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FOXP3 Gene Variants in Systemic Lupus Erythematosus Patients: Association with Disease Susceptibility in Men and Relationship with Abortion in Women

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ABSTRACT

Background: FOXP3, an important transcription factor of regulatory T cells has shown a contribution to the development of various autoimmune diseases.

Objectives: To investigate the influence of FOXP3 polymorphisms (rs3761548 and rs2294021) on systemic lupus erythematosus (SLE) susceptibility and patients' characteristics.

Methods: Genotyping was performed on 265 patients with SLE and 404 healthy controls using PCR-RFLP. Patients' demographic, laboratory, and clinical information were all documented. The relationship between the SNPs and patients' characteristics was statistically analyzed.

Results: The frequency of C/- genotype in male patients was significantly higher than in the healthy male controls, whereas the frequency of A/- genotype was lower (OR=0.53; 95% CI=0.28-1.00, P=0.05). Analysis of the correlation between these SNPs and the patients' characteristics showed a longer disease duration in the rs3761548 C/- carriers and a correlation with arthralgia in both SNPs. In the females, there was a significant association between CC haplotype and disease susceptibility (OR=0.6, CI=0.38-0.94, P=0.027). A significant association of both SNPs with the history of abortion was also detected. The frequencies of the rs3761548 AA (P=0.006) and the rs2294021 CC genotypes (P=0.038) and AC/AC combination (P=0.033) were higher in women who had an abortion. We found a correlation between the rs3761548 AC genotype and the decreased C4 level and cardiovascular involvement, and the rs2294021 CC genotype with ESR, neurological involvement, and photosensitivity.

Conclusions: FOXP3 rs3761548 C/- genotype association with disease susceptibility in male patients, an association of both SNPs with the abortion risk in female patients, and the correlation between these SNPs and several clinical features of the patients suggest their association with the disease development and pathology.

Keywords: Autoimmune Disease, FOXP3, Lupus, Polymorphisms, Regulatory T Cell

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the presence of pathogenic autoantibodies, the formation of immune complexes, and their deposition in various organs (1). The overall incidence of the disease varies and is approximately 0.3-2.7 per 100 000 people per year (2). The disease can affect persons of various ages and races, and it can affect both sexes, but it is more frequent in females, with approximately 90% of new patients being females of childbearing potential.

Regulatory T cells (Treg) are a subset of $CD4^+$ T cells that consist of about 5-6% of the population of these cells (3). Treg cells can inhibit and suppress the activation and expansion of various types of immune cells. These cells can inhibit the activation, proliferation, and production of cytokines by $CD4^+$ and $CD8^+$ T cells (4). They also inhibit B cell proliferation and immunoglobulin production. The mechanisms by which Treg cells perform their inhibitory function include the release of inhibitory cytokines, induction of tryptophan catabolism via cytotoxic T-lymphocyte-associated protein (CTLA4), and inducing cell death (5). In most autoimmune diseases, such as type 1 diabetes, myasthenia gravis, multiple sclerosis, SLE and rheumatoid arthritis, there have been reported functional defects in the number and function of Treg cells (6).

An important transcription factor involved in the development, maintenance, and function of Treg cells is FOXP3 (4). The *FOXP3* gene belongs to the Forkhead/Winged-helix family and is located in the X chromosome (XP11.23). This gene has different single nucleotide polymorphisms (SNP) and their relationship with various diseases has been reported (7). Polymorphisms in the *FOXP3* gene can lead to the development of improperly Treg cells, and impaired Treg cells number and function have been reported in a range of autoimmune diseases such as type 1diabetes, rheumatoid, and autoimmune thyroid diseases. One of the SNPs in the *FOXP3* gene is the rs3761548 C/A, which is located in the intron region and appears to affect gene expression (7). In this functional SNP, A allele is associated with lower expression of *FOXP3* gene compared with C allele (8). The association between the rs3761548 polymorphism has been reported in various autoimmune diseases such as Crohn's disease, Graves' and Hashimoto's diseases, Behcet's disease, and psoriasis (8-11). The rs2294021 T/C is another SNP, which is located in the intron region of the Coiled-coil domain, containing the 22 (CCDC22) gene. According to the PubMed database, this gene is also located in the X chromosome (Xp11.23) and encodes a protein containing a coiled-coil domain which is involved in the regulation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB), therefore, this gene plays a role in regulating the inflammatory response, also, this SNP is close to *FOXP3* gene and may be involved in its function (12). Results showed that this SNP has been related to the risk of various diseases such as colitis and Crohn's disease (11), pemphigus foliaceus (13), Graves' disease, and Addison's disease (14). Both of these SNPs have minor allele frequencies (MAF) of more than 5% and their functionality, combined with the presence of scientific evidence for their participation in autoimmune diseases, make them ideal research targets.

From the etiological point of view, genetic factors play key roles in the development of autoimmune diseases such as SLE. Considering the important role of Treg cells and their transcription factor, coupled with FOXP3 in the development of autoimmune diseases, in this study we decided to investigate the relationship between the two mentioned *FOXP3* SNPs (rs3761548 and rs2294021) with susceptibility to SLE as well as their link to the patients' clinical characteristics and laboratory indicators.

MATERIALS AND METHODS

Patients and Sampling

Patients were selected from those referred

to Hafez Clinic of Lupus, Shiraz. To adhere to ethical issues, the study participants were provided the essential information on how to perform the study prior to the study. Blood samples were obtained after getting consent from the patients and the healthy individuals. The study included 265 people with SLE who had been diagnosed and confirmed to have the condition by a specialized physician. These patients had met the revised classification criteria of the American College of Rheumatology (ACR) (15). We excluded the patients who did not specify the SLE diagnostic criteria or had other autoimmune diseases associated with the SLE. Demographic characteristics, as well as the clinical and laboratory manifestations of the patients were recorded. Disease activity was determined by estimating the SLE disease activity index (SLEDAI) according to the revised criteria of the American College of Rheumatology (ACR) (15). The control group consisted of 404 healthy individuals with no history of autoimmune diseases or other immunological disorders. Female patients were compared with healthy females (sex- match, not age-matched) and men patients were compared with healthy men (sex-matched, not age-matched). The study protocol was approved by the Shiraz University of Medical Sciences Ethics Committee (IR.SUMS.RE.1399.193).

Five milliliters of peripheral blood were taken from the patients and the controls in a tube containing EDTA anticoagulant to extract the genomic DNA using the salting-out technique. Extracted DNA was examined for the quality and then the appropriate concentration was used for the polymerase chain reaction (PCR) test.

Genotyping

PCR-fragment length polymorphism (PCR–RFLP) was used to determine the genetic variations of *FOXP3* rs3761548 and the rs2294021 SNPs. Amplification of the polymorphic sequences was carried out using specific primers by PCR. The primers were designed based on previous studies and checked by BLAST (13, 16). The sequences of primers used were F: 5'- CCTCTCCGTGCTCAGTGTAG-3', R: 5'- GGGTGTTACAAGGAAAGGTTGGG AC-3' (rs3761548) and F: 5'-CACACA CAATCCATCCCAGTCACCC-3', R: 5'-ATCTCCATGCCCTAAGAAGGC CCAC-3' (rs2294021). The amplification process was performed in a total volume of 7 µl containing Parstous PCR buffer, 1 µl $MgCl_2$, 0.7 µl DNA Taq polymerase, 0.1 µl dNTP, 0.3 µl sterile distilled water, 3.5 µl 0.7 µl of each primer (Cinnagen, Iran), and 3 µl genomic DNA. The program for PCR conditions for two of the polymorphisms started with denaturation at 95 °C (5 min) and continued with 31 cycles of denaturation (95 °C, 30 s) for the rs3761548 and 33 cycles of denaturation (95 °C, 30 s) for the rs2294021, annealing (58 °C, 45 s for rs3761548 and 60 °C, 35 s for rs2294021), extension (72 °C, 55s) and final extension (72 °C, 5 min). The size of PCR products for the rs3761548 was 487 bp and for the rs2294021 was 429 bp. After verifying the PCR reaction using electrophoresis on agarose gel, enzymatic digestion was carried out using Thermo Scientific PstI (for the rs3761548) and HaeIII (for the rs2294021) at 37 °C for 16 hrs. The produced fragments were separated by electrophoresis and then visualized under UV light.

Statistical Analysis

Statistical Package for the Social Sciences software version 19 (SPSS, Chicago, IL) was used for the statistical analysis. Pearson's chi-square and Fisher's exact tests were used to compare the distribution frequency of the FOXP3 genotypes and alleles between the groups. Deviation from Hardy–Weinberg equilibrium was determined by Arlequin software. The relationship between the patients' characteristics and genotypes was determined using Mann-Whitney and Kruskal Wallis non-parametric tests. Haplotype analysis was performed by Arlequin and Epi-

Info software. P values equal to or less than 0.05 were considered significant.

RESULTS

Patients' Characteristics

To evaluate the *FOXP3* gene polymorphism in patients with SLE, 265 patients and 404 healthy individuals were selected as the controls. Of the patients, 209 (78.9%) were females and 56 (21.1%) were males. The control group included 221 females and 183 males. Demographics, clinical manifestations, and laboratory parameters of the total male and female patients are given in Tables 1 and 2.

Table 1. Demographic features of the patients with SLE

Characteristics	Patients (total)	Female	Male
Number of patients	265	209 (78.9)	56 (21.1)
Age (year)	35.2±9.5	35.6±9.1	33.3±11.2
Age of onset (year)	28.2±9.8	28.4±9.7	27.2±10.6
Disease duration (month)	80.9±78.2	83.4±74.9	70.4±90.7
Family history of SLE	64 (25.8)	54 (26.3)	10 (23.3)
Family history of other AID	101 (40.4)	84 (40.8)	17 (38.6)
Smoking	29 (11.6)	15 (7.3)	14 (31.8)
Abortion history	55 (26.8)	55 (26.8)	-

Data are presented as mean±SD or number (%). AID, autoimmune diseases

Table 2. Clinical and paraclinical features of the patients with SLE

Clinical features	Patients (total)	Female	Male
Organ involvement			
-Cardiac	8 (3.2)	7 (3.5)	1 (2.1)
- Otolaryngological	34 (14.8)	29 (14.4)	5 (17.2)
-Gastrointestinal	27 (10.8)	20 (9.8)	7 (15.6)
-Joint			
-Arthritis	80 (32)	70 (34.5)	10 (21.3)
-Arthralgia	189 (75)	157 (77)	32 (66.7)
-Neurological	17 (6.7)	12 (5.9)	5 (10.4)
-Pulmonary	22 (8.8)	16 (7.8)	6 (13.3)
-Renal	113 (49.3)	94 (47.2)	19 (63.3)
-Skin			
-Malar rash	132 (52)	108 (52.4)	24 (50)
-Photosensitivity	162 (64)	133 (64.9)	29 (60.4)
-DLE	24 (9.8)	14 (6.9)	10 (23.8)
-Other forms	128 (51.2)	105 (51.2)	23 (51.1)
SLEDAI, median (range)	8.0 (0-44)	6.0 (0-44)	12.0 (0-22)
-C3			
Normal	177 (78.3)	159 (80.7)	18 (62.1)
Decreased	49 (21.7)	38 (19.3)	11 (37.9)
-C4			
Normal	161 (71.6)	143 (73)	18 (62.1)
Decreased	64 (28.4)	53 (27)	11 (37.9)
-Anti-dsDNA positive	123 (52.1)	98 (50)	25 (62.5)
-ANA positive	193 (81.1)	158 (81.4)	35 (79.5)
Increased ESR	100 (42)	85 (42.7)	15 (38.5)
CRP positive	39 (16.5)	35 (17.5)	4 (11.1)

Data are presented as number (%). SLEDAI, SLE disease activity index; ANA, antinuclear antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Of the total patients, 75% had arthralgia and 49.3% had kidney involvement. Skin involvement in the form of malar rash was observed in 52% and photosensitivity in 64% of the patients. Other organ involvements

such as cardiovascular, pulmonary, ocular, otolaryngological, gastrointestinal ,and arthritis were also present in the patients. Of the female patients, 26.8% had a history of at least one abortion. The range of patients' disease activity (SLEDAI) was 0 to 44 (mean, 8.7±7.5) with a median of 8. Anti-dsDNA antibodies were present in 123 patients (52.1%) and antinuclear antibody (ANA) in 193 patients (81.1%). Of the patients, 42% had increased erythrocyte sedimentation rate (ESR) (22.7±20.2 mm/h) and 16.5% were positive for C-reactive protein (CRP). Data analyzing was performed based on the patients' available information

Genotypic Frequency of the rs3761548 and the rs2294021 Polymorphisms in the Male Patients and the Healthy Men Controls

Genotype frequencies in both male and female patients and controls were in the Hardy-Weinberg equilibrium (P>0.05). As the *FOXP3* gene is an X-linked gene, the distribution of genotypes in the male and female patients was separately evaluated and compared with related gender in the healthy group. In a study of the genotypic distribution of the rs3761548 SNP, we found that 18 patients had A/- genotype and 38 patients had C/- genotype, and in the control group, 86 had A/- genotype and 97 had C/- genotype. A comparison of these data showed that the frequency of C/- genotype in the male patients was significantly higher than in the healthy male controls, whereas the frequency of A/- genotype was lower (OR=0.53; 95% CI=0.28-1.00, P=0.05).

The genotypic distribution of the patients and the controls for the rs2294021 showed that 30 patients had T/- genotype and 26 had C/- genotype. In the control group, 80 people had T/- genotype and 103 people had C/- genotype. A comparison of these data did not show any significant differences in the frequency distribution of genotypes in this SNP among the male patients and the controls (P=0.195). The genotypic frequencies of these two SNPs in the men are shown in Table 3.

Genotypic and Allelic Frequencies of the rs3761548 and the rs2294021 in the Female Patients and the Healthy Women Controls

In the study of the genotype distribution of the rs3761548 in women with SLE, we observed that 35 patients had AA genotype, 76 had CC genotype, and 98 had AC genotype (Table 4). Corresponding data for the control group was 32 subjects with AA genotype, 91 subjects with CC genotype, and 98 subjects with AC genotype which showed no significant difference. A comparison of the genotype combination, the frequencies of alleles in patients (40.2% A allele and 59.8% C allele) and in the controls (36.7% A allele and 63.3% C allele) showed no significant results.

For the rs2294021 distribution of genotypes and alleles we demonstrated that 59 patients had TT genotype, 49 had CC and 101 had

Table 3. FOXP3 polymorphism in male patients with SLE

Genotype	Patients (n=56)	Controls (n=183)	P	OR	95% CI
		rs3761548			
A/-	18 (32.1)	86 (47)	0.050*	0.53	0.28-1.00
C/-	38 (67.9)	97 (53)		1.0 (ref)	
		rs2294021			
T/-	30 (53.6)	80 (43.7)		1.0 (ref)	
C/-	26 (46.4)	103 (56.3)	0.195	0.673	0.36-1.22

Data are represented as number (%). OR, odds ratio, CI, confidence interval, P: P value. *P≤0.05

Table 4. FOXP3 polymorphism in female patients with SLE

	Patients (n=209)	Controls (n=221)	P	OR	95%CI
		rs3761548			
Genotypes					
CC	76 (36.4)	91 (41.2)		1.0 (ref)	
AA	35 (16.7)	32 (14.5)	0.563	1.30	0.74-2.3
AC	98 (46.9)	98 (44.3)		1.19	0.79-1.8
AA+AC	133 (63.6)	130 (58.8)	0.306		
CC	76 (36.4)	91 (41.2)			
CC+AC	174 (83.3)	189 (85.5)	0.517		
AA	35 (16.7)	32 (14.5)			
AA+CC	111 (53.1)	123 (55.7)	0.596		
AC	98 (46.9)	98 (44.3)			
Alleles					
C	250 (59.8)	280 (63.3)		1.0 (ref)	
A	168 (40.2)	162 (36.7)	0.286	1.16	0.88-1.5
		rs2294021			
Genotype					
TT	59 (28.2)	69 (31.2)		1.0 (ref)	
CC	49 (23.4)	61 (27.6)	0.320	0.93	0.56-1.56
CT	101 (48.3)	91 (41.2)		1.29	0.82-2.03
TT+CT	160 (76.6)	160 (72.4)	0.323		
CC	49 (23.4)	61 (27.6)			
CC+CT	150 (71.8)	152 (68.8)	0.498		
TT	59 (28.2)	69 (31.2)			
TT+CC	108 (51.7)	130 (58.8)	0.136		
CT	101 (48.3)	91 (41.2)			
Alleles					
T	219 (52.4)	229 (51.8)		1.0 (ref)	
C	199 (47.6)	213 (48.2)	0.864	0.97	0.74-1.27

Data are represented as number (%). OR, Odds ratio; CI, confidence interval. P: P value. *P≤0.05

CT genotype. In comparison, 69 control individuals had TT genotype, 61 had CC genotype, and 91 had CT genotype. Of the patients, 52.4% had a T allele and 47.6% had a C allele. In the control individuals, 51.8% had a T allele and 48.2% had a C allele. Statistical analysis did not show any significant differences in the frequency distribution of genotypes, genotypic combination, and frequency distribution of alleles of this SNP among the patients and the controls.

Haplotype Analysis in the Patients with SLE and the Controls

In the male group, haplotype analysis showed a higher frequency of CT genotype (the rs3761548/rs2294021) in the patients *versus* the controls (OR, 0.53, 95% CI, 0.28-1.00, P=0.05).

In the female group, a significant difference in the frequency of CC haplotype was observed between the patients (7.8%) and the controls (12.4%) (OR=0.6, CI=0.38-0.94, P=0.027). The frequencies of haplotype combinations of AC/AC, CT/CT, CC/CC, AC/CT, AC/CC, AC/AT, and CT/CC between the female patients and the controls did not show any significant differences.

The Relationship between FOXP3 Polymorphisms with Demographic, Clinical, and Paraclinical Features of the Patients

In men with SLE, as shown in Table 5, a significant relationship was observed between genotypes of the rs3761548 and disease duration (P=0.03). In this group, the mean

Table 5. FOXP3 polymorphism in relation to demographic, clinical and paraclinical features of female and male patients with SLE

Variable	rs3761548				rs2294021			
	Female patients							
	AA (N=35)	CC (N=76)	AC (N=98)	P	TT (N=59)	CC (N=49)	CT (N=101)	P
Abortion history,								
Yes	17 (30.9)	16 (29.1)	22 (40)	0.006*	14 (25.5)	20 (36.3)	21 (38.2)	0.038*
No	18 (12)	58 (38.7)	74 (49.3)		44 (29.3)	29 (19.3)	77 (51.4)	
C4								
-Normal	31 (21.7)	51 (35.7)	61 (42.6)	0.029*	40 (28)	39 (27.3)	64 (44.7)	0.320
-Decreased	3 (5.7)	21 (39.6)	29 (54.7)		16 (30.2)	9 (17)	28 (52.8)	
ESR (mm/h)	24.1±24.1	21.2±18.4	24.4±18.2	0.347	22.7±19.6	18.5±19.6	25.8±18.8	0.011*
-Increased	15 (17.6)	29 (34.2)	41 (48.2)	0.917	25 (29.4)	14 (16.5)	46 (54.1)	0.089
-Normal	20 (17.4)	42 (36.8)	52 (45.6)		30 (26.3)	34 (29.8)	50 (43.9)	
Cardiac involvements								
Yes	0	6 (85.7)	1 (14.3)	0.039	4 (57.1)	2 (28.6)	1 (14.3)	0.103
No	35 (17.9)	68 (34.7)	93 (47.4)	*	54 (27.5)	47 (24)	95 (48.5)	
Epilepsy								
Yes	3 (25)	2 (16.7)	7 (58.3)	0.377	0	4 (33.3)	8 (66.7)	0.044*
No	32 (16.6)	72 (37.3)	89 (46.1)		58 (30.1)	45 (23.3)	90 (46.6)	
Photosensitivity								
Yes	21 (15.8)	43 (32.3)	69 (51.9)	0.141	32 (24)	28 (21.1)	73 (54.9)	0.022*
No	14 (19.4)	31 (43.1)	27 (37.5)		26 (36.1)	21 (29.2)	25 (34.7)	
Variables	**rs3761548**				**s2294021**			
	Male patients							
	A/- N=18	**C/- N=38**		**P**	**T/- N=30**	**C/- N=26**		**P**
Disease								
Duration (m)	39.4±61.5	84.5±98.8		0.03*	95.04±109	43.7±55.2		0.091
-Arthralgia								
Yes	7 (21.9)	25 (78.1)		0.048*	20 (62.5)	12 (37.5)		0.041*
No	8 (50)	8 (50)			5 (31.3)	11 (68.7)		

Data are represented as number (%) or mean±SD. ESR, erythrocyte sedimentation rate; m, month. P: P value. *P<0.05. The percentages are based on the patients' available information

duration of the disease was higher in those who had the C/- genotype (84.5±98.8 month) compared with those with A/- genotype (39.4±61.5 month). A significant relationship was found between the rs3761548 genotypes and arthralgia (P=0.048), so the majority of patients with arthralgia (78.1%) were C carriers. Similarly, a significant relationship between the rs2294021genotypes and arthralgia was detected (P=0.041) and most of the male patients with arthralgia (62.5%) had the T/- genotype.

In the female patients, the rs3761548 SNP was significantly associated with the history of at least one abortion (P=0.006). Of the patients who had experienced abortion, 30.9% had an AA genotype, while 12% of the women without abortion had this genotype.

Regarding the rs2294021, there was also a significant relationship between genotypes and the history of abortion in the patients (P=0.038). Thirty-six percent of those with a history of abortion had CC genotype compared with 19.3% CC genotype in those without a history of abortion.

In the female patients, a significant association was found between the rs3761548 genotypes and serum C4 level (P=0.029). Most of those patients with decreased C4 level had the AC genotype (54.7%).

Concerning the rs2294021, a correlation was found between ESR and genotypes (P=0.011). Also, the mean ESR level was the highest in individuals with CT genotype (25.8±18.8 mm/h) and was the lowest in the individuals with CC genotype (18.5±19.6 mm / h) (Table 5).

As for the clinical features, as shown in Table 5, for the rs3761548, statistical analysis showed a significant relationship between the genotypes of this SNP and the clinical presentation of heart disease (P=0.039). In this group, the rate of CC genotype in the patients with cardiovascular disease was 51% higher than in the group that did not have this organ involvement.

In terms of the rs2294021, there was a significant difference between the genotypes of this SNP and the incidence of epilepsy (P=0.044). The CT genotype was the highest in patients with epilepsy (66.7%) while the TT genotype was the lowest (0%). This SNP also showed a significant association with photosensitivity (P=0.022) and most patients with this manifestation had the CT genotype (54.9%).

There was no significant association between the rs3761548 and the rs2294021 genotypes and the disease activity in the male and female patients.

The Relationship between Haplotypic Combinations and Disease Characteristics in Women with SLE

In this part of the study, the possible relationship between demographic characteristics, clinical manifestations, and laboratory parameters with seven haplotypic combinations of AC/AC, CT/CT, CC /CC, AC/CT, AC/CC, AC/AT, and CT/CC in affected women were checked. In terms of demographic characteristics, a significant relationship was seen with the history of abortion (P=0.033). In this group the AC/AC

Table 6. Haplotype combination and relation to demographic, clinical and paraclinical features of female patients with SLE

Variable	Haplotype combination	Other combinations	P
	AC/CT	Other combinations	
Age of onset			
<25	30 (36.6)	52 (63.4)	0.022*
25-50	49 (41.2)	70 (58.8)	
>50	4 (66.7)	2 (33.3)	
	AC/AC	Other combinations	
Abortion			
Yes	16 (29.1)	39 (70.9)	0.033*
No	17 (11.3)	133 (88.7)	
	CT/CT	Other combinations	
Epilepsy			
Yes	0	12 (100)	0.018*
No	58 (30.1)	135 (69.9)	
	CT/CT	Other combinations	
Cardiac involvement			
Yes	4 (57.1)	3 (42.9)	0.002*
No	54 (27.6)	142 (72.4)	
	AC/CT	Other combinations	
ESR			
Increased	40 (47.1)	45 (52.9)	0.021*
Normal	40 (35.1)	74 (64.9)	
	AC/AT	Other combinations	
ESR (mm/h)	63±21.2	18.74±5.92	0.001*

Data are represented as number (%) or mean±SD. ESR, erythrocyte sedimentation rate. P: P value. *P<0.05. The percentages are based on the patients' available information

haplotypic combination was more frequent in patients with abortion compared with the other combinations.

In terms of clinical manifestations, a significant relationship was observed in the incidence of epilepsy (P=0.018) and cardiovascular disease (P=0.002). The frequency of CT/CT haplotype combination was lower in patients with epilepsy (0%) and higher in patients with cardiovascular disease (57.1%) compared with the other combinations (Table 6).

In terms of laboratory parameters, a significant relationship between AC/CT haplotype combination with increased ESR (P=0.021) and ESR level (P=0.001) was detected. In this group, the AC/CT haplotype combination was lower (47.1%) in those with increased ESR level. The highest ESR level was seen for the AC/AT haplotype combination (63±21.2 mm/h).

DISCUSSION

SLE is an important chronic autoimmune disease that mainly affects young women. Various environmental and genetic factors contribute to the development of this disease (17). Among the genetic factors those that are related to the immune system play critical roles. FOXP3, the master regulator of Treg cells has been shown to contribute to the development of various autoimmune diseases such as thyroid autoimmune diseases, skin autoimmune diseases, and gastrointestinal autoimmune diseases (8, 9, 11, 13). The *FOXP3* gene have several variants, some of which are functional. In the present study, we have investigated the impact of two *FOXP3* polymorphisms on disease susceptibility and characteristics in a group of Iranian patients with SLE. Up to this point, and to the best of our knowledge, there is solely a report in which the role of *FOXP3* polymorphisms in a Taiwanese population has been studied (18). Lin et al. studied the impact of five polymorphisms including the rs3761548 on genetic predisposition to SLE. They found no association between the rs3761548 and other studied SNPs with disease development, but found a lower quantity of anti-dsDNA in females carrying the rs3761548 A allele.

The *FOXP3* gene is an X-linked gene, thus we have reported the results of this study in male and female patients separately. Moreover, according to the studies it is documented that the number of men affected by SLE is much lower than females (ratio, 1 to 9), therefore, we tried to increase the number of male patients, and to enhance the validity of results we used more samples from healthy men as the control to compare.

We found a significant relationship between the rs3761548 gene variants and risk of SLE so that the frequency of C/- genotype in male patients was greater than that in the healthy men controls. This result was in contrast to the Lin et al. study who did not find any association between the rs3761548 and the risk of SLE (18).

We analyzed the relation of this SNP to demographic, clinical, and paraclinical features of the patients and found that the C carrier patients had longer disease duration and the percentage of arthralgia development was greater in this group of patients. These data suggested that *FOXP3* rs3761548 C/- genotype in addition to having a relationship with disease susceptibility, may have a role in the pathogenesis of SLE.

With respect to rs2294021 in men, no significant association with the risk of SLE was detected, however, similar to the rs3761548, this SNP showed a relationship with the presence of arthralgia in the male patients. We could not really locate any reviews on it in the literature on how the *FOXP3* gene can influence the arthralgia; however, it has been reported that FOXP3 deficiency arthralgia, in addition to the development of several autoimmune diseases such as type 1 diabetes mellitus and thyroid diseases, arthralgia is one of the presenting features of the patients (19). Further research is needed to determine whether FOXP3 gene

variations are involved in this manifestation.

In the females, the frequencies of the rs3761548 and the rs2294021 genotypes and alleles did not differ between the patients and the controls, and thus these SNPs were not associated with the disease development; however, they showed various relationships with disease characteristics of the patients. An interesting finding of this study was the significant association of both SNPs with the history of abortion in the female patients. We found that in women who had an abortion, the frequency of the rs3761548 AA genotype was significantly higher than those without abortion and the frequency of CC genotype was lower in patients with an abortion history. It has been shown that the rs3761548 A allele is associated with decreased FOXP3 expression which could result in decreased Treg cell number or function (20).

As Treg cells have a critical role in maternal tolerance and suppression of immune response against the fetus, their impairment may affect a normal pregnancy (21, 22). In various studies decreased Treg cell development associated with FOXP3, diminished expression in patients with unexplained recurrent spontaneous abortion (URSA) have been reported (23, 24). Regarding the *FOXP3* polymorphism and abortion, Mishra et al. found an association of *FOXP3* 924 A/G and 459T/C but not the rs3761548 with URSA in an Indian population (24). Similarly, Naderi-Mahabady reported no association of the rs3761548 with URSA in 195 Iranian patients (7). In contrast, other investigators found a relationship between the rs3761548 and URSA in Chinese Han and Indian populations (12, 25).

As for the rs2294021 variants, we found that the frequency of the rs2294021 CC genotype was significantly higher in the group of women with a history of abortion than those without abortion. It has been shown that the rs2294021 CC genotype was associated with increased FOXP3 expression (26), thus, the increased Treg cell numbers or function. This result may be contrary to the above finding. To resolve this contradiction, we studied the relationship of various haplotype combinations for the two SNPs with the abortion history in the females. Interestingly, we found a significant association of abortion history with the AC/AC haplotype combination which showed that a combination of A alleles from the rs3761548 and C alleles from the rs2294021 can result in a higher risk of abortion in the patients.

In the female patients, we also found a relationship between decreased C4 level and cardiovascular involvement so that the rs3761548 AC genotype was associated with more decreased C4 level compared with AA genotype, and the rate of cardiovascular disease was lower in patients with AA genotype compared with CC genotype. The rs2294021 CC genotype frequency was less in patients with increased ESR level, and the rs2294021 CT genotype was observed more in patients with neurological involvement and with photosensitivity. These data suggested the relationship of these two SNPs with disease manifestations and pathology.

In the haplotype combination study, significant relationship of various rs3761548 and the rs2294021 combinations with different characteristics of the female patients was detected which included; AC/CT with increased ESR, CT/CT with cardiovascular and neurological involvement, AC/AC with abortion and pulmonary involvement, AC/CT with the age of onset and AC/AT with increased ESR. These data showed that a combination of *FOXP3* gene variants may contribute to disease manifestations in SLE patients.

CONCLUSION

As the results of this study showed, *FOXP3* rs3761548 C/- genotype was associated with the risk of SLE in the male patients. Moreover, this genotype had relationships with longer disease duration and with the presence of arthralgia which suggested its possible role

in the pathogenesis of SLE.

In the female patients, genotypes and alleles of the two SNPs were not associated with the disease development, but the rs3761548 CC haplotype was less seen in the patients. Significant association of both SNPs and the AC/AC haplotype combination with the history of abortion was detected which suggested the influence of *FOXP3* variants on the risk of abortion in SLE patients. The relation of these SNPs with several clinical and paraclinical features of the patients showed the impact of these two SNPs on disease manifestations and pathology.

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