ORIGINAL ARTICLE

Association of Signal Transducer and Activator of Transcription 4 rs10181656 Polymorphism With Rheumatoid Arthritis and Systemic Sclerosis in Khuzestan Province in Southwestern Iran

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ABSTRACT

Objectives: This study aims to investigate the association of polymorphism rs10181656 (C>G) of signal transducer and activator of transcription 4 (STAT4) gene with rheumatoid arthritis (RA) and systemic sclerosis (SSc) in the southwest of Iran as well as the probable relationship between the polymorphism with clinical features and disease activity parameters in both diseases.

Patients and methods: A total of 200 patients (120 with RA [21 males, 99 females; mean age 44.83 years; range, 16 to 75 years] and 80 with SSc [13 males, 67 females; mean age 44.3 years; range, 30 to 75 years]) and 120 healthy controls (25 males, 95 females; mean age 46.93 years; range, 30 to 75 years) were recruited in this study. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism. A set of genotypes was confirmed by sequencing.

Results: A statistically significant association was detected between STAT4 rs10181656 polymorphism and RA (p=0.007). No significant correlation was detected between STAT4 rs10181656 polymorphism and SSc (p=0.357). None of the clinical features (anti-cyclic citrullinated peptide, rheumatoid factor) or disease activity parameters (limited cutaneous SSc, diffuse cutaneous SSc) showed any correlation with the genotype distribution of the STAT4 rs10181656 polymorphism in RA or SSc patients.

Conclusion: Our findings suggest an association between RA susceptibility and STAT4 rs10181656 polymorphism. However, no significant association was found between the mentioned polymorphism and SSc. Clinical features and disease activity parameters did not show any association with the polymorphism.

Keywords: Anti-cyclic citrullinated peptide, rheumatoid arthritis, rheumatoid factor, STAT4, systemic sclerosis.

Autoimmune diseases are a various group of complex diseases caused by loss of immunologic tolerance to self-antigens leading to immune-mediated destruction of tissues and organs. The diseases affect up to about 5% of the world population.[1] The pathogenesis of autoimmune diseases has been widely considered to be multifactorial, with genetics, epigenetics, and environmental factors involved in disease onset and progression.[2] These diseases constitute a group of several complex disorders, such as rheumatoid arthritis (RA), systemic sclerosis (SSc), psoriasis and celiac disease.

Recent studies have shown that many autoimmune diseases share similar immune pathogenesis.[3] RA and SSc are autoimmune diseases characterized by the development of immune response against self-antigens and

inflammatory factors. Although their etiology remains a mystery, RA and SSc share a few similar pathogenic mechanisms.[4]

Rheumatoid arthritis is phenotypically heterogeneous. It is a systemic autoimmune disease characterized by chronic destructive inflammation in synovial joints, leading to progressive joint damage.[5] The disease can be subdivided into two groups (autoantibody positive or autoantibody negative) according to the presence or absence of either rheumatoid factor (RF) or anti-cyclic citrullinated peptide (anti-CCP).[5] RA affects about 0.5-1% of the adult population worldwide and is higher among females than males.[6] The etiology of RA is still unknown, but it is thought to have both genetic and environmental basis.[7]

Systemic sclerosis is a rare multifaceted autoimmune disease. Its main manifestations are immune activation, vasculopathy, and ultimately extensive fibrosis of the skin and internal organs.[8] One of the central events responsible for SSc development is the dysregulation of the immune system. The altered immune response in SSc is marked by an increased T cell activation and the secretion of pro-inflammatory mediators that contribute to the generation of fibrosis and endothelial alterations. Based on the extent of skin involvement, SSc is classified into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc).[8,9] It should be noted that the relative risk of developing SSc remains rather low, 1.6% in families versus 0.026% in the general population.[10]

Until now, shared susceptibility genetic loci have been documented to be associated with multiple autoimmune diseases, including the genetic major histocompatibility complex (MHC) and non-MHC polymorphisms.[11] However, except for MHC alleles associated with nearly all known autoimmune diseases in diverse populations, identifying non-MHC susceptibility genetic variants has remained to be challenging. Among the second group, the association between the Arg620→Trp variant of the PTPN22 gene and RA has already been documented in several studies.[12]

The signal transducer and activator of transcription 4 (STAT4) maps to chromosome 2q33. This gene produces a transcription factor which is involved in intracellular signals activated by some cytokines such as interleukin (IL)-12,

IL-23, IL-27 and type I interferons. The gene is expressed in different cells such as dendritic cells, activated peripheral blood monocytes, and macrophages at the sites of inflammation.[12,13]

Cytosolic STAT4 can be phosphorylated to form a homodimer which translocates to the nucleus.[14,15] STAT4 has a deoxyribonucleic acid (DNA)-binding domain and specifically binds to several DNA sequences among the promoter regions of cytokine genes as well as receptors and signal factors.[16]

To date, four polymorphisms in the third intron of STAT4 gene (rs11889341, rs7574865, rs8179673, and rs10181656) have been found to be associated with increased risk of diverse complex autoimmune diseases even in different ethnic populations. The diseases include SSc,[17] RA, and Sjögren's syndrome.[18] Studies suggest that STAT4 may be an "autoimmune disease susceptibility gene" and support the concept of common dysregulated pathways across multiple autoimmune diseases.

To our knowledge, this single nucleotide polymorphism (SNP) has not been previously tested in the Khuzestan province in Southwestern Iran. Thus this population study can be of great importance. Therefore, in this study, we aimed to investigate the association of polymorphism rs10181656 (C>G) of STAT4 gene with RA and SSc in the southwest of Iran as well as the probable relationship between the polymorphism with clinical features and disease activity parameters in both diseases.

PATIENTS AND METHODS

This study was conducted at Shahid Chamran University of Ahvaz between August 2017 and August 2018. The study included 200 patients (120 with RA [21 males, 99 females; mean age 44.83 years; range, 16 to 75 years] and 80 with SSc [13 males, 67 females; mean age 44.3 years; range, 30 to 75 years]) and 120 healthy controls (25 males, 95 females; mean age 46.93 years; range, 30 to 75 years). Participants of the study were matched by geographic region, age and sex. The population was composed of a case-control set from Khuzestan province in the Southwest of Iran including Arab and non-Arab populations.

The study protocol was approved by the Shahid Chamran University of Ahvaz Ethics Committee. A written informed consent was obtained from each participant. The study was conducted in accordance with the principles of the Declaration of Helsinki.

The RA and SSc patients were classified according to the American College of Rheumatology (ACR) criteria. Detailed clinical and laboratory information was provided from the patients of the Rheumatology Clinic at Golstan Hospital in Ahvaz, Iran. A questionnaire was supplied regarding parameters such as ethnicity, sex, positive family history and age. Clinical parameters were estimated using the commercially available enzyme linked immunosorbent assay kits for anti-CCP and RF. RF measured to be >20 IU/mL was considered as RF positive. The presence of anti-CCP was determined and a cut-off point of >5 U/mL was used as a stringent criterion for anti-CCP positivity.

Clinical symptoms of SSc were diagnosed based on the ACR criteria by a specialist. Preliminary criteria for disease classification included having at least three of the five features of the CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias). SSc patients were further classified based on diffuse or limited skin involvement and by autoantibody status. A questionnaire was supplied for the SSc patients, too.

Subjects of the control group were selected from the Golstan Hospital. None of the subjects had any family history of autoimmune diseases.

They were recruited from the same geographic region to be genotyped and compared with the patients in the designed study. A questionnaire was filled for each subject.

Whole blood was collected in ethylenediaminetetraacetic acid containing tubes. Genomic DNA was isolated using standard salting-out method. The quality and quantity of genomic DNA were determined by agarose gel electrophoresis and ultraviolet (UV) spectrophotometer measuring absorption at 260 and 280 nm wavelengths. DNA was stored at -20°C until analysis. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. A set of genotypes was confirmed by direct DNA sequencing. The upstream primer 5'-AGTTTTCAAAGTCTAACACTGTG-3' and downstream primer 5'-TACTCTCGACATGGCAGC-3' were used to amplify the region of interest (TAG Copenhagen, Frederiksberg, Denmark).

The PCR was carried out in a total volume of 25 μL containing 100-200 ng of genomic DNA, 12.5 μL mastermix, 1 μL of each primer (10 μM), 2 μL deoxynucleotide triphosphate (5 mM) and 8.5 μL water. Amplification was performed with a denaturation step at 95°C for three minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 45 seconds and extension at 72°C for 45 seconds. The final extension step at 72°C was performed for five minutes. Three to five μL of PCR products were run on 1.5%

Table 1. Distribution of STAT4 rs10181656 polymorphism frequency in RA patients and controls

	RA patients (n=120)		Controls (n=120)				
	n	%	n	%	*p*	OR	95% CI
Genotype							
CC	74	56.5	57	43.5	-	-	-
CG	32	36.8	55	63.2	0.005	0.448	0.257-0.781
GG	14	63.6	8	36.4	0.531	1.348	0.529-3.433
Allele							
C	180	51.6	169	48.4	-	1	-
G	60	45.8	71	54.2	0.260	1.261	0.842 -1.886

STAT4: Signal transducer and activator of transcription 4; RA: Rheumatoid arthritis; OR: Odds ratio; CI: Confidence interval. Values are presented as frequency in percentage and number of genotypes or alleles. Frequency comparison between groups was analyzed by Chi-square test. Statistical significance was set at p<0.05.

Table 2. Distribution of STAT4 rs10181656 polymorphism frequency in SSc patients and controls

	SSc patients (n=120)		Controls (n=120)				
	n	%	n	%	*p*	OR	95% CI
Genotype							
CC	37	46.3	57	47.5	-	-	-
CG	33	41.3	55	45.8	0.796	0.924	0.508 -1.681
GG	10	12.5	8	6.7	0.207	1.926	0.696 -5.328
Allele							-
C	107	66.9	169	70.4	-	1	-
G	55	33.1	71	29.6	0.435	0.848	0.551-1.304

STAT4: Signal transducer and activator of transcription 4; SSc: Systemic sclerosis; OR: Odds ratio; CI: Confidence interval. Values are presented as frequency in percentage, number of genotypes or alleles data. Frequency comparison between groups was analyzed by chi-square test. Statistical significance was set at p<0.05.

agarose gel to observe the target band before performing the restriction digestion reaction. Digestion was performed in a total volume of 31 μL, containing 10 μL of PCR products, 1 unit of DdeI enzyme (10 Units/μL, Thermo Fisher Scientific Inc., Waltham, Massachusetts, ABD), 2 μL 10× enzyme buffer and 10 μL of nuclease-free water. Digestion reaction was incubated at 37°C overnight. Digested products were analyzed by running on 3% agarose gel next to ultra-low range DNA ladder (Fermentas, Hennigsdorf, Germany), stained by ethidium bromide, visualized under UV light and photographed. Direct sequencing of PCR products was performed by Macrogen Inc. (Seoul, South Korea).

Statistical analysis

Hardy-Weinberg equilibrium was tested using the Hardy-Weinberg package (http://CRAN. Rproject.org/package=HardyWeinberg). For

Table 3. Demographic and clinical characteristics of RA and SSc patients

	RA patients (n=120)			SSc patients (n=80)			Controls (n=120)		
	n	%	Mean±SD	n	%	Mean±SD	n	%	Mean±SD
Demographic characteristics									
Age (year)			44.8±11.9			44.3±11.3			46.9±11.9
Sex									
Females	99	82.5		67	63.7		95	79.2	
Males	21	17.5		13	16.3		25	20.8	
Clinical characteristics									
Disease duration (year)			7.2±6.3						
Anti-CCP Ab positive	79	66							
Total patients	120								
Anti-CCP Ab negative	41	34							
Total patients	120								
RF positive	42	35							
Total patients	120								
RF negative	78	65							
Total patients	120								
lcSSc (lcSSc/total patients)				31	38.75				
				80					
dcSSc (lcSSc/total patients)				49	61.25				
				80					

RA: Rheumatoid arthritis; SSc: Systemic sclerosis; SD: Standard deviation; Anti-CCP: Anti-cyclic citrullinated peptide; Ab: Antibody; RF: Rheumatoid factor; lcSSc: Limited cutaneous systemic sclerosis; dcSSc: Diffuse cutaneous systemic sclerosis.
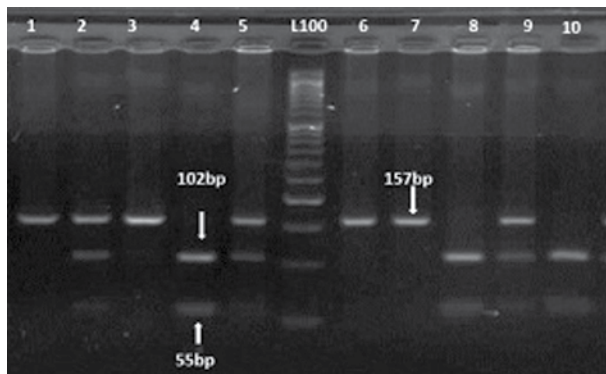
Figure 1. Restriction fragment length polymorphism analysis including rs10181656 column L100 is 100 bp marker, columns 4, 8 and 10 are GG wild homozygote samples shown at 102 bp and 55 bp, columns 2, 5 and 9 are GC heterozygote samples and column 1, 3, 6 and 7 are CC mutated homozygote genotypes that appear at 157 bp.

genotypic and allelic frequencies, Chi-square test and Spearman's correlation test were used (the PASW version 18.0, SPSS Inc., Chicago, IL, USA). Results were given as mean values, standard deviation and minimum and maximum scores. Odds ratio (OR) and 95% confidence intervals (CIs) were also calculated. In each test, a p value of less than 0.05 was considered statistically significant.

RESULTS

The genotypic and allelic frequencies of the rs10181656 STAT4 gene polymorphism in RA, SSc patients and controls are shown in Tables 1 and 2. Genotype frequency for the STAT4 rs10181656 polymorphism was in Hardy-Weinberg equilibrium in SSc patients and control cohorts. Our results showed a statistically significant association between STAT4 rs10181656 polymorphism and RA (p=0.007). Moreover, no significant difference in allele frequency was detected between RA patients and control group (p=0.260). The comparison between genotypes showed that only CG genotype of STAT4 rs10181656 polymorphism had a significant association with RA (p=0.005); however, our findings suggest that this genotype decreases the risk of susceptibility to RA (OR=0.448, 95% CI=0.257-0.781). Our results showed no more association of the STAT4 rs10181656 polymorphism with SSc in this population (p=0.357). Also, none of the GG, GC, CC genotypes or G or C alleles showed any significant differences between the lcSSc and dcSSc patient groups.

The demographic and clinical data of the RA patients are shown in Table 3. Agarose gel electrophoresis regards to RFLP is shown in Figure 1. There was no significant genotypic association with age, race and sex (p>0.05) (data not shown). Of the 120 RA patients tested for anti-CCP antibodies, 45.8% were positive and 54.2% were negative for the antibody. Also, 32.5% of RA patients were positive and 67.5% were negative for RF. The RA patients were classified according to anti-CCP status and no statistically significant difference was observed in genotype distributions (p=0.873) in

Table 4. Comparison of STAT4 rs10181656 genotypes in RA patients stratified according to anti-CCP antibody

	Anti-CCP antibody positive		Anti-CCP antibody negative				
	n	%	n	%	p	OR	95% CI
Genotype							
CC	50	67.6	24	32.4	-	-	-
CG	20	62.5	12	37.5	0.613	0.80	0.337 -1.901
GG	9	64.3	5	35.7	0.811	0.864	0.261 -2.859
Allele							
C	120	66.7	60	33.3	-	1	-
G	38	63.3	22	36.7	0.637	1.158	0.629 -2.130

STAT4: Signal transducer and activator of transcription 4; RA: Rheumatoid arthritis; Anti-CCP: Anti-cyclic citrullinated peptide; OR: Odds ratio; CI: Confidence interval. Referred as subgroups of rheumatoid arthritis patients.

Table 5. Comparison of STAT4 rs10181656 genotypes in RA patients stratified according to RF antibody

	RF antibody positive		RF antibody negative				
	n	%	n	%	p	OR	95% CI
Genotype							
CC	31	41.9	43	58.1	-	-	-
CG	8	25.0	24	75.0	0.102	0.462	0.184-1.165
GG	3	21.4	11	78.6	0.160	0.378	0.097 -1.470
Allele							
C	70	38.9	110	61.1	-	1	-
G	14	23.3	46	76.7	0.031	0.478	0.244-0.933

STAT4: Signal transducer and activator of transcription 4; RA: Rheumatoid arthritis; RF: Rheumatoid factor; OR: Odds ratio; CI: Confidence interval. Referred as subgroups of rheumatoid arthritis patients.

positive patients compared with negative patients (Table 4). The RA patients were stratified into RF positive and negative groups and were analyzed in regard to their genotypes. No correlation was found except for G allele (p=0.031, OR=0.478, 95% CI=0.244-0.933) (Table 5). Comparison of STAT4 rs7574865 genotypes in RA patients stratified with ACCP+/RF-, ACCP-/RF+ and ACCP+/RF+ groups did not show any statistically significant association (Table 6).

When the SSc patients were divided into lcSSc and dcSSc patient groups, no statistically significant association was found between the two groups (p=0.694) (Table 7).

Table 6. Comparison of STAT4 rs10181656 genotypes in RA patients stratified according to RF antibody

	ACCP+/RF-		ACCP-/RF+		ACCP+/RF+		ACCP+/RF-		ACCP-/RF+		ACCP+/RF+	
	n	%	n	%	n	%	p	OR	p	OR	p	OR
Genotype												
CC	24	45.28	4	7.55	26	47.17	-	-	-	-	-	-
CG	14	61.92	3	9.5	6	28.58	0.76	1.17	0.60	1.52	0.19	0.46
GG	6	66.67	0	0	3	33.33	0.75	1.25	0.67	0.51	0.50	0.58

STAT4: Signal transducer and activator of transcription 4; RA: Rheumatoid arthritis; RF: Rheumatoid factor; ACCP: Anti-cyclic citrullinated peptide; RF: Rheumatoid factor; OR: Odds ratio; Referred as subgroups of rheumatoid arthritis patients.

Table 7. Comparison of STAT4 rs10181656 genotypes in SSc patients stratified into lcSSc and dcSSc types

	lcSSc		dcSSc				
	n	%	n	%	p	OR	95% CI
Genotype							
CC	18	58.1	19	38.8	-	-	-
CG	11	35.5	22	44.9	0.196	1.895	0.719 -4.994
GG	2	6.5	8	16.3	0.120	3.789	0.707 -20.297
Allele							
C	47	75.8	60	61.2	-	1	-
G	15	24.2	38	38.8	0.058	0.504	0.247-1.02

STAT4: Signal transducer and activator of transcription 4; SSc: Systemic sclerosis; lcSSc: Limited cutaneous systemic sclerosis; dcSSc: Diffuse cutaneous systemic sclerosis; OR: Odds ratio; CI: Confidence interval. Referred as subgroups of systemic sclerosis patients.

DISCUSSION

One of the main reasons for the development of RA and SSc is the dysregulation of the immune system. The altered immune response in these two diseases is marked by an increased T cell activation and the secretion of pro-inflammatory mediators. Several mechanisms regulate T cell activation differentiation, one of the more important of the mechanisms being the specific activation of gene transcription after cytokine stimulation.[19] Signal transducers and activators of transcription (STAT) are a family of transcription factors that have been demonstrated to exert a fundamental role in driving T cell differentiation and signaling.[20] Among the six described STAT proteins, STAT4 is implicated in the differentiation of type 1 and 17 helper T cells, two of the T cell subsets that are implicated in RA and SSc pathogenesis.[9]

Third intron of the STAT4 encompasses a few polymorphisms (rs18889341, rs7574865, rs8179673, and rs10181656) which have been reported to be associated with RA and SSc in some populations worldwide.[21-25] However, this association remains to be challenging based on some studies conducted on the Southwestern Chinese Han, Turkish, African American and Southwestern Iran populations.[26-28]

Persistent and strong association between the STAT4 polymorphisms and RA and SSc in multiple independent and different ethnic populations suggested[25] an important role of STAT4 gene in pathogenesis of SSc and RA in European and Asian populations and is considered a risk factor for both diseases.

In the current study, we confirmed the genetic association of STAT4 rs10181656 polymorphism with RA which was consistent with the previous reports[28] in other ethnic populations. Comparison of genotypes showed that only CG genotype of STAT4 rs10181656 polymorphism had a statistically significant association with RA (p=0.005, OR=0.448, 95% CI=0.257-0.781). We also did not find any statistically significant difference in allele frequency between RA patients and control group. Although contribution of the variant has been replicated, contradictory results have been reported (decreased G allele in Khuzestan province population and increased G allele in other populations). Due to the

lack of power to detect OR below 1.5, the absence of an association may more likely be suggested. Also, the results indicated that rs10181656 polymorphism in the STAT4 gene was not associated with susceptibility to SSc in the Khuzestan province population. Therefore, the rs10181656 polymorphism is unlikely to be an influential variant related with susceptibility to RA and SSc in this population. RA and SSc are complex disorders and several factors would complicate their molecular genetics. Thus, the conflict between our finding and studies on some other populations might be due to the genetic background of the studied groups, small sample sizes, or an inadequate definition of the phenotypes. Furthermore, it should be noted that different environmental factors may have an effect on the development of diseases. Consequently, STAT4 gene may have different degrees of genetic risk among ethnic groups under different environmental exposure.

Although it seems that STAT4 rs10181656 polymorphism plays an effective role in several pathways involved in RA and SSc pathogenesis, the functional role of this polymorphism needs to be discovered. Due to its position in intron of STAT4 gene, it may be hypothesized that the SNP may be involved in the regulation of the splicing process. However, it is known that there are many ways by which introns can affect gene expression. Due to different interconnections between the various complexes involved in splicing and other post-transcriptional regulations, there are multiple opportunities for introns to affect expression by different mechanisms. For example, the presence of introns can increase the levels of mature messenger ribonucleic acid in the cytosol by impacting on the rate of transcription, nuclear export, and transcript stability. Also, intron-mediated enhancement is a phenomenon in which introns can elevate expression when present in the transcribed region.[29-31] The mechanisms which are involved in this process of eukaryotic gene expression are not fully understood.

Our study has some limitations. Firstly, the small sample size may have limited the statistical power to detect any existing association. Thus, a larger number of patients and control subjects could be used to verify the results. Secondly, additional polymorphisms exist in the STAT4

gene that might contribute to the susceptibility or activity of RA and SSc in this population.

In conclusion, our findings suggest an association between RA susceptibility and STAT4 rs10181656 polymorphism. However, no significant association was found between this polymorphism and SSc. Moreover, clinical features and disease activity parameters did not show any association with the polymorphism. Further studies can reveal the functional and molecular role of this polymorphism as well as its association with inflammatory diseases.[32]

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