The *STAT4* SNP (rs7574865) and systemic lupus erythematosus

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Abstract

Introduction: Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease affecting several systems and organs in the body. The association of STAT4 transcription factor with SLE risk remains unclear.
Objectives: The aim of this study was to investigate the association of STAT4 gene polymorphism (rs7574865) with the incidence of SLE.
Patients and Methods: One hundred and sixty participants (80 patients with SLE and 80 healthy individuals) were included in this study. Gene analysis was conducted by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) in peripheral blood samples.
Results: Fifty-seven percent (n=45) of patients with SLE had SLE disease activity index (SLEDAI) above six and had active disease. In the SLE group, the frequency of G and T alleles were 81% and 19%, respectively. Moreover, 72.50% (n=58) of patients carried the GG genotype, 17.5% (n=14) had the GT genotype and 10.1% (n=8) carried the TT genotype. There was no significant difference between allele frequency and genotypic distribution for rs7574865 polymorphism ($P>0.05$) between SLE and control groups. Significant differences were observed between the distribution of genotypes and clinical manifestations including leukopenia ($P=0.04$), pulmonary ($P=0.01$) and ophthalmic ($P=0.049$) problems. The T allele with an odd ratio of 1.47 and confidence interval of 0.80 to 2.6 could increase the risk of SLE; however, it was not statistically significant ($P=0.20$).
Conclusion: The T allele and TT genotype of the STAT4 rs7574865 polymorphism could increase the risk of lupus; however, these observations were not statistically significant.

Introduction

Systemic lupus erythematosus (SLE) is a clinically heterogeneous and multifarious autoimmune disease described by the autoantibodies production and the immune complexes deposition, influencing a wide range of organs (1). Different genetic, hormonal and environmental factors are involve in the etiologies of the SLE; however, its pathogenesis is complex and remains unknown. It is postulated that specific gene and environment interactions are the most significant mechanisms contributing to the SLE (2). Observational and genome-wide association studies have identified more than 100 genes that can increase vulnerability to the SLE (2,3) including signal transducer and activator of transcription (STAT4).

The *STAT4* gene encodes a transcription factor that has essential roles in the proliferation and differentiation of T cells (4,5). The STAT4 is needed for the inflammatory response after stimulation of IL-12 receptor (IL-12R),

Key point

Studying population differences in genetic susceptibility factors help us to expand our understanding of the disease mechanisms. In this study, we found that the distribution of GT and TT genotypes was higher in SLE patients. In addition, there were significant differences between the distribution of genotypes and clinical manifestations including leukopenia, pulmonary and ophthalmic problems.

wherein STAT4 translocates to the nucleus and binds to DNA and then, promotes the expression of pro-inflammatory genes, mainly interferon gamma (IFN-γ). Further, the STAT4 induces type 1 *IFN* and IL-23 signals in monocytes and T cells, resulting in activation of monocyte, differentiation of T helper 17 (Th17) and Th1 and production of IFN-γ (6).

Since Th17 and Th1 cells are critical effectors in chronic inflammatory, STAT4 gene variations may associate with susceptibility to different autoimmune diseases (7) and

exert a significant role in their pathogenesis. However, the molecular mechanisms by which these variants contribute to the autoimmune disease are unidentified. A variation of STAT4 gene (a single-nucleotide polymorphism (SNP) located in the 3rd intron; rs7574865) probably affects the expression of this protein at both translation and splicing levels (6).

Objectives
In SLE patients, the rs7574865 SNP has been connected with a severe disease phenotype and cardiovascular morbidity (8,9). Studying population differences in genetic susceptibility factors help us to expand our understanding of the disease mechanisms and accordingly develop clinical intervention for the complex SLE disease. In this study, we aimed to investigate the association of the *STAT4* gene polymorphism (rs7574865) with the incidence of SLE.

Patients and Methods
Subjects
In this cross-sectional study, 80 SLE patients were enrolled. The included patients had over 15 years old without underlying diseases. Patients with diabetic nephropathy or other rheumatic diseases such as rheumatoid arthritis were excluded from the study. In addition, 80 healthy controls were subjected as normal control for comparing the results. After recording clinical information, disease activity was determined based on Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2k) criteria in which organic brain syndrome, psychosis, seizures, visual disturbances, neurological disorders, vasculitis, cerebrovascular accidents and lupus headache was considered eight points. Accordingly arthritis, myositis, urinary casts, hematuria, proteinuria, considered four points; pericarditis, pleurisy, mucosal ulcers, alopecia, rash, low-complement levels and increased DNA binding was considered two points; while fever, thrombocytopenia and leukopenia got one point. Based on the Anić study (10), score higher than six is considered as active SLE.

DNA extraction and qualification
Whole blood sample (2 mL) was collected from SLE patients and healthy individuals in the same condition and prepared for DNA extraction. DNA extraction was conducted for each sample using magnetic nanoparticles (ZiAViZ kit, Iran). Qualification of samples were confirmed by loading in 1.5% gel electrophoresis.

DNA amplification
DNA amplification was conducted by amplifcation refractory mutation system-polymerase chain reaction (ARMS-PCR) for genotyping using thermocycler. DNA was amplified in a 25-µL PCR resection including 5 µL 10× PCR buffer, 0.75 µL MgCl2 50 mM, 0.5 µL dNTP, 0.625 µL specific primer 10 mM (Table 1), 1-2 µL DNA solution, 2.5 U taq DNA polymerase and DEPC up to 25 µL. Prepared samples were incubated at 94°C for two minutes and 30 cycles in 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 40 seconds. At last, the samples were incubated for five minutes at 72°C.

Data analysis
Quantitative variables were expressed as mean ±standard deviation or median (min-max) based on normal data distribution. Qualitative variables were expressed as frequency (percentage). Chi-square test was employed to compare the observed genotypes with the expected values. Genotype and allele frequencies in both healthy and SLE groups were calculated and the effect of each genotype and allele for SLE risk were assessed by chi-square test and logistic regression with odds ratio (OR) and 95% confidence interval (CI). Data was analyzed using SPSS software version 23.0. In evaluating the results, a *P* value < 0.05 was considered meaningful.

Results
This study was conducted on eighty SLE patients; 87.7% of whom were female (n = 71). Moreover, eighty healthy controls were included in the study to compare the obtained results. It was observed that the mean age of patients was 37.91 ± 11.75 years and the mean age of healthy individuals was 52.4 ± 6.54 years.

The median disease activity based on SLEDAI criteria among SLE patients was 8 (min 0-max 22). Our study showed that the SLEDAI scores of 45 patients (57%) were higher than six and they had active disease. Twenty-five patients had proteinuria and renal involvement and 5% of them had proteinuria in the nephrotic range and 12 patients (14.8%) had hematuria. The median of serum creatinine in the SLE group was 1.07 (0.06-7.6) mg/dL which was significantly different from the control group (*P* = 0.01). It was also observed that twenty patients (45%) with active SLE had significant renal involvement (*P* = 0.005). In addition, 84.6% (66 patients) and 67.1% (53 patients) had positive antinuclear antibody (ANA) and

Table 1. Primer sequences of *STAT4* (rs7574865) gene polymorphism

STAT4 (rs7574865)	Primers (5' to 3')	PCR product Size
Outer primer F & R	ATCCACAAATTATATCTTTTCCATTTTTT GGAATTTTTGTTATGATGAAAATAGCTT	410 bp
F inner T allele	AGTATGAAAAGTTGGTGACCAAAATATT	179 bp
R inner G allele	AATTCCACTGAAATAAGATAACCACTACTC	288 bp

F: Forward, R: Reverse.

anti-double stranded DNA (anti-dsDNA), respectively. The involvement of different organs and tissues among SLE patients was represented in Table 2. The most common clinical manifestations were anemia (66.7%) and rash (53.2%).

In SLE patients, the frequency of the G and T alleles were 81% and 19%, respectively. There were no statistically significant differences in the frequency of G and T alleles between SLE patients and controls ($P=0.207$; Figure 1A). In addition, 72.50% of SLE patients (n = 58) had GG genotype, 17.5% of them (n = 14) had GT genotype, and 10.1% of them (n = 8) had TT genotype. There was no significant difference in genotype distribution of the rs7574865 polymorphism between the SLE and control groups ($P=0.566$; Figure 1A).

The genotype distribution (GG, GT and TT) of rs7574865 polymorphism was evaluated based on the clinical variables. There was no significant difference between the distribution of genotypes and positive complement, anti-dsDNA, C-reactive protein (CRP), ANA and proteinuria ($P \geq 0.461$, Table 2). The organ involvement and genotype distribution of rs7574865 polymorphism genotypes were also investigated. There were significant differences between the genotype distribution of rs7574865 and leukopenia ($P=0.045$) and pulmonary involvement ($P=0.011$) with ophthalmic ($P=0.049$) involvement. However, the genotype distribution of rs7574865 was not statistically affected other clinical manifestations (Table 3). Based on the chi-square test, allele frequency and genotype distribution of rs7574865 gene polymorphism in the lupus nephritis (LN) group were not significantly different from those SLE patients without renal involvement ($P \geq 0.497$; Figure 2A and B).

The results of logistic regression analysis showed that the frequency of the T allele in the lupus group was higher compared to the control group (19% versus 13%) and with OR=1.47 and confidence interval of 0.80 to 2.68, it increased the risk of lupus, however, it was not statistically
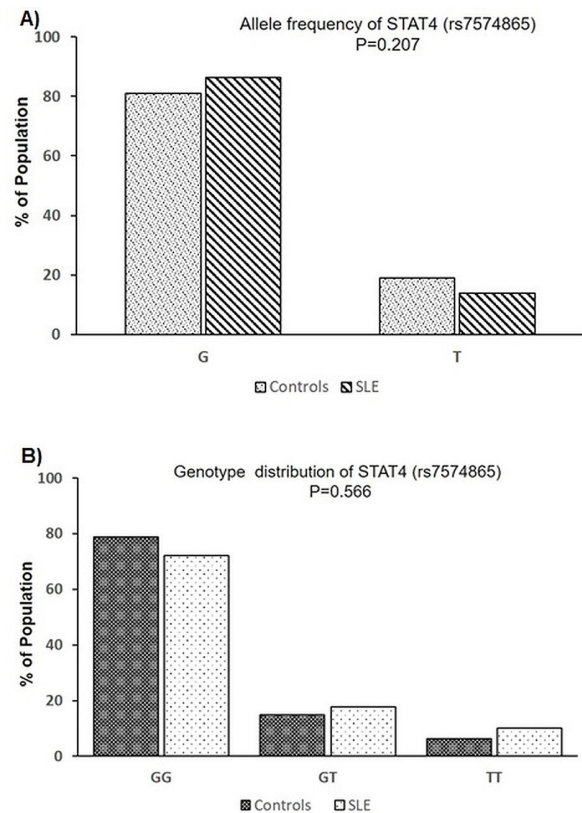

Figure 1. Genotype distribution and allele frequency of *STAT4* gene polymorphism among SLE patients. Allele (A) and genotype (B) distribution of *STAT4* gene polymorphism are represented among SLE patients in compared to healthy individuals. SLE: Systemic lupus erythematosus, STAT4: Signal transducer and activator of transcription 4.

significant ($P=0.208$). Similarly, the TT (OR=1.73, CI 0.58-5.68; $P=0.33$) and GT (OR=1.26, CI 0.54-2.64; $P=0.58$) genotypes increased the risk of SLE. The higher frequent distribution of GT and TT genotypes in the lupus group than the healthy group was not statistically significant. Furthermore, GT+TT genotype could increase the risk of SLE (OR=1.43, CI 0.69-2.9; $P=0.33$).

Table 2. Genotype distribution of *STAT4* gene polymorphism and clinical variables in SLE

Variables		Genotype frequencies, n (%)			Total	P value*
		GG	GT	TT		
Gender	Men	6 (7.6%)	2 (2.5%)	-	8 (10%)	0.555
	Women	51 (64.4%)	12 (15.2%)	8 (10.1%)	72 (90%)	
CRP	Positive	26 (32.9%)	6 (7.6%)	5 (6.3%)	37 (46.8%)	0.634
	Negative	31 (39.2%)	8 (10.1%)	3 (3.8%)	42 (53.2%)	
ANA	Positive	49 (62.8%)	11 (14.1%)	6 (7.6%)	66 (84.6%)	0.787
	Negative	8 (10.1%)	3 (3.8%)	1 (1.3%)	12 (15.4%)	
anti-dsDNA	Positive	40 (50.6%)	9 (11.4%)	4 (5.1%)	53 (67.1%)	0.508
	Negative	17 (21.5%)	5 (6.3%)	4 (5.1%)	26 (32.9%)	
Blood complement	Positive	30 (38%)	7 (9%)	6 (7.6%)	43 (54.4%)	0.461
	Negative	27 (34.2%)	7 (9%)	2 (2.6%)	36 (45.6%)	
Proteinuria	Positive	19 (24.4%)	4 (5.1%)	2 (2.6%)	25 (31.6%)	0.861
	Negative	38 (48.7%)	10 (12.8%)	6 (7.6%)	54 (68.4%)	

CRP: C-reactive protein.

Table 3. The correlation between genotype distribution of *STAT4* gene polymorphism and clinical manifestations in SLE patients

Diagnostic markers		Genotypes			Total	P value*
		GG	GT	TT		
Leukopenia	Yes	17 (21.5%)	-	1 (1.3%)	18 (22.8%)	**0.045**
	No	41 (51.2%)	14 (17.5%)	7 (8.8%)	62 (77.5%)	
Hematuria	Yes	6 (7.6%)	3 (3.8%)	3 (3.8%)	12 (15.2%)	0.107
	No	52 (65%)	11 (14.1%)	5 (6.4%)	68 (85.2%)	
Anemia	Yes	37 (47.4%)	10 (12.8%)	7 (9%)	54 (69.2%)	0.461
	No	19 (24.4%)	4 (5.1%)	1 (1.3%)	24 (30.8%)	
Thrombocytopenia	Yes	15 (19.2%)	2 (2.6%)	3 (3.8%)	20 (25.6%)	0.517
	No	42 (53.8%)	11 (14.1%)	5 (6.4%)	58 (74.4%)	
CNS involvement	Yes	7 (8.9%)	-	-	7 (8.9%)	0.227
	No	50 (63.3%)	14 (17.7%)	8 (10.1%)	72 (91.1%)	
Dermatological involvement	Yes	29 (36.7%)	10 (12.8%)	3 (3.8%)	42 (53.2%)	0.249
	No	28 (35.4%)	4 (5.1%)	5 (6.3%)	37 (46.8%)	
Hematological problems	Yes	19 (24.1%)	3 (3.8%)	4 (5.1%)	26 (32.9%)	0.387
	No	38 (48.1%)	11 (13.9%)	4 (5.1%)	53 (67.1%)	
Vasculitis	Yes	2 (2.6%)	1 (1.3%)	-	3 (3.8%)	0.685
	No	55 (69.4%)	13 (16.5%)	8 (10.1%)	76 (96.2%)	
Ophthalmic involvement	Yes	-	1 (1.3%)	1 (1.3%)	2 (2.6%)	**0.049**
	No	57 (72.2%)	13 (16.5%)	7 (8.9%)	77 (97.5%)	
Musculoskeletal involvement	Yes	15 (19.2%)	4 (5.1%)	2 (2.6%)	21 (26.6%)	0.980
	No	42 (53.2%)	10 (12.8%)	6 (7.6%)	58 (73.4%)	
Pulmonary involvement	Yes	-	-	1 (1.3%)	1 (1.3%)	**0.011**
	No	57 (72.2%)	14 (17.7%)	7 (8.9%)	78 (98.7%)	

CNS: Central nervous system.
* Data presented as number (percentage). *P* value < 0.05 was considered as significant result.

Discussion

In this study, the association of the STAT4 rs7574865 polymorphism with SLE was investigated. About 57% of patients with lupus had the active disease (SLEDAI >6). In the SLE group, the frequencies of G and T alleles were 81% and 19%, respectively. In addition, 17.5% and 10.1% of patients had GT and TT genotypes, respectively. The T allele increased the risk of lupus; however, it was not statistically significant. The higher distribution of GT and TT genotypes, carrying at least one T allele in SLE patients was not statistically significant. No significant difference was observed between lupus and control groups regarding allelic frequency and genotype distribution of rs7574865 polymorphism.

Studies indicate that expression of the TNFSF4 and STAT4 genes along with IL-10 and TNF-α levels can be useful as biomarkers for assessing the SLE activity and managing treatment protocols (11). The STAT4 haplotype has been reported to be linked with an increased risk of SLE and rheumatoid arthritis, indicating a common pathway for these diseases (8).

A pervious meta-analysis on forty studies (2012) revealed that the *STAT4* rs7574865 T allele increases vulnerability to primary Sjögren's syndrome, autoimmune thyroid, inflammatory bowel diseases, type 1 diabetes, SLE and rheumatoid arthritis (7). Moreover, the STAT4 gene polymorphisms are associated with susceptibility to the

SLE in different populations. The TT genotype of STAT4 rs7574865 polymorphism was significantly associated with an increased risk of SLE in the Japanese population (12). In a Chinese population, the T allele of this polymorphism was a risk allele in the SLE (13).

Another meta-analysis on 24 research papers from the different populations including 17 389 lupus patients and 29 273 controls showed a significant relationship between T allele and risk genotypes (TT, TG and TT + TG) of rs7574865 polymorphism and susceptibility to the SLE. Subgroup analysis of this study showed an association between rs7574865 polymorphism and ethnicities; the rs7574865 T allele has significant correlation with SLE in the Asian population (14). The genetic contribution of this gene to the SLE may be higher in the Japanese population than in European Americans (15). The results of these studies suggest that STAT4 acts as a genetic risk factor in the pathogenesis of SLE. Likewise, we found that the distribution of GT and TT genotypes and the frequency of the T allele were higher in SLE patients compared to controls. Moreover, statistically insignificantly, the studied polymorphism could increase the risk of lupus in adults. However, the mechanism of this gene to increase the risk of SLE is still unknown. In addition, the question of how STAT4 polymorphisms regulate this complex disease needs to be clarified. It should be noted that in some ethnic populations and autoimmune diseases STAT4 rs7574865
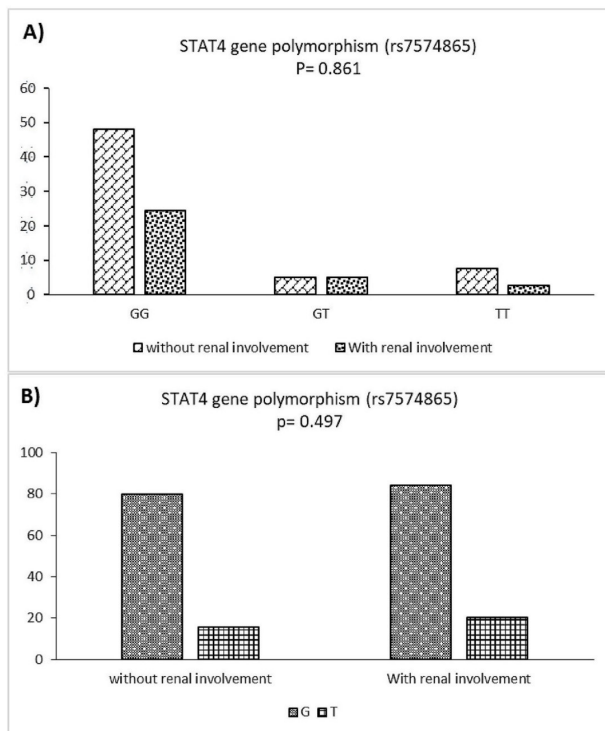
Figure 2. The relationship between renal involvement and *STAT4* gene polymorphism in SLE patients. The correlation between renal involvement and different observed alleles (A) and genotypes (B) of *STAT4* gene polymorphism in SLE patients are represented. SLE: Systemic lupus erythematosus, STAT4: Signal transducer and activator of transcription 4.

does not have a role in susceptibility, suggesting that some of the SLE susceptibility genes might be population-specific (16).

The SLE patients demonstrate different clinical phenotypes. The *STAT4* SNPs are correlated with definite clinical manifestations of SLE including presence of anti-dsDNA, an earlier onset of disease, a severer disease phenotype, and an increased risk for lupus nephritis (LN) and stroke (9, 17, 18). In individuals with *STAT4* risk allele, an increased IFN-γ production is induced by IL-12, speculating that the increased risk for LN might be driven by IFN-γ overproduction (19). Yang et al observed a strong association between SNP rs7574865 and anti-dsDNA positive antibodies in SLE patients (16). In addition, rs7574865 risk allele carriers had higher levels of C3, C4 and anti-dsDNA (17). Additionally, an increase in the frequency of the rs7574865 T allele was tested in SLE patients with rash, hematologic disorders, proteinuria, and anti-dsDNA-positive antibodies (20). Patients with the *STAT4* CC genotype had significantly increased SLE activity index (SLEAI) and injury index compared to the GG genotype and these homozygous patients have a high risk for severe disease manifestations (21). El-Saadany et al found an association between *STAT4* rs7574865 polymorphism and increased susceptibility to lupus. The frequency of *STAT4* (TT) genotype in patients was much higher than control group. The T allele carriers

had a higher risk of developing the disease and had lower complement (C3, C4), proteinuria and anti-dsDNA (22). In the present study, we found significant differences between the distribution of GG, GT, and TT genotypes and leukopenia, pulmonary and eye involvement. No significant correlation was observed for the rest of the clinical, demographic data and the manifestations of SLE. Further studies is necessary to these polymorphisms contributing in different clinical SLE phenotypes.

Conclusion
Overall, the T allele and TT genotype of the STAT4 rs7574865 polymorphism could increase the risk of developing lupus; however, this observation was not statistically significant, which may be due to the small sample size. Genotype distribution of the studied polymorphism could significantly impact leukopenia and pulmonary and ophthalmic problems.

Limitations of the study
Small sample size was the main limitation of this study, during the COVID-19 pandemic, patients did not agree to participate in this study.

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