Ethnic Differences in Cytotoxic T Lymphocyte Associated Antigen 4 Genotype Associations with Systemic Sclerosis

LORI L. HUDSON, RICHARD M. SILVER, and JANARDAN P. PANDEY

ABSTRACT. ***Objective.*** To determine the role of cytotoxic T lymphocyte associated antigen 4 (CTLA-4) genetic polymorphisms in susceptibility to systemic sclerosis (SSc, scleroderma).

Methods. The study population consisted of 293 African American and Caucasian patients with SSc and matched controls. Subjects were genotyped for allelic determinants at 4 polymorphic sites: 3 in the promoter region (positions –318, –1661, –1722) and one in the first exon (position +49) of the *CTLA-4* gene, using polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) methods. Genotype frequencies were compared using Pearson's chi-square or Fisher's exact test.

Results. In African American patients, the frequency of AG heterozygotes at position +49 was significantly higher than in controls (71% vs 36%, p = 0.003; OR = 4.37), while the frequency of AA homozygotes was significantly lower in patients than in controls (29% vs 61%, p = 0.007; OR = 0.26). The distribution of *CTLA-4* alleles at other loci did not differ significantly between patients and controls. *CTLA-4* genotypes were not associated with SSc in Caucasians. No differences in *CTLA-4* genotype distributions were observed between patients with the limited and diffuse forms of the disease.

Conclusion. Our data show that the exon 1 (+49) polymorphism of the *CTLA-4* gene is associated with systemic sclerosis in African Americans. (J Rheumatol 2004;31:85–7)

Key Indexing Terms:
SCLERODERMA T CELL ACTIVATION AUTOIMMUNITY
SINGLE NUCLEOTIDE POLYMORPHISMS LINKAGE DISEQUILIBRIUM

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4, CD152) (CD 152), a T cell surface glycoprotein, plays an important role in the maintenance of peripheral tolerance. Several studies have shown that CTLA-4 negatively regulates T cell function[1,2]. For instance, blocking the activity of CTLA-4 by monoclonal antibodies in vitro promotes T cell proliferation[3,4]. Mice lacking the *CTLA-4* gene (CTLA-4 –/–) develop a lethal lymphoproliferative disease due to uncontrolled T cell expansion[5].

Because of its pivotal role in immune homeostasis, *CTLA-4* has been designated by some as a general susceptibility gene for autoimmune diseases[6]. Studies in both experimental animal models and humans support this contention. For instance, in experimental allergic encephalomyelitis, a model of human multiple sclerosis, inhibition of the CTLA-4 action by monoclonal antibodies results in disease exacerbation[7]. Particular alleles of the *CTLA-4* gene have been implicated in several autoimmune diseases such as systemic lupus erythematosus, insulin dependent diabetes mellitus, multiple sclerosis, and Graves' disease[8-12]. Its potential role in the etiology of scleroderma (systemic sclerosis, SSc), an autoimmune disease characterized by inflammation and fibrosis of the skin and visceral organs, has been investigated in a Japanese population[13]. No significant associations with SSc were found. Due to ethnic differences in allele frequencies, it is imperative to study *CTLA-4* polymorphisms in other population groups. Here we report associations of particular *CTLA-4* genotypes with SSc in African Americans.

MATERIALS AND METHODS

Subjects. Blood was collected from Caucasian (100 patients, 122 controls) and African American (37 patients, 34 controls) subjects presenting at the Rheumatology Clinic of the Medical University of South Carolina. All patients fulfilled the American College of Rheumatology criteria for SSc[14]. Controls consisted of patients with osteoarthritis, fibromyalgia, gout, or regional musculoskeletal pain syndromes attending the same clinic as patients. Controls with conditions associated with autoimmune or connective tissue diseases were excluded. All subjects provided written informed consent.

CTLA-4 genotyping. DNA was isolated from whole blood using standard isolation procedures. Four single nucleotide polymorphisms of the *CTLA-4* gene were examined in this study: an adenine to guanine substitution (+49 A→G) in exon 1, and 3 promoter region substitutions: cytosine to thymine (–318 C→T), adenine to guanine (–1661 A→G), and thymine to cytosine (–1722 T→C). The polymerase chain reaction-restriction fragment length

From the Department of Microbiology and Immunology, and the Department of Medicine, Medical University of South Carolina, Charleston, South Carolina, USA.

Supported in part by funds from the U.S. Department of Energy cooperative agreement DE-FC02-09CH11109.

L.L. Hudson, BSc, Graduate Student; R.M. Silver, MD, Professor of Medicine; J.P. Pandey, PhD, Professor of Microbiology and Immunology.

Address reprint requests to L.L. Hudson, Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425-2230, USA. E-mail: hudsonll@musc.edu

Submitted January 28, 2003; revision accepted June 9, 2003.

polymorphism (PCR-RFLP) methods used to determine these polymorphisms have been described[12,15,16]. (For technical reasons, some subjects could not be typed for certain determinants, causing a slight variation in the sample sizes for different genotypes in Tables 1 and 2.)

Statistical analysis. The distribution of genotype frequencies was analyzed using Pearson's chi-square test, except when expected cell counts were less than or equal to 5; in the latter case, data were analyzed by Fisher's 2-tailed exact test. Statistical significance was defined as $p < 0.05$. Odds ratios (OR) were calculated to measure the strength of the associations observed. Calculations were made using the internet programs http://home.clara.net/sisa/two2hlp.htm and http://www.matforsk.no/ola/fisher.htm.

RESULTS

The distribution of *CTLA-4* genotypes in African American patients with SSc and controls is given in Table 1. The *CTLA-4* (+49) genotypes were significantly associated with SSc. The frequency of the AA homozygotes was lower (29 vs 61%, p = 0.007; OR = 0.26) and the frequency of the AG heterozygotes was higher (71 vs 36%, p = 0.003; OR = 4.37) in patients than in controls.

Table 1. Distribution of *CTLA-4* genotypes among African American patients with SSc and controls.

Genotype	Patients	Controls
Exon 1 + 49	n = 35 (%)	n = 33 (%)
AA	10 (29)	20 (61)*
AG	25 (71)	12 (36)**
GG	0	1 (3.0)
Promoter–318	n = 37 (%)	n = 34 (%)
CC	36 (97)	33 (97)
CT	1 (3.0)	1 (3.0)
TT	0	0
Promoter–1661	n = 34 (%)	n = 31 (%)
AA	24 (70)	22 (71)
AG	10 (30)	9 (29)
GG	0	0
Promoter–1722	n = 33 (%)	n = 31 (%)
TT	30 (90)	24 (77.5)
TC	3 (9.0)	5 (16)
CC	0	2 (6.5)

* p = 0.007; OR = 0.26; 95% CI 0.09–0.71. ** p = 0.003; OR = 4.37; 95% CI 1.57–12.13

Table 2. Distribution of *CTLA-4* genotypes among Caucasian patients with SSc and controls.

Genotype	Patients	Controls
Exon 1 + 49	n = 88 (%)	n = 104 (%)
AA	29 (33)	34 (33)
AG	52 (59)	63 (60)
GG	7 (8.0)	7 (7.0)
Promoter–318	n = 100 (%)	n = 122 (%)
CC	85 (85)	108 (88.5)
CT	15 (15)	14 (11.5)
TT	0	0
Promoter–1661	n = 96 (%)	n = 122 (%)
AA	67 (70)	88 (72.1)
AG	28 (29)	33 (27.1)
GG	1 (1.0)	1 (0.8)
Promoter–1722	n = 95 (%)	n = 118 (%)
TT	84 (88.4)	108 (91.5)
TC	9 (9.5)	10 (8.5)
CC	2 (2.1)	0

The distribution of *CTLA-4* genotypes in Caucasian patients with SSc and controls is given in Table 2. No significant associations were found. Also, no differences in *CTLA-4* genotype distributions were observed between patients with the limited and diffuse forms of the disease (data not shown).

DISCUSSION

Our results show strong associations between particular *CTLA-4* genotypes and SSc. Furthermore, there are clear ethnic differences: The *CTLA-4* (+49) locus was implicated in African Americans but not in Caucasians. The risk of SSc in African Americans homozygous for the A allele at the *CTLA-4* (+49) locus was 74% less than that in AG heterozygotes and GG homozygotes. Conversely, the risk of SSc in AG heterozygotes was over 4 times greater than that in individuals with the other 2 genotypes.

As SSc patients have increased numbers of activated helper and cytotoxic T lymphocytes in affected tissues and organs, a functional relationship between particular *CTLA-4* genotypes and T cell proliferation may explain the associations we observed[17,18]. Indeed, a few studies have shown associations between certain *CTLA-4* genotypes and levels of CTLA-4 as well as T cell proliferation[19-21]. A mechanism for the involvement of *CTLA-4* genotypes in SSc pathogenesis may be through its possible contribution to the fibrosis of skin and visceral organs, a hallmark feature of this disorder. Cross-linking of CTLA-4 on CD4+ T cells results in marked increase in transforming growth factor (TGF)-ß production[22]. This immunoregulatory cytokine has pleomorphic effects, including the ability to stimulate extracellular matrix components such as collagen I and III, which contribute to the extensive fibrosis in SSc[23,24]. It is possible that the CTLA-4 molecules encoded by genotypes associated with SSc susceptibility are more efficient in cross-linking, leading to an increase in TGF-ß secretion and concomitant increase in fibrosis.

As mentioned above, the *CTLA-4* genotype associations we observed are ethnically restricted. The reasons for this restriction are not clear. Involvement of a gene in susceptibility to a disease in one ethnic group, but not in another, may be a reflection of genetic heterogeneity, a well-documented phenomenon in polygenic diseases[25]. Although we did not find any associations between *CTLA-4* genes and SSc in Caucasians, it is possible that these genes do contribute to the disease in this ethnic group as well, but that their role in this population is minor and detectable only in the presence of another major disease susceptibility gene, perhaps an allele of the *HLA* system. Such interactions between *CTLA-4* and *HLA* alleles have been shown in rheumatoid arthritis[26]. Additionally, *CTLA-4* alleles may be

affecting particular clinical aspects of the disease, such as specific organ involvement or autoantibody production rather than disease risk, as found in a Japanese population[13].

Alternatively, the observed associations may be due to linkage disequilibrium between *CTLA-4* alleles and alleles of an as-yet-unidentified locus for susceptibility to SSc. One way to examine the validity of this explanation would be to analyze data from patients' sibs by the transmission disequilibrium test[27]. If linkage is confirmed, one can employ additional single-nucleotide polymorphisms in the *CTLA-4* region to better localize the putative susceptibility gene[28]. In addition to the genetic heterogeneity in SSc, potential differences in linkage disequilibrium among populations originating from different continents may also contribute to the ethnically-restricted genotype associations observed here[29,30]. Furthermore, it is likely that alleles at several loci epistatically interact to cause SSc, and racial differences in gene frequencies at these loci result in differences in relative risk to develop the disease in various groups. Evidence for such epistatic interactions in SSc has been presented[31].

Although the *CTLA-4* genotype associations with SSc we found are strong and make biological sense, the best way to determine whether these observations have universal applicability would be to extend these studies using another large, multiethnic study population.

REFERENCES

1. Greenwald RJ, Latchman YE, Sharpe AH. Negative co-receptors on lymphocytes. Curr Opin Immunol 2002;14:391-6.
2. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 2001;19:225-52.
3. Brunner M, Chambers C, Chan F, Hanke J, Winoto A, Allison J. CTLA-4 mediated inhibition of early events of T-cell proliferation. J Immunol 1999;162:5813-20.
4. Oosterwegel MA, Greenwald RJ, Mandelbrot DA, Lorsbach RB, Sharpe AH. CTLA-4 and T cell activation. Curr Opin Immunol 1999;11:294-300.
5. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3:541-7.
6. Kristiansen OP, Larsen ZM, Pociot F. CTLA-4 in autoimmune diseases — a general susceptibility gene to autoimmunity? Genes Immun 2000;1:170-84.
7. Perrin PJ, Maldonado JH, Davis TA, June CH, Racke MK. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. J Immunol 1996;157:1333-6.
8. Ahmed S, Ihara K, Kanemitsu S, et al. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. Rheumatology 2001;40:662-7.
9. Ligers A, Xu C, Saarinen S, Hillert J, Olerup O. The CTLA-4 gene is associated with multiple sclerosis. J Neuroimmunol 1999;97:182-90.
10. Marron MP, Raffel LJ, Garchon H, et al. Insulin-dependent diabetes mellitus is associated with CTLA-4 polymorphisms in multiple ethnic groups. Hum Mol Gen 1997;6:1275-82.
11. Yanagawa T, Hidaka Y, Guimaracs V, Soliman M, DeGroot LJ. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. J Clin Endocrinol Metab 1995;80:41-5.
12. Hudson LL, Rocca K, Song YW, Pandey JP. CTLA-4 gene polymorphisms in systemic lupus erythematosus: a highly significant association with a determinant in the promoter region. Hum Genet 2002;111:452-5.
13. Takeuchi F, Kawasugi K, Nabeta H, Mori M, Tanimoto K. Association of CTLA-4 with systemic sclerosis in Japanese patients. Clin Exp Rheumatol 2002;20:823-8.
14. Subcommittee for SSc criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 23:581-90.
15. Deichmann K, Heinzmann A, Bruggenolte E, Forster J, Kuehr J. An Mse I RFLP in the human CTLA4 promotor. Biochem Biophys Res Commun 1996;225:817-8.
16. Heward J, Gordon C, Allahabadia A, Barnett AH, Franklyn JA, Gough SCL. The A-G polymorphism in exon 1 of the CTLA-4 gene is not associated with systemic lupus erythematosus. Ann Rheum Dis 1999;58:193-5.
17. Sapadin AN, Esser AC, Fleischmajer R. Immunopathogenesis of scleroderma- evolving concepts. Mt Sinai J Med 2001;68:233-42.
18. Artlett CM, Smith JB, Jimenez SA. New perspectives on the etiology of systemic sclerosis. Mol Med Today 1999;5:74-8.
19. Ligers A, Teleshova N, Masterman T, Huang W-X, Hillert J. CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. Genes Immun 2001;2:145-52.
20. Kouki T, Sawai Y, Gardine CA, Fisfalen ME, Alegre ML, DeGroot LJ. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. J Immunol 2000;165:6606-11.
21. Maurer M, Loserth S, Kolb-Maurer A, et al. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (exon 1 +49) alters T-cell activation. Immunogenetics 2002;54:1-8.
22. Chen W, Jin W, Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4(+) T cells. J Exp Med 1998;188:1849-57.
23. Quefeld C, Eckes B, Huerkamp C, Krieg T, Sollberg S. Expression of TGF-ß1, -ß2, -ß3 in localized and systemic scleroderma. J Dermatol Sci 1999;21:13-22.
24. Ihn H, Yamane K, Kubo M, Tamaki K. Blockade of endogenous transforming growth factor ß signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts. Arthritis Rheum 2001;44:474-80.
25. Wanstrat A, Wakeland E. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. Nat Immunol 2001;2:802-9.
26. Yanagawa T, Gomi K, Nakao EI, Inada S. CTLA-4 gene polymorphism in Japanese patients with rheumatoid arthritis. J Rheumatol 2000;27:2740-2.
27. Spielman RS, Ewens WJ. A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test. Am J Hum Genet 1998;62:450-8.
28. Goldstein DB. Islands of linkage disequilibrium. Nat Genet 2001;29:109-11.
29. Goddard KAB, Hopkins PJ, Hall JM, Witte JS. Linkage disequilibrium and allele frequencies for 114 single-nucleotide polymorphisms in five populations. Am J Hum Genet 2000;66:216-34.
30. Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ. Linkage disequilibrium in the human genome. Nature 2001;411:199-204.
31. Kameda H, Pandey JP, Kaburaki J, Inoko H, Kuwana M. Immunoglobulin allotype gene polymorphisms in systemic sclerosis: interactive effect of MHC class II and KM genes on anticentromere antibody production. Ann Rheum Dis 1998;57:366-70.