

Coding Sequence 1 and Promoter Single Nucleotide Polymorphisms in the CTLA-4 Gene in Wegener's Granulomatosis

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ABSTRACT. Objective. To analyze the association of Wegener's granulomatosis (WG) with 2 single nucleotide polymorphisms (SNP), a +49 A/G polymorphism in coding sequence (CDS) 1 and a C/T base exchange in the promoter region at position -318.

Methods. Restriction enzyme digestion of PCR amplified genomic DNA was used to analyze the CTLA-4 SNP in 32 patients with WG and 100–122 ethnically matched healthy controls.

Results. Patients were more often heterozygous for C/T in the promoter region (31% of the patients vs 14% of controls; $p < 0.05$). Homozygosity for C was less frequent in patients (69% of patients vs 86% of controls; $p < 0.05$). There was no association with the A/G SNP in CDS 1. There was a linkage disequilibrium between allele A of CDS 1 and the shortest allele, 86 bp, in the (AT)_n of the 3' untranslated region in controls but not in patients.

Conclusion. The CTLA-4 SNP in the promoter region at position -318 is associated with WG. The loss of linkage disequilibrium between allele A of CDS 1 and the short 86 bp in the (AT)_n in patients indicates that the promoter SNP and the (AT)_n polymorphism are independent genetic risk factors. (J Rheumatol 2002;29:950–3)

Key Indexing Terms:

WEGENER'S GRANULOMATOSIS

GENE POLYMORPHISM

CTLA-4

Wegener's granulomatosis (WG) is a necrotizing systemic vasculitis that primarily involves the upper and lower respiratory tracts and the kidneys, although virtually any organ can be affected. Its etiology is unknown. Involvement of T cells is suggested by the presence of T cells in the local lesion and by our finding that T cells in most patients have a biased T cell receptor AV/BV receptor usage. These expanded T cells are activated and clonally expanded^{1,2}. A prominent characteristic of the disease is a high and persistent expression of activation markers on the peripheral lymphocytes^{3,4}, which is evident not only in the acute stage but also when the patient is in complete remission.

There is no clear association of WG to MHC genes⁵, although patients with HLA-DRB1*04 had a higher prevalence of renal vasculitis⁶. Among possible candidates for non-MHC susceptibility genes are those encoding proteins that regulate the immune response, such as the cytotoxic T lymphocyte antigen 4 (CTLA-4). The CTLA-4 protein is a receptor displayed on activated T cells that receives a nega-

tive signal upon binding to CD80 and CD86 on the antigen presenting cells⁷. CTLA-4 is thus of importance for the normal downregulation of T cell activation, and polymorphisms of the CTLA-4 gene (*Ctla-4*) that affect the function or the expression of this gene could thus have implications for the immune response. Three *Ctla-4* polymorphisms have been described: a single nucleotide polymorphism (SNP) in the promoter region [cytosine (C) or thymidine (T)] at position -318⁸, a SNP [adenine (A) or guanine (G)] in the coding sequence (CDS) 1 at position +49⁹, and a microsatellite (AT)_n marker at position 642 of the 3' untranslated region (UTR) of exon 3^{9–11}. The SNP in CDS 1 at +49 has been associated to Graves' disease¹² and multiple sclerosis¹³. The (AT)_n polymorphism has been linked to insulin dependent diabetes mellitus^{14,15}, Graves' disease¹⁵, Hashimoto's thyroiditis¹⁶, and myasthenia gravis with thymoma¹⁷.

We have recently reported a strong association of WG to the length of (AT)_n in the 3' UTR¹⁸. This study describes 2 SNP in the *Ctla-4* in WG.

MATERIALS AND METHODS

Patients and controls. Thirty-two unrelated Swedish Caucasian patients and 100–122 ethnically matched healthy individuals were studied. The age of the patients ranged from 30 to 81 years (mean 58 yrs). Twenty-nine patients had circulating antibodies (ANCA) against proteinase 3 (PR3). Twenty-one patients were male and 11 female. Nine patients had localized disease involving the upper respiratory tracts and/or eye only, 23 had the generalized form with renal, lung, heart, and/or central and peripheral nervous system involvement.

DNA extraction and genotyping. DNA was extracted from EDTA preserved

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peripheral blood from patients and controls using a standard proteinase K digestion and phenol/chloroform method.

The *Ctla-4* promoter SNP at position -318 was defined employing a polymerase chain reaction (PCR) with oligonucleotides forward primers 5'-AAATGAATTGGACTGGATGGT-3' and reverse primers 5'-TTACGA-GAAAGGAAGCCGTG-3', designed according to the published protocol¹⁵. A 247 bp fragment was amplified by PCR using 0.01 µg genomic DNA, 1 U Taq polymerase (Pharmacia Biotechnical, Uppsala, Sweden), and 20 pmol of each primer and 8 mM dNTP. Twenty microliters volume/tube was applied in a programmed thermocycler (Techne, PHC-3 Dri-Block, Cambridge, UK).

A 152 bp fragment containing the +49 A/G SNP in CDS 1 was amplified using a forward primer 5'-AAGGCTCAGCTGAACCTGGT-3' and a reverse primer 5'-CTGCTGAAACAAATGAAACCC-3'. The forward primer was designed with a single base mismatch for the last nucleotide, which corresponds to the +47 position. The SNP in the CDS 1 (+49) and the promoter (-318) were determined by digestion of the amplified products with the restriction enzymes BstE 2 and MseI, respectively. The digestion products were analyzed by electrophoresis in a 2% agarose gel. The *Ctla-4* (AT)n was determined as described¹⁸.

Statistical analysis. The frequencies of the alleles and genotypes were compared by Fisher's exact test with 2 sided p values. A p value < 0.05 was considered significant.

RESULTS

The prevalence of homozygosity for C at the -318 allele of the promoter SNP was decreased and the prevalence of the C/T genotype increased in patients (p < 0.05) (Table 1). There was no difference between patients with localized and generalized diseases.

There was no difference between patients and controls in the SNP in CDS 1 at +49.

The SNP in the promoter region and in CDS 1 were compared. Patients had less often the combination of homozygosity for C/C in the promoter and A/A in CDS 1 (p < 0.05).

The promoter SNP and the length of the (AT)n of the 3' UTR, from our previous study of the same patient group¹⁸, were compared. Patients were less often homozygous for C/C in combination with homozygosity for 86/86 bp. This pattern was found in 9% of patients and 31% of controls (p < 0.05) (Table 2). Consequently, the combination of genotype C/T and homozygosity for the longer (AT)n alleles was more common in patients, 28% in patients vs 8% in controls (p < 0.01) (Table 2). There was no association between allele T of CDS 1 and allele 86 of (AT)n microsatellite in patients or in controls.

A comparison between SNP at CDS 1 and length of

(AT)n showed that patients less often had the combination of homozygosity for both A and the short allele 86 bp. This combination was present in 3% of the patients and in 26% of controls (p < 0.005). Homozygosity for the A allele and for (AT)n > 86 bp was present in 13% of the patients and in 1% of controls (p < 0.05). Heterozygosity for A/G and homozygosity for (AT)n alleles > 86 bp was over-represented in patients compared with controls, 41 vs 12%, respectively (p < 0.005).

Allele A of CDS 1 was in strong linkage disequilibrium with allele 86 in the (AT)n in controls but not in patients (p < 0.0001) (Table 3).

DISCUSSION

The CTLA-4 is an important negative regulator of T cell proliferation, and deficient expression/function of CTLA-4 leads to T cell hyperactivity¹⁹⁻²¹. CTLA-4 deficient mice exhibit a lethal phenotype with massive polyclonal expansion of peripheral T cells with increased expression of CD25 and CD69²²⁻²⁴, whereas treatment of mice with anti-CTLA-4 enhances the clonal expansion of antigen-specific T cells after immunization²⁵. WG is characterized by expanded T cells that persist for a long time even when the disease is in complete remission^{1,4}. A remarkable feature of these expanded T cells is their continuous high expression of the activation markers HLA-DR and CD25⁴, suggesting an ongoing active immune response despite the absence of clinical or humoral evidence of inflammatory activity. In an earlier study, we have shown a strong association of WG to the length of the (AT)n microsatellite in the *Ctla-4*¹⁸. We have also shown that cells from individuals with long (AT)n have an increased spontaneous activation, as evidenced by increased levels of interleukin 2 receptor in serum and increased telomerase activity¹⁹. Thus, this genetic variant might lead to deficient T cell inhibition upon antigen stimulation and consequently to a hyperactive immune response.

In this study, we analyzed 2 other polymorphic sites in *Ctla-4*. We found an association also with the C/T SNP in the promoter region at -318, but no association with the A/G SNP in CDS 1. The primary risk marker among the 3 polymorphic sites within the *Ctla-4* is unknown. Our data suggest a linkage between these regions. The allelic association with allele A in CDS 1 and the short 86 bp allele of the (AT)n of the 3' UTR in healthy controls but not in patients

Table 1. *Ctla-4* promoter SNP at -318 in 32 patients with WG and 122 healthy controls. The prevalences of genotypes C/C and C/T were compared.

Promoter	-318	C/C, n (%)	C/T, n (%)	T/T	p	OR	95% CI
WG		22 (69)	10 (31)	NP	< 0.05	0.36	0.14-0.88
Controls		105 (86)	17 (14)	NP			

NP: not present.

Table 2. Association between promoter (−318) SNP and (AT)n microsatellite of the *Ctla-4* in 32 patients with WG and 100 healthy controls.

Promoter	(AT)n	WG, n (%)	Controls n (%)	p	OR	95% CI
C/C	86/86	3 (9)	31 (31)	< 0.05	0.23	0.07–0.81
C/C	86/> 86	7 (22)	26 (26)	NS	0.80	0.31–2.10
C/C	> 86/> 86	12 (38)	27 (27)	NS	1.62	0.70–3.76
C/T	86/86	NP	6 (6)			
C/T	86/> 86	1 (3)	2 (2)	NS	1.58	0.14–18.0
C/T	> 86/> 86	9 (28)	8 (8)	< 0.01	4.50	1.56–132

NP: not present; NS: not significant.

Table 3. Association between allele A of CDS 1 and allele 86 of (AT)n microsatellite in *Ctla-4* in healthy controls but not in patients with WG.

		(AT)n		p
		86+	86−	
CDS1	HC	A+	63	< 0.0001
		A−	2	
WG	A+	10	17	NS
		A−	1	

CDS1: Coding sequence 1; NS: not significant; A: adenine

suggests that the (AT)n might be the variant primarily associated to WG.

It is unknown whether the 2 other *Ctla-4* dimorphisms are implicated in susceptibility to WG. Studies of other autoimmune diseases have described the T allele of the promoter SNP at −318 to be less common in patients with Graves' disease and Hashimoto's thyroiditis¹⁵ and in patients with multiple sclerosis¹³, but more prevalent in patients with peripheral neuropathy²⁶. Other studies have not verified these associations²⁷. Although this SNP occurs in the promoter region, it is unlikely to be of functional importance since it does not affect any known consensus sequence in the regulatory region of the promoter⁸.

More investigations have been performed regarding the A/G SNP in the CDS 1 at position +49. A higher prevalence of the G allele was described in systemic lupus erythematosus²⁸, rheumatoid arthritis²⁹, type 1 diabetes mellitus³⁰, primary biliary cirrhosis³¹, multiple sclerosis¹³, and Graves' disease³². However, other studies failed to confirm these results^{33–35}. The A/G SNP results in an amino acid exchange (Thr to Ala) in the leader sequence. It was recently reported that carriage of the G allele reduced the inhibitory function of CTLA-4 and thus contributes to the pathogenesis of Graves' disease³⁶. In our study, there was no association of WG with this A/G SNP, but a strong linkage disequilibrium between allele A of the A/G SNP and the shortest allele, 86 bp, of the (AT)n of the 3' UTR in healthy controls, but not in patients. Since the G allele and alleles > 86 bp of the

(AT)n are both associated with a decreased inhibitory function of CTLA-4^{19,36}, this loss of linkage disequilibrium might be of functional importance for the development and persistence of the T cell activation characteristically present in patients with WG^{3,4}.

We reported earlier a strong association of WG to (AT)n of the 3' UTR in the *Ctla-4*¹⁸. This study shows an association also with one of the other polymorphic sites, C/T −318 allele of the promoter region, but not with the A/G SNP in CDS 1. The loss of linkage disequilibrium between allele A of the A/G SNP and the shortest allele, 86 bp, of the (AT)n of the 3' UTR in patients might have functional consequences and predispose to the increased T cell activation in WG.

REFERENCES

- Giscombe R, Grunewald J, Nityanand S, Lefvert AK. T cell receptor V gene usage in patients with systemic necrotizing vasculitis. *Clin Exp Immunol* 1995;101:213-9.
- Grunewald J, Halapi E, Wahlström J, et al. T-cell expansions with conserved T-cell receptor b chain motifs in the peripheral blood of HLA-DRB1*0401 positive patients with necrotizing vasculitis. *Blood* 1998;92:3737-44.
- Schlesier M, Kaspar T, Gutfleisch J, Peter HH. Activated CD4+ and CD8+ T cell subsets in Wegener's granulomatosis. *Rheumatol Int* 1995;14:213-9.
- Giscombe R, Nityanand S, Lewin N, Grunewald J, Lefvert AK. Expanded T cell populations in patients with Wegener's granulomatosis: Characteristics and correlates with disease activity. *J Clin Immunol* 1998;18:404-13.
- Papiha SS, Murty GE, Ad'Hia A, Mains BT, Venning M. Association of Wegener's granulomatosis with HLA antigens and other genetic markers. *Ann Rheum Dis* 1992;51:246-8.
- Gencik M, Borgmann S, Zahn R, et al. Immunogenetic risk factors for anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis. *Clin Exp Immunol* 1999;117:412-7.
- Noel PJ, Boise LH, Green JM, Thompson CB. CD28 co-stimulation prevents cell death during primary T cell activation. *J Immunol* 1996;157:636-42.
- Deichmann K, Heinzmann A, Bruggenolte E, Forster J, Kuehr J. An Mse I RFLP in the human CTLA-4 promoter. *Biochem Biophys Res Commun* 1996;225:817-8.
- Harper K, Balzano C, Rouvier E, Mattei MG, Luciani MF, Golstein P. CTLA-4 and CD28 activated lymphocytes molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol*

- 1991;147:1037-44.
10. Dariavach P, Mattei MG, Golstein P, Lefranc MP. Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. *Eur J Immunol* 1988;18:1901-5.
 11. Polymeropoulos MH, Xiao H, Rath DS, Merrill CR. Dinucleotide repeat polymorphism at the human CTLA4 gene. *Nucleic Acids Res* 1991;19:4018.
 12. Heward JM, Allahabadia A, Armitage M, et al. The development of Graves' disease and the CTLA-4 gene on chromosome 2q33. *J Clin Endocrinol Metab* 1999;84:2398-401.
 13. Ligiers A, Xu C, Saarinen S, Hillert J, Olerup O. The CTLA-4 gene is associated with multiple sclerosis. *J Neuroimmunol* 1999; 97:182-90.
 14. Donner H, Rau H, Walfish PG, et al. CTLA-4 alanine-17 confers genetic susceptibility to Graves' disease and to type 1 diabetes mellitus. *J Clin Endocrinol Metab* 1997;82:143-6.
 15. Braun J, Donner H, Siegmund T, Walfish PG, Usadel KH, Badenhoop K. CTLA-4 promoter variants in patients with Graves' disease and Hashimoto thyroiditis. *Tissue Antigen* 1998;51:563-6.
 16. Donner H, Braun J, Seidl C, et al. Codon 17 polymorphism of the cytotoxic T lymphocyte antigen 4 gene in Hashimoto's thyroiditis and Addison's disease. *J Clin Endocrinol Metab* 1997;82:4130-2.
 17. Huang D, Liu L, Norén K, et al. Genetic association of CTLA-4 to myasthenia gravis with thymoma. *J Neuroimmunol* 1998;88:192-8.
 18. Huang D, Giscombe R, Zhou Y, Lefvert AK. Polymorphisms in CTLA-4 but not tumor necrosis factor- α or interleukin 1b genes are associated with Wegener's granulomatosis. *J Rheumatol* 2000;27:397-401.
 19. Huang D, Giscombe R, Zhou Y, Pirskanen R, Lefvert AK. Dinucleotide repeat expansion in the CTLA-4 gene leads to T cell hyper-reactivity via the CD28 pathway in myasthenia gravis. *J Neuroimmunol* 2000;105:69-77.
 20. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995; 182:459-65.
 21. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4 mediated inhibition of early events of T cell proliferation. *J Immunol* 1999;162:5813-20.
 22. Khattry R, Auger JA, Griffin MD, Sharpe AH, Bluestone JA. Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 responses. *J Immunol* 1999;162:5784-97.
 23. 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.
 24. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctl α -4. *Science* 1995;270:985-8.
 25. Kearney ER, Walunas TL, Karr RW, et al. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol* 1995;155:1032-6.
 26. Masterma T, Ligiers A, Olerup O, Vrethem M, Hillert J. CTLA-4 dimorphisms in gammopathy-associated peripheral neuropathy. *Eur J Neurol* 1999;6:491-3.
 27. Gonzalez-Escribano MF, Rodriguez R, Valenzuela A, Garcia A, Garcia-Lozano JR, Nunez-Roldan A. CTLA4 polymorphisms in Spanish patients with rheumatoid arthritis. *Tissue Antigens* 1999;53:296-300.
 28. Pullmann R Jr, Lukac J, Skerenova M, et al. Cytotoxic T lymphocyte antigen 4 (CTLA-4) dimorphism in patients with systemic lupus erythematosus. *Clin Exp Rheumatol* 1999;17:725-9.
 29. Seidl C, Donner H, Fischer B, et al. CTLA4 codon 17 dimorphism in patients with rheumatoid arthritis. *Tissue Antigens* 1998;51:62-6.
 30. Abe T, Takino H, Yamasaki H, et al. CTLA4 gene polymorphism correlates with the mode of onset and presence of ICA512 Ab in Japanese type 1 diabetes. *Diabetes Res Clin Pract* 1999;46:169-75.
 31. Agarwal K, Jones DE, Daly AK, et al. CTLA-4 gene polymorphism confers susceptibility to primary biliary cirrhosis. *J Hepatol* 2000;32:538-41.
 32. Vaidya B, Imrie H, Perros P, et al. The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus. *Hum Mol Genet* 1999;8:1195-9.
 33. Matsushita M, Tsuchiya N, Shiota M, et al. Lack of a strong association of CTLA-4 exon 1 polymorphism with the susceptibility to rheumatoid arthritis and systemic lupus erythematosus in Japanese: an association study using a novel variation screening method. *Tissue Antigens* 1999;54:578-84.
 34. Heinzmann A, Plesnar C, Kuehr J, Forster J, Deichmann KA. Common polymorphisms in the CTLA-4 and CD28 genes at 2q33 are not associated with asthma or atopy. *Eur J Immunogenet* 2000;27:57-61.
 35. Barton A, Myerscough A, John S, Gonzalez-Gay M, Ollier W, Worthington J. A single nucleotide polymorphism in exon 1 of cytotoxic T-lymphocyte-associated-antigen-4 (CTLA-4) is not associated with rheumatoid arthritis. *Rheumatology (Oxford)* 2000;39:63-6.
 36. 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.