

CTLA-4 gene polymorphism of exon 1(+49 A/G) in Turkish systemic lupus erythematosus patients

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Summary

Cytotoxic T lymphocyte-associated antigen-4 is a cell-surface molecule providing a negative signal for T cell activation. CTLA-4 gene polymorphisms are known to be related with genetic susceptibility to various autoimmune diseases, including systemic lupus erythematosus (SLE). However, the effects of this polymorphism on clinical features of SLE have not been defined. We analysed the CTLA-4 gene +49 A/G polymorphisms in patients with SLE by using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and investigated the effect of polymorphisms on clinical outcomes. Blood was collected from 47 unrelated Turkish SLE patients, all fulfilling the American College of Rheumatology criteria for SLE, and 100 ethnically matched healthy volunteers. The AA genotype was a predominant genotype in the Turkish population and genotype frequencies of CTLA-4 AA were significantly higher in SLE patients (70%) than healthy controls (47%) ($P = 0.015$). There was a statistically significant difference in the AA genotype [odds ratio (OR): 2.66, confidence interval (CI) 95%: 1.27–5.56, $P = 0.014$] distribution among patients and controls. There was also an increase in A allele frequency in SLE and controls, but the difference was not statistically significant (81% vs. 70%, $P = 0.068$, OR = 1.8, CI 95%: 0.99–3.28). Interestingly, mean age and mean age of onset disease was higher in AA homozygote SLE patients compared to non-AA (39.2 ± 11.5 vs. 31.6 ± 10.6, $P = 0.044$; 32.38 vs. 24.31, $P = 0.046$, respectively). There was no association between genotype and the other clinical features of SLE. Our results suggested that CTLA-4 +49 AA genotype

might be a risk factor for the development of SLE in Turkish population and G allele might be involved in early development of SLE. No association with clinical features was found for polymorphism of the promoter region in CTLA-4 +49.

Introduction

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease with frequent exacerbations and remissions. The etiology is not known yet and the pathogenesis is complex, involving immunological, genetic, hormonal and environmental factors (Mills, 1994). Immune complex formations are considered to be responsible for tissue damage in SLE (Deshmukh *et al.*, 2006). Experimental animal models and genetic investigations helped to know more about immunopathogenesis of the disease over the last 40 years.

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a costimulatory molecule expressed on T lymphocytes and belonging to the immunoglobulin (Ig) superfamily. It plays two roles in immune regulation: (i) the transduction of negative control to activated T cells, (ii) the activation of suppressive function of regulatory T cells (Bluestone, 1997). CTLA-4 is important in self-tolerance and development of autoimmune diseases (Salomon & Bluestone, 2001). Expression of CTLA-4 increases in patients with active SLE and increase of CTLA-4 expression implies a fundamental role in the pathogenesis of SLE (Lee *et al.*, 2006).

The CTLA-4 gene is located on chromosome 2q33 (Harper *et al.*, 1991). CTLA-4 gene polymorphism has been shown to affect the inhibitory function of CTLA-4. The G allele at position 49 of the CTLA-4 gene has been found to be associated with the impaired control of T cell proliferation and it has been proposed that the G allele might contribute to the pathogenesis of autoimmune diseases (Kristiansen *et al.*, 2000; Ueda *et al.*, 2003). CTLA-4 gene polymorphisms have been shown to be associated with Graves' disease (Kouki *et al.*, 2000), type 1 diabetes mellitus (Nistiko *et al.*, 1996), rheumatoid arthritis (Cornelis *et al.*, 1998; Jawaheer *et al.*, 2003; Osorio *et al.*, 2004), Behcet disease (Sallakci *et al.*, 2005; Gunesacar *et al.*, 2007). Several reports demonstrate an association between variants of the CTLA-4 and SLE in different ethnical populations (Pullmann *et al.*,

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1999; Ahmed *et al.*, 2001; Lee *et al.*, 2001; Hudson *et al.*, 2002; Barreto *et al.*, 2004; Fernandez-Blanco *et al.*, 2004). Besides, there are also reports which do not support the association of CTLA-4 with SLE in the literature (Heward *et al.*, 1999; Matsushita *et al.*, 1999; D'Alfonso *et al.*, 2000; Liu *et al.*, 2001; Aguilar *et al.*, 2003; Takeuchi *et al.*, 2003). A meta-analysis including these studies found a significant association between SLE and the GG genotype of exon-1 +49 in Asians, but not in Europeans (Lee *et al.*, 2005). Recently, a case report showed that disorders of the CTLA-4 gene, especially a GG genotype in exon 1 at +49, might be involved in early development of SLE in Japanese children (Sugimoto *et al.*, 2008). This genetic polymorphism may partly influence disease expression or severity in SLE. Hence, our aims in this study were (i) to examine the frequency of the CTLA-4 gene +49 A/G polymorphism in Turkish SLE patients compared with controls and, (ii) to investigate whether there is any association between polymorphisms and the clinical features of SLE.

Material and methods

A total of 47 patients (40 females, 7 males: mean \pm SD age: 36.9 ± 11.98 years), who have at least four American College of Rheumatology criteria for SLE (Tan *et al.*, 1982) and 100 ethnically matched healthy controls (51 females, 49 males: mean \pm SD age: 32.9 ± 2.83 years) were enrolled in the study. The control group had neither family history nor symptoms related to SLE. The study was approved by the institutional review board and written informed consent was taken from all patients. The study was in compliance with the Helsinki declaration.

Most of the patients were using oral corticosteroids (4–8 mg/day). Also some patients were being treated with other immunosuppressive therapies such as azothioprine or mycophenolate mofetil. The mean \pm SD duration of the disease was 6.76 ± 4.20 years and mean age of onset disease was 29.82 years. Disease activity was evaluated with the SLE Disease Activity Index (SLEDAI) score (Bombardier *et al.*, 1992). Patients with SLEDAI = 4 were considered as active and SLEDAI < 4 were accepted to have inactive disease. In addition, we noted the clinical features of patients. Patients with hepatitis C, AIDS, sarcoidosis, anticholinergic drug using, graft vs. host disease, lymphoma and patients getting anticholinergic drugs were not included in the study.

Genotyping

CTLA-4 gene polymorphism in exon 1 position 49 (codon 17) was detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Genomic DNA was extracted from peripheral mononuclear cells from each subject with a DNA extraction kit (Fermentas, Hanover, MD, USA). PCR–RFLP method was defined using specific primers forward 5'-GCTCTACTTCCTGAAGACCT-3' and reverse 5'-AGTCTCACTCACCTTTGCAG-3' as described by Harper *et al.* (1991).

PCR was performed with 100–500 ng genomic DNA, 2 U *Taq* polymerase (Fermentas), 20 pmol of each primer, 0.2 mM each of dNTP under following conditions: initial denaturation for 4 min at 95 °C, denaturation for 60 s at 95 °C, annealing for 60 s at 55 °C, extension for 60 s at 72 °C (30 cycles), and a final extension for 4 min at 72 °C in a PerkinElmer thermocycler (Gene Amp PCR System 2400). The amplified DNA was 162 bp.

Restriction fragment length polymorphism analysis of CTLA-4

The amplified products were digested with restriction enzyme Bbv1 (New England BioLabs, Beverly, MA, USA), which cleaved the sequence if a G allele was present at position 49, resulting in 88/74-bp fragments. A sequence with an A allele at position 49 was not digested and resulted in a 162-bp fragment. PCR product was digested in a final volume of 20 μ L with 6 U BbvI enzyme at 65 °C over 3 h. The resulting digestion products were then visualized on 3% agarose gels stained with ethidium bromide.

Statistical analysis

Statistical analysis was conducted with SPSS software (version 11.0, SPSS, Chicago, IL, USA). Allele and genotype frequencies were calculated by direct counting. A significant association was determined by chi-squared test. Hardy–Weinberg equilibrium was tested by the chi-squared test for goodness-of-fit. Odds ratios (OR) together with their 95% confidence intervals (CI) were used to assess the strength of association between the CTLA-4 exon-1 +49A/G polymorphism and SLE. *P* values were corrected with Bonferroni correction for multiple comparisons. We calculated the power of the study for 47 SLE patients and 100 healthy controls. The result illustrated that the power was 0.71. Chi-squared or Mann–Whitney *U*-test, which was appropriated, were used to detect the effect on clinical parameters. A *P* value less than 0.05 was considered statistically significant.

Results

Thirty-three of the 47 patients were AA homozygotes, 10 patients were AG heterozygotes, 4 patients were GG homozygotes. The CTLA-4 +49 AA genotype was a predominant genotype of the CTLA-4 exon 1 polymorphism in both SLE patients and healthy controls (70%, 47%, respectively). CTLA-4 +49 AA genotype frequency was found to be higher in SLE patients (70%) compared to healthy controls (47%) and this difference was statistically significant (*P* = 0.015). The CTLA-4 +49 GG genotype frequency was low in both SLE patients and healthy controls (8.5%, 7%, respectively). SLE patients and the control group were divided in to two groups as AA and non-AA genotypes. The AA genotype was higher than non-AA genotype in SLE patients (*P* = 0.014). There was no significant difference in the frequency of A allele and G allele between groups (*P* = 0.068). OR of AA genotype

Table 1. Comparison of genotype and allele distributions of in SLE patients and controls

	AA	AG	GG	AA	non-AA	Allels	A	G
SLE (n, %)	33 (70)	10 (21.5)	4 (8.5)	33 (70)	14 (30)		76 (81)	18 (19)
Control (n, %)	47 (47)	46 (46)	7 (7)	47 (47)	53 (53)		140 (70)	60 (30)
	$P = 0.015$			$P = 0.014$		$P = 0.068$		
				OR: 2.66		OR: 1.8		
				(95% CI: 1.27–5.56)		(95% CI: 0.99–3.28)		

OR: Odds ratio, CI: Confidence interval.

Table 2. The clinical features of SLE patients and the association between CTLA-4 +49 genotype and clinical findings

	Overall (n = 47)	AA (%) (n = 33)	non-AA (%) (n = 14)	P
Gender (F/M)	40/7	29/4	11/3	0.251
Age (mean ± SD)	36.9 ± 11.98	39.2 ± 11.5	31.6 ± 10.6	0.044
Initiation age of disease (mean ± SD)	29.8 ± 5.2	32.4 ± 7.2	24.3 ± 5.4	0.046
Duration of disease (mean ± SD)	6.76 ± 4.3	6.15 ± 4.7	8.10 ± 6.2	0.548
SLEDAI (mean ± SD)	7.60 ± 9.7	7.16 ± 7.7	9.3 ± 13.6	0.601
Clinical features	Percentage			
Oral ulcers	35.7	11 (33)	4 (28)	0.739
Mucocutaneous	65.9	22 (66)	9 (64)	0.713
Malar rash	51.1	16 (48)	8 (57)	0.748
Discoid rash	14.3	5 (15)	1 (7)	0.647
Photosensitivity	42.9	13 (39)	5 (35)	0.748
Renal involvement	40.5	10 (30)	6 (42)	0.510
Arthritis/arthralgia	66.7	21 (63)	7 (50)	0.298
Serositis	19	6 (18)	3 (21)	NA
Pericarditis	14.3	3 (9)	3 (21)	NA
Pleurisy	16.7	5 (15)	2 (14)	NA
Haematological involvement	50	14 (42)	7 (50)	1.00
Anemia	62.5	14 (42)	6 (42)	1.00
Leukopenia	28.1	6 (18)	3 (21)	1.00
Thrombocytopenia	34.4	8 (24)	3 (21)	1.00
Neurological involvement	28.6	9 (27)	3 (21)	0.722
Immunological involvement	69	20 (60)	9 (64)	1.00
ANA (+)	95.2	27 (81)	13 (92)	1.00
Anti ds-DNA	59.5	16 (48)	9 (64)	0.598
Inactive disease (n)		18	7	
Active disease (n)		13	6	0.564

F, female; M, male; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; ANA, antinuclear antibody; NA, not available.

was 2.66 (CI 95%: 1.27–5.56) and OR of A allele was 1.8 (CI 95%: 0.99–3.28) (Table 1, Fig. 1).

We compared the genotypes and clinical manifestations of SLE patients. Clinical findings such as oral ulcers, malar rash, discoid rash, photosensitivity, arthritis, pericarditis, pleurisy, haematological, renal and neurologic involvement, ANA and anti ds-DNA positivity were similar in patients with AA, AG and GG genotypes (data not shown). Because of the low number of patients in each subgroup, we made two groups of patients with the AA and non-AA genotypes. No associations were seen between CTLA-4 genotypes and clinical features (Table 2). CTLA-4 polymorphisms were also not associated with duration of the disease and disease activity. The mean age of patients with the AA genotype was higher than patients with non-AA [(mean ± SD), 39.2 ± 11.5 vs. 31.6 ± 10.6, $P = 0.044$].

Moreover, the age of initiation of the disease in AA homozygote patients was significantly higher compared to non-AA patients (32.38 years; 24.31 years, respectively; $P = 0.046$) (Table 2).

Discussion

Genetic risk factors are important at many steps in pathogenesis of SLE. There are several studies reporting associations between CTLA-4 polymorphisms and SLE. Exon 1 codon 49 A/G polymorphisms were identified as playing a significant role in the development of SLE. Allele G and genotype GG of the exon 1 +49 polymorphism were defined as risk factors for SLE development in Asians but not in Europeans (Lee *et al.*, 2005). In contrast to this, our study showed a relationship between the AA genotype

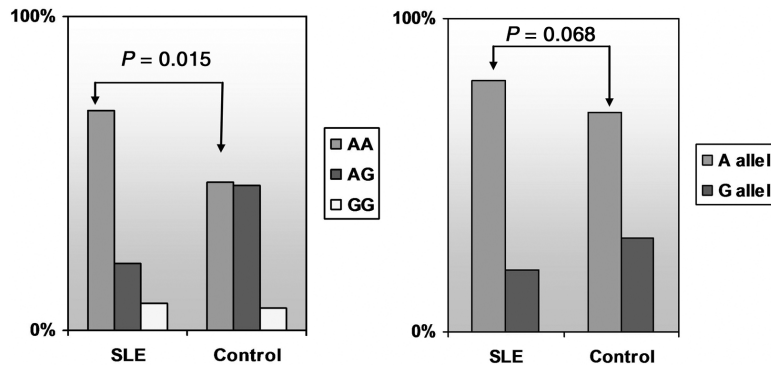


Figure 1. Distribution of gene and allele in SLE patients and controls.

and development of SLE in our cohort. Another result of our study was the lower number of patients with GG genotype compared to other genotypes in both SLE and control group (8.5%, 7%, respectively). GG genotype was reported in 6.8–10% of SLE patients and 8.6–8.8% of healthy population in Europe (Nistico *et al.*, 1996; Aguilar *et al.*, 2003; Barreto *et al.*, 2004). In Turkey, Gunesacar *et al.* showed that CTLA-4 GG genotype in healthy individuals was 10.6% (Gunesacar *et al.*, 2007). The results of studies show that CTLA-4 GG genotype in the Japanese population was higher than in Turks and Europeans (48% of SLE patients and 31% of healthy controls were detected to have GG genotype in a Japanese population) (Ahmed *et al.*, 2001). Although the first studies revealed that the prevalence of SLE is more common in Asians than in Caucasians (Petri, 2002), new epidemiological studies on SLE show that the lowest overall incidence is in Iceland and Japan, and highest in the USA and France, and there is a trend towards a higher incidence and prevalence of SLE in Europe (Danchenko *et al.*, 2006). According to the results of these studies, frequency of the G allele in Turkish population was lower than the Japanese population, similar to Europeans, but there is no data on incidence of SLE in Turkish population. A trend towards higher incidence and prevalence of SLE in Europe may be associated with the high frequency of A allele and AA genotype. Epidemiologic studies are required to understand the frequency of SLE in Turkey and relationship between SLE and AA genotype in Turkish and European population. This is a pilot study with only 47 participants.

SLE is a prototype of systemic autoimmune diseases and is a highly heterogeneous disease with regard to severity, organ involvement, and autoantibody profile. Autoantibodies against a variety of autoantigens are known to be associated with organ involvement. The anti-dsDNA titers most often correlate with disease activity and renal involvement (Okamura *et al.*, 1993). Antiribosomal-P is associated with neuropsychiatric symptoms (Iverson, 1996), anticardiolipin antibodies are associated with thrombosis (Afeltra *et al.*, 2005). Although it was suggested that some polymorphisms were associated with increased risk of disease, the relationship between the genetic factors and different clinical features are not known exactly.

Genetic factors may influence the process of autoantibody production.

Genotype–phenotype associations between risk alleles and disease subtypes may give insight into disease etiology and mechanisms. Poly (ADP-ribose) polymerase (PARP) polymorphisms were found significantly associated with nephritis and arthritis (Hur *et al.*, 2006); The Fc gamma RIIIA-V/F158 polymorphism had a significant impact on the development of lupus nephritis (Karassa *et al.*, 2003); Polymorphic variants of the CRP and Fc-receptor genes were associated with the clinical phenotype in SLE (Jonsen *et al.*, 2007). There is even stronger evidence in this subset for relationships between the *STAT4* gene and nephritis, autoantibodies to double-stranded DNA, and early age at diagnosis (Taylor *et al.*, 2008). CTLA-4 is cell-surface molecule on T lymphocyte and interacts with the B7 cell-surface molecule on the antigen-presenting cells. If the negative signal caused by the CTLA-4-B7 interaction dominates, T cell activation is suppressed, which is important in immune modulation (Salomon & Bluestone, 2001). Because SLE is an extremely heterogeneous autoimmune disease, we sought to investigate whether or not *CTLA-4* contributes to this phenotypic heterogeneity in SLE patients. Our study illustrated that there was no correlation between CTLA-4 exon 1 position 49 polymorphisms and clinical features, activity of disease and duration of disease in SLE patients. But, mean age and mean age at onset of disease in patients with non-AA genotype was younger than with AA. This finding is very interesting because Sugimoto *et al.* (2008) has recently reported that disorders of the CTLA-4 exon 1 position 49 GG genotype may be associated with early onset of SLE in two Japanese families with SLE. Both patients presented by Sugimoto were boys and their families had highly active SLE complicated with severe lupus nephritis or fibrinous pneumonia. Four patients with GG genotype in our study (two women, two men) had no family history, and severity of disease or organ involvement were no different from the other patients. These findings suggest that G allele may only be a risk factor for early development of SLE.

Although several studies reported CTLA-4 exon 1 position + 49 gene polymorphisms in SLE, this is the first study to report a search for an association between polymorphisms

at CTLA-4 and clinical features of SLE patients. In the present study, we observed that these polymorphisms didn't effect clinical features of SLE, except the age at onset of disease. We observed that CTLA-4 +49(A/G) gene polymorphisms were not associated with organ involvements and severity of the disease. However, interpretation of these results should be carefully performed since there are several limitations in this study. The main limitation of this study is the low number of patients, especially patients with GG genotype, which is small for statistical analyses. The results, therefore, need to be confirmed by studies including a large number of patients with SLE.

Consequently, our study results showed that CTLA-4 +49 AA genotype might be associated with development of SLE in Turkish population. On the contrary, G allele may be involved in early development of SLE. More studies with large patient numbers are needed to understand the effect of polymorphisms exon-1(+49 A/G) on the clinical spectrum of SLE.

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