

ORIGINAL ARTICLE

# No evidence of association between *CTLA-4* polymorphisms and systemic lupus erythematosus in Iranian patients

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## Abstract

**Aim:** Cytotoxic T lymphocyte-associated antigen-4 (*CTLA-4*) is an important negative regulator of T-cell responses. *CTLA-4* polymorphisms have been confirmed to be associated with several autoimmune diseases such as systemic lupus erythematosus (SLE). We analyzed the role of *CTLA-4* polymorphism at positions –1661 and –1722 in Iranian patients suffering from SLE.

**Methods:** One hundred and eighty SLE patients and 304 ethnically and age-matched healthy controls were studied. Polymerase chain reaction restriction fragments length polymorphism (PCR-RFLP) was used to analyze the genotype and allele frequencies of these polymorphisms.

**Results:** There was no significant association between the studied genotypic and allelic frequencies between SLE patients and the controls. Although the TC genotype in 1722TC polymorphism was more common among the control group, the correlation was not statistically significant.

**Conclusion:** Our results suggest that the –1661AG and –1722TC polymorphisms in the promoter region of the *CTLA-4* gene does not play any role in genetic susceptibility to SLE. However, further studies on larger sample sizes are needed to approve our results.

**Key words:** *CTLA-4*, polymorphism, promoter, systemic lupus erythematosus.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex inflammatory disease characterized by autoantibody production.<sup>1</sup> The disease is more common in women but found in different racial and ethnic groups. It is more frequently reported in individuals in the second,

third or fourth decades of life.<sup>2</sup> SLE afflicts more than 1 million individuals in the US and 3.2–14.1 cases per 100 000 in women of European descent.<sup>3,4</sup> The disease is reported in 40 per 100 000 of the Iranian population.<sup>5</sup> The etiology of the disease is unknown but is thought to be caused by both genetic and environmental factors.<sup>6</sup> The expression of Cytotoxic T lymphocyte-associated antigen-4 (*CTLA-4*) is increased in patients with active SLE.<sup>7</sup> *CTLA-4* is an important negative regulator of T-cell responses, and its dysregulation has the potential to affect the pathogenesis of SLE by altered activation of T-cells to self-antigens.<sup>1</sup> Inappropriate T-cell-dependent

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expansion of autoreactive B cells is considered to play a role in the production of pathogenic autoantibodies<sup>8</sup> in multiple organs, including kidneys, heart, lung, joints and immune system.<sup>9</sup> The *CTLA-4* gene is located within the risk region on chromosome 2q33 and several polymorphisms have been reported in this gene,<sup>10</sup> *CTLA-4* polymorphisms have been confirmed to be associated with several autoimmune diseases, such as Graves' disease, type I diabetes, celiac disease, autoimmune thyroid disease, rheumatoid arthritis and multiple sclerosis and SLE.<sup>11</sup> Two of these polymorphisms are located within the promoter region: A/G transition at position -1661<sup>12-14</sup> and T/C transition at position -1722.<sup>13,15</sup> Using a case-control study design, we have determined the role of *CTLA-4* gene polymorphisms in the promoter regions in SLE pathogenesis.

## MATERIALS AND METHODS

### Patients

One hundred and eighty SLE patients (15 males and 165 females) with a mean age of  $10.45 \pm 32.99$  years (range 13–70 years) were enrolled in the study. Three hundred and four ethnically and age-matched healthy controls (23 males and 281 women) with no history of any autoimmune diseases were also recruited from the Azar Fifth Teaching Hospital affiliated to Gorgan University of Medical Sciences, Gorgan, Iran. All the SLE patients fulfilled the American College of Rheumatology 1997 revised criteria for SLE.<sup>16</sup> The study was approved by the local ethics committee and a written informed consent was obtained from each patient.

### DNA extraction and genotyping

The DNA from the patients and controls were extracted from their peripheral blood with a DNA extraction kit (Roche Applied Science, Penzberg, Germany) according to the standard protocol from the manufacturer. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyze the polymorphisms at positions -1661 and -1722. Promoter-region polymorphisms were genotyped as follows using a single primer set: forward 5'-CTAAGAGCATCCGCTGCACCT-3' and reverse 5'-TTGGTGTGATGCACAGAAGCCTTT-3'. Amplification was carried out after initial denaturation at 94°C (5 min), followed by 30 cycles at 94°C (15 s), 58°C (30 s), 72°C (45 s), and a final extension at 72°C (7 min). The PCR products were digested using restriction enzyme MseI or BbvI (New England BioLabs, Hitchin, UK) at 37°C for 4 h and then were analyzed on 2% agarose gel using

ethidium bromide staining. The amplified DNA for 1722TC was 486 bp fragment (T allele) or two fragments of 270 and 216 bp (C allele). The 1661AG polymorphism was determined by detecting a 486 bp fragment (G allele) or two fragments of 347 and 139 bp (A allele).

### Statistical analysis

The frequency of alleles and genotypes were assessed using direct counting. Chi-square and Fisher's exact test were used to compare the distributions and association of alleles and genotypes in the patients and the controls. *P*-values < 0.05 were considered statistically significant. The strength of the association between different groups and alleles or genotypes of polymorphism was estimated using odds ratios (OR) and 95% confidence intervals (CI). Statistical analysis was conducted with STATA (v8) software (StataCorp., College Station, TX, USA).

### Linkage disequilibrium and haplotype analysis

Estimated haplotype frequencies and testing for linkage disequilibrium between pairs of polymorphisms in the cases and controls were calculated using the EHPLUS program,<sup>17</sup> which provides log likelihood, chi-square and the number of degrees of freedom. To test for heterogeneity in haplotype frequencies between the cases and controls, the likelihood ratio test was used.

## RESULTS

Blood samples from 180 SLE patients and 304 controls were successfully genotyped for the -1661AG and -1722TC in the promoter regions of the *CTLA-4* gene. Genotype and allele frequencies of the -1661AG and -1722TC polymorphisms are shown in Tables 1 and 2, respectively. There was no statistically significant difference in the genotype and allele frequencies at the -1661 and -1722 sites between SLE patients and healthy controls. There was no significant linkage disequilibrium (LD) between the markers both in cases and controls (*P* > 0.05) in our population. The estimated haplotype frequencies in cases and controls are given in Table 3. No significant differences for estimated haplotype frequencies were found between cases and controls.

## DISCUSSION

Although the definite etiopathogenesis of SLE remains unclear, evidence indicates that *CTLA-4* polymorphisms

**Table 1** Genotypic distribution and allelic frequencies of –1661 AG *CTLA-4* polymorphisms in Iranian SLE patients and healthy controls

Promoter –1661	SLE (%) <i>n</i> = 180	Control (%) <i>n</i> = 304	<i>P</i> -value	OR (95% CI)
Genotype				
AA	66 (36.7)	112 (36.8)	0.49	0.97 (0.66–1.43)
AG	59 (32.8)	103 (33.9)	0.42	0.94 (0.63–1.39)
GG	55 (30.6)	89 (29.3)	0.49	1.02 (0.68–1.53)
Allele				
A	190 (52.8)	327 (53.8)	0.32	0.89 (0.59–1.32)
G	170 (47.2)	281 (46.2)	0.5	0.98 (0.67–1.44)

**Table 2** Genotypic distribution and allelic frequencies of –1722 TC *CTLA-4* polymorphisms in Iranian SLE patients and healthy controls

Promoter –1722	SLE (%) <i>n</i> = 180	Control (%) <i>n</i> = 304	<i>P</i> -value	OR (95% CI)
Genotype				
TT	160 (88.9)	270 (88.8)	0.55	1 (0.56–1.81)
TC	13 (7.2)	21 (6.9)	0.46	0.9 (0.45–1.83)
CC	7 (3.9)	13 (4.3)	0.51	0.9 (0.35–2.31)
Allele				
T	333 (92.5)	561 (92.3)	0.59	1.01 (0.39–2.62)
C	27 (7.5)	47 (7.7)	0.52	0.96 (0.53–1.76)

**Table 3** Estimated haplotype frequencies of *CTLA* polymorphisms in patients and controls

Allele at locus 1	Allele at locus 2	Haplotype frequency	
		Cases	Control
1	1	0.485022	0.499411
1	2	0.040401	0.038418
2	1	0.444356	0.426576
2	2	0.030220	0.035595

play an important role in susceptibility to SLE. Some studies have reported a significant correlation between *CTLA-4* polymorphisms and SLE.<sup>12,13,15,18,19</sup> On the other hand, several studies have failed to show any association between SLE and *CTLA-4* polymorphisms.<sup>14,20–23</sup> In this study no association was observed between *CTLA-4* polymorphisms and SLE in the Iranian population. The results demonstrated no difference between the genotype and allele frequencies for the –1661 site between patients and controls. In line with our results, studies conducted in China,<sup>19</sup> Korea,<sup>13</sup> Japan,<sup>14</sup> Malaysia<sup>21</sup> and the US<sup>20</sup> did not find a positive correlation between the polymorphism and

SLE. Our results and that of previous studies indicate that ethnicity and geography do not affect the 1661AG polymorphism as different populations showed similar results on the test. Other polymorphisms such 1722TC were also not associated with SLE. This is compatible with the results of other reports.<sup>14,20–22</sup>

Our study showed a insignificantly higher frequency for the TC genotype among the controls. Khalaf *et al.* reported a strong association between 1722TC polymorphism and SLE.<sup>12</sup> An-ping *et al.* also studied the 1722TC polymorphism and showed significantly higher presence of TC genotype in SLE patients.<sup>19</sup> On the contrary, corroborating with our results, Hudson *et al.* reported that the TC genotype was more frequently reported among the healthy controls, adding that the correlation was statistically significant.<sup>13</sup>

The LD analysis performed on –1661AG and –1722TC polymorphisms revealed no differences between the cases and controls in our study. In association with 1722TC polymorphism, our results indicate that our population might be different from other Asian people such as the Chinese and the Koreans, as the results were inconsistent across different ethnic populations.<sup>12,13,18,19</sup> While several studies have reported

–1661AG and –1722TC polymorphisms to be more common in SLE patients, this is the first study to show the association among Iranians. Hence, further studies on larger sample sizes are needed to approve our results.

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