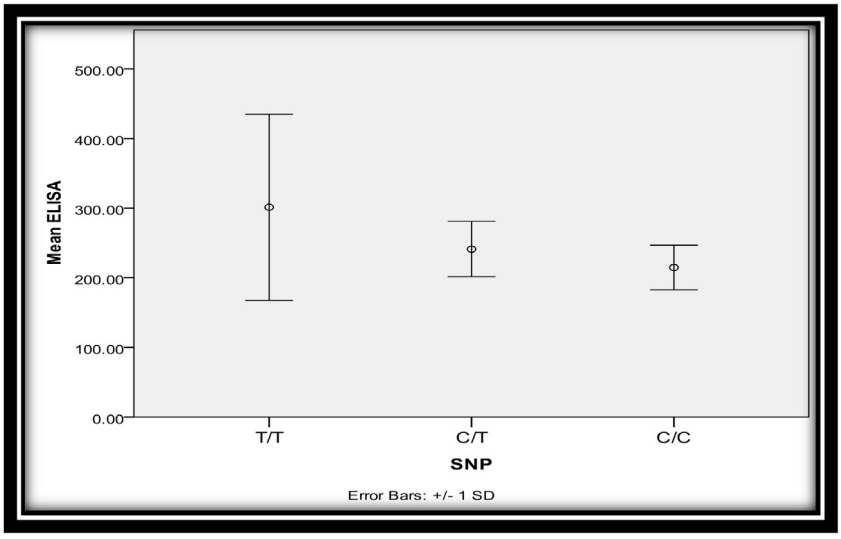

Figure 5 Correlation between SNP in IL-21 rs2221903 and serum IL-21 levels.

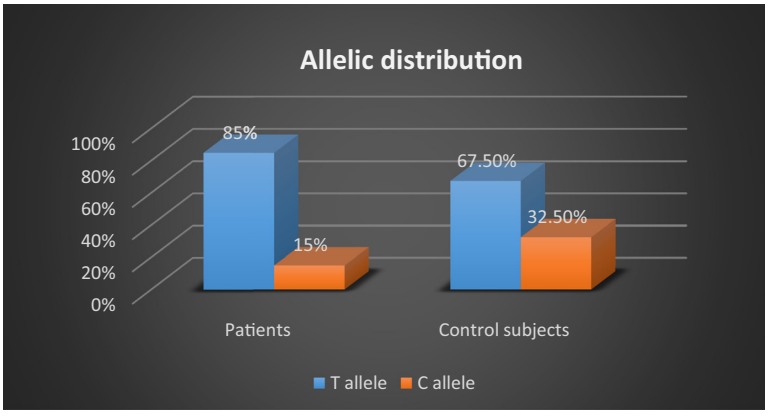

Figure 6 Allelic distribution of SNP in IL-21 rs2221903 in the studied groups. *One Way ANOVA.

SLEDAI. This elevation may be contributed to the generation of memory and plasma cells [22,24].

Approximately 90% of DNA polymorphisms are SNPs due to single base substitutions. Some have effects on regulation of gene expression or on the function of the coded protein. These functional polymorphisms, despite being of low penetrance, could contribute to the differences between individuals in susceptibility to and severity of disease [28,29].

In this study, a statistically significant association was found between the **T** allele of SNP rs2221903 and SLE. The **T** allele was found to be the major allele in both patients and controls (85% vs 67.5%), while the **C** allele was 15% and 32.5% in patients and controls respectively. The frequency of **T** allele was significantly elevated in SLE patients in comparison to controls.

These results are in accordance with those reported by Ding et al.; the **T** allele frequency was significantly higher in SLE patients compared to controls (89.4% vs 86.8%), while the **C** allele frequency was 10.6% in SLE patients and 13.2% in the controls. They concluded that the **T** allele was a risk factor for SLE [8]. Swalha et al. genotyped three SNPs located within the IL-21 gene in several ethnic groups. The **T** allele of SNP rs2221903 was found to be the major allele in both patients and controls, but they found that the **T** allele frequency was significantly lower in SLE patients compared to controls of both European American and African-American groups. In African American participants, they found that the **T** allele frequencies were 90.6% and 94% in the patients and control groups respectively [5].

Another study was done in USA by Hughes et al. in 2 large independent lupus sample sets. In participants of European descent, the **T** allele SNP rs2221903 was found to be the major allele in both patients and controls, and its frequency was significantly lower in SLE patients compared to controls. The **T** allele frequency was 68% in patient group, while in the control group the **T** allele frequency was 70% [30]. Lan et al. failed to detect significant association between any of the two alleles and the disease [9]. Qi et al. reported significant differences in the SNP of IL-21 rs2221903 allelic distribution between SLE patients and healthy controls in patients with European ancestry; however the **C** allele was the risk allele [4]. The discrepancy with their results may be due to genetic variation between different ethnic groups, and small sample size in this study.

In this study we failed to find an association between SNP of IL-21 rs2221903 genotypes and SLE, In agreement, Ding et al. and Lan et al. reported no significant association between the SNP of IL-21 rs2221903 genotypes and SLE [8,9]. However, Swalha et al. reported that a significant association was found between the SNP of IL-21 rs2221903 genotypes and SLE in European-American group, while no association was found in the African-American group [5]. Qi et al. observed that no significant association was found between different genotypes and SLE in African-American or Asian populations. On the other hand, SNP of IL-21 rs2221903 might be associated with SLE in Caucasian population [4]. The results from these studies were inconsistent and IL-21 rs2221903 gene polymorphisms were distinct in different population, this could be attributed to ethnic variation.

In this study we failed to find a statistically significant association between the SNP of IL-21 rs2221903 and serum IL-21 levels. Similar result was reported by Lan et al. [9]. The IL-21 rs2221903 SNP is located in the second intron of IL-21 gene. Intronic SNPs may be associated with functional consequences i.e. a variant that influences the translation of m RNA. However, the significance of non-coding SNPs is evolving [31]. Therefore, larger sample size may be needed to prove such an association.

6. Conclusion

IL-21 plays an important role in the immune-pathogenesis of SLE, this was proven by its elevated serum levels in patients and its association with activity score index. Thus, IL-21 may represent a novel target for the treatment of SLE and could be used as a marker for disease activity and treatment follow up. The **T** allele of SNP rs2221903 suggests that the IL-21 gene may contribute to an inherited predisposition to SLE although additional studies with larger sample sizes will be necessary to confirm our findings.

Conflict of interest

The authors declare no conflicts of interest.

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