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 genes[5], although patients with HLA-DRB1*04 had a higher prevalence of renal vasculitis[6]. 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 negative signal upon binding to CD80 and CD86 on the antigen presenting cells[7]. 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 –318[8], a SNP [adenine (A) or guanine (G)] in the coding sequence (CDS) 1 at position +49[9], 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' disease[12] and multiple sclerosis[13]. The (AT)n polymorphism has been linked to insulin dependent diabetes mellitus[14,15], Graves' disease[15], Hashimoto's thyroiditis[16], and myasthenia gravis with thymoma[17].

We have recently reported a strong association of WG to the length of (AT)n in the 3' UTR[18]. 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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Supported by grants from the Swedish Heart-Lung Foundation, the King Gustaf V 80 Years Foundation, the Börje Dahlin Foundation, the Swedish Rheumatism Association, and the Swedish Medical Research Council.

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Submitted May 23, 2001; revision accepted November 6, 2001.

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 protocol[15]. 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 Mse1, respectively. The digestion products were analyzed by electrophoresis in a 2% agarose gel. The *Ctla-4* (AT)n was determined as described[18].

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 group[18], 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 hyperactivity[19-21]. CTLA-4 deficient mice exhibit a lethal phenotype with massive polyclonal expansion of peripheral T cells with increased expression of CD25 and CD69[22-24], whereas treatment of mice with anti-CTLA-4 enhances the clonal expansion of antigen-specific T cells after immunization[25]. 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 CD25[4], 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 activity[19]. 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 86+	86–	*p*
CDS1				
HC	A+	63	17	< 0.0001
	A–	2	22	
WG	A+	10	17	NS
	A–	1	4	

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 thyroiditis[15] and in patients with multiple sclerosis[13], but more prevalent in patients with peripheral neuropathy[26]. Other studies have not verified these associations[27]. 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 promoter[8].

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 erythematosus[28], rheumatoid arthritis[29], type 1 diabetes mellitus[30], primary biliary cirrhosis[31], multiple sclerosis[13], and Graves' disease[32]. 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' disease[36]. 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.

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