Association Between Toll-like Receptor 4 Gene Polymorphism and Biopsy-proven Giant Cell Arteritis

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ABSTRACT. ***Objective.*** Dendritic cells localized at the adventitia-media border of the normal medium-sized arteries play a pivotal role in the initiation of giant cell arteritis (GCA). These cells express a singular surface receptor profile, including a series of Toll-like receptors (TLR). Ligands of TLR-4 promote activation and differentiation of adventitial dendritic cells and are directly implicated in the pathogenesis of GCA. We aimed to assess the potential implication of the *TLR4-(+896 A/G)* gene polymorphism in the susceptibility to GCA.

Methods. A total of 210 patients diagnosed with biopsy-proven GCA and 678 matched controls were included in our study. DNA from patients and controls was obtained from peripheral blood. Samples were genotyped for the *TLR4-(+896 A/G)* (rs4986790) gene polymorphism by polymerase chain reaction, using a predesigned TaqMan allele discrimination assay.

Results. The *TLR4 +896* G allele was significantly increased in biopsy-proven GCA patients compared to controls [p = 0.01; odds ratio (OR) 1.65; 95% confidence interval (CI) 1.08-2.52]. The increase was due to a significantly increased frequency of heterozygosity for the *TLR4* –896 A/G genotype in the group of patients with biopsy-proven GCA compared to controls (*TLR4* –896 A/G heterozygous in patients with GCA 18.1% compared to 11.4% in controls: p = 0.01; OR 1.72; 95% CI 1.10-2.69). However, no significant differences were observed when patients with GCA were stratified according to the presence of specific clinical features of the disease.

Conclusion. Our results show for the first time an association of *TLR4-(+896 A/G)* gene polymorphism with susceptibility to biopsy-proven GCA. (First Release June 15 2009; J Rheumatol 2009;36: 1501–6; doi:10.3899/jrheum.081286)

Key Indexing Terms:
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Giant cell arteritis (GCA), a large and medium-sized blood vessel granulomatous systemic vasculitis characterized by the involvement of the aorta and especially its cranial branches, is the most common systemic vasculitis in elderly individuals from Western countries[1,2]. Inflammation of the arterial wall and vessel occlusion through fast and concentric intimal hyperplasia leads to the severe ischemic complications observed in patients with this vasculitis[3]. Dendritic cells (DC) localized at the adventitia-media border of normal medium-sized arteries play a pivotal role in the initiation of this vasculitis[4]. These cells produce chemokines and recruit and locally activate T cells[4]. Moreover, DC express a singular surface receptor profile, including a series of Toll-like receptors (TLR). Ligands of TLR-4 promote activation and differentiation of adventitial DC into chemokine-producing effector cells with high-level expression of both CD83 and CD86 and mediated T cell recruitment through release of interleukin-18[4-6].

Human TLR participate in the innate response and signal the activation of adaptive immunity. Therefore, these TLR may be important in inflammatory autoimmune diseases. Interestingly, TLR-4, one of the most important pattern recognition receptors, recognizes lipopolysaccharide (LPS) of gram negative bacteria, products from fungal pathogens, a soluble component of *Mycobacterium tuberculosis* and

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endogenous ligands, such as fibronectin and several heat shock proteins[7].

Susceptibility to autoimmune disorders may be the result of the interaction of multiple genetic factors that regulate the threshold of autoreactivity. In this regard, GCA is a complex polygenic disease[8,9]. Besides a strong association of GCA with genes that lie within the major histocompatibility complex (MHC)[10-12], many other studies have shown the implication of genetic variants in key components of immune and inflammatory pathways in GCA susceptibility or clinical expression of this vasculitis[13-27].

The *TLR4* gene, which has been mapped to chromosome 9 (9q32-q33), is involved in innate immune recognition with subsequent proinflammatory cytokine release[28,29]. A single nucleotide polymorphism (SNP)*(+896A/G)* resulting in the amino acid substitution aspartic acid/glycine (Asp299Gly) (rs4986790), in high linkage disequilibrium with other non-synonymous polymorphisms of TLR4 (rs4986790, $r^2 >$ 0.99) in Caucasian populations, has been proposed to interrupt TLR-4 mediated signaling[28,29] (Figure 1).

This polymorphism occurs with an allelic frequency of less than 6% in most European populations[30]. Arbour, *et al* showed that the *TLR4-(+896 A/G)* gene polymorphism exhibited a blunted response to inhaled LPS[28]. Moreover, transfected cells with the mutant *TLR4* allele have a reduced nuclear factor (NF)-κB activity compared to the wild-type TLR4 allele, leading to reduced cytokine production. The *TLR4-(+896 A/G)* gene polymorphism functional variant was associated with decreased susceptibility to rheumatoid arthritis (RA) in a Dutch population[31]. However, no association with susceptibility to RA has been reported in other studies[32,33].

To the best of our knowledge, there are no data on the potential influence of this functional *TLR4-(+896 A/G)* gene polymorphism in the susceptibility to primary systemic vasculitides. We analyzed the potential role of this

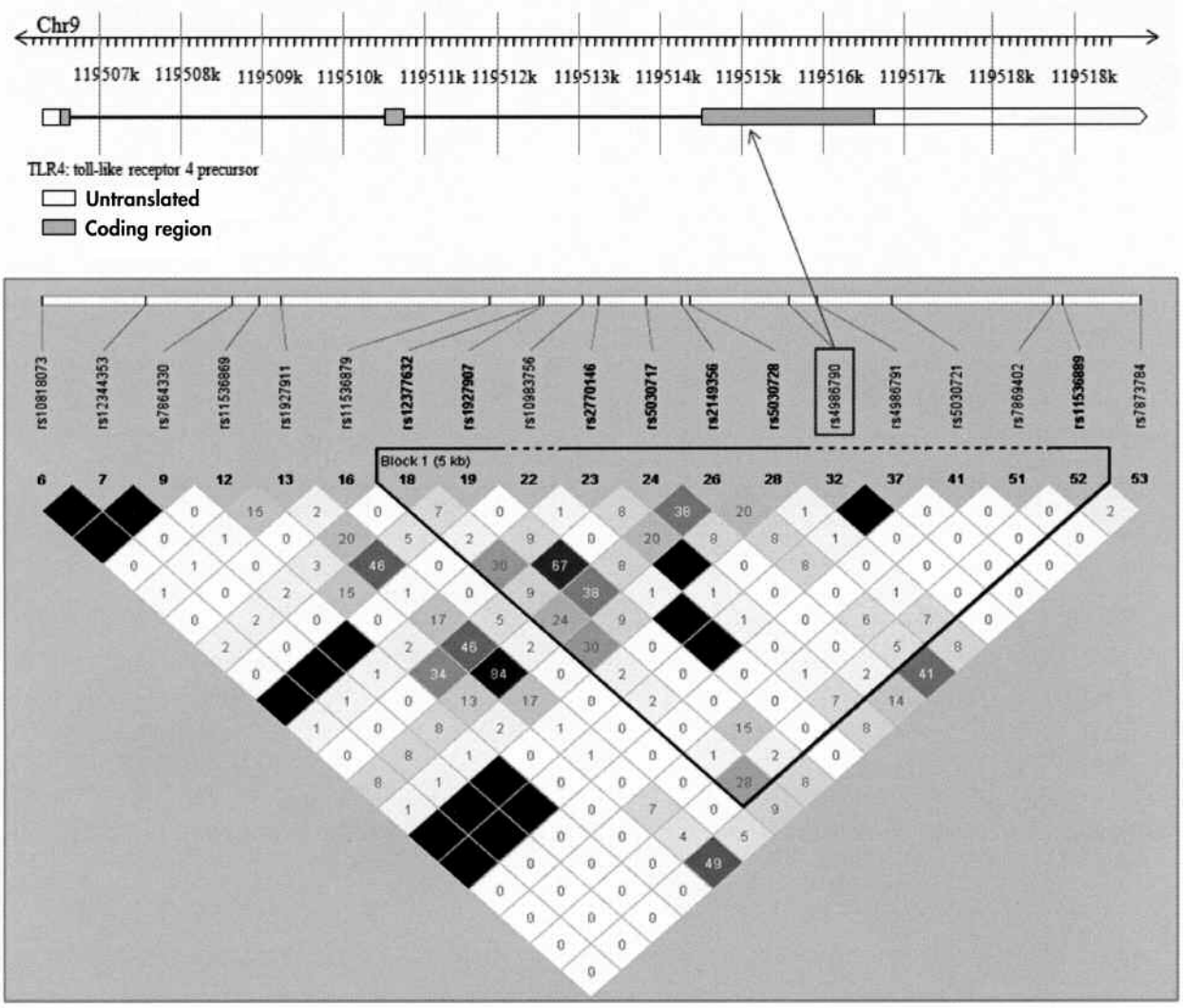

Figure 1. Map of the whole *TLR4* gene, viewed relative to common single nucleotide polymorphisms on HapMap in Caucasian population. Squares indicate pairwise r^2 on grayscale (black = 1, white = 0).

gene polymorphism in a large series of individuals with biopsy-proven GCA.

MATERIALS AND METHODS

Patients. A total of 210 patients diagnosed with biopsy-proven GCA were included in this study. Most of them (n = 126) were diagnosed in the Division of Rheumatology of the Hospital Xeral-Calde (Lugo, Northwest Spain). The remaining patients were diagnosed in Madrid (Hospital Clínico San Carlos and Hospital de la Princesa; n = 73) and Granada (Hospital Clínico San Cecílio; n = 11). A control population composed of 678 healthy controls from the corresponding cities matched by age, sex, and ethnicity with GCA patients was also assessed. However, we did not find significant differences between populations when we compared clinical features and genotyping and allele frequencies in the *TLR4 +896 A/G* variant (data not shown).

All patients had a positive temporal artery biopsy showing disruption of the internal elastic laminae with infiltration of mononuclear cells into the arterial wall with or without giant cells[34]. In addition, all of them fulfilled the 1990 American College of Rheumatology criteria for the classification of GCA[35].

Patients with GCA were considered to have associated polymyalgia rheumatica (PMR) if they had severe bilateral ache and pain involving the neck, the shoulder, and/or the pelvic girdles, associated with morning stiffness[36,37]. Patients were considered to have visual ischemic complications in the context of GCA if they experienced transient visual loss including amaurosis fugax, permanent visual loss, or diplopia[38]. Severe ischemic manifestations were considered to be present if patients with GCA suffered at least 1 of the following complications: visual manifestations, cerebrovascular accidents (stroke and/or transient ischemic attacks), jaw claudication, or large-artery stenosis of the extremities that caused signs of occlusive manifestations[39,40]. Patients and controls gave prior written informed consent, and ethical committee approval was obtained.

TLR4-(+896 A/G) *gene genotyping*. DNA was obtained from peripheral blood mononuclear cells, using standard methods. The genotyping of the *TLR4-(+896 A/G)* (rs4986790) polymorphisms was performed using a predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM, respectively. The polymerase chain reaction (PCR) was carried out in a total reaction volume of 5 μl, containing 50 ng genomic DNA as template, 2.5 μl of TaqMan genotyping master mix, 0.25 μl of 20× assay mix, and ddH_2O up to 5 μl of final volume. The amplification protocol used was the following: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 s, and annealing/extension at 60°C for 1 min. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic-specific fluorescence on the ABI PRIM 7900 Sequence Detection Systems using SDS 2.3 software for allelic discrimination (Applied Biosystems). Duplicate samples and negative controls were included to check the accuracy of genotyping.

Statistical analysis. We used the chi-squared test for Hardy-Weinberg equilibrium and statistical analysis to compare allelic and genotypic distributions. Genotype distribution was assessed using chi-squared test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated according to Woolf´s method using the Statcalc program (Epi Info 2002, Centers for Disease Control and Prevention, Atlanta, GA, USA). p values less than 0.05 were considered statistically significant.

RESULTS

This study included 210 biopsy-proven GCA patients from 3 different cities in Spain: 140 women and 70 men (median age at disease diagnosis 74 yrs; range 52-93 yrs). From the onset of GCA symptoms to 1 month after the onset of corticosteroid therapy, 96 (46%) experienced PMR manifestations and 52 had (25%) visual ischemic complications. Twenty (10%) had irreversible (permanent) visual loss. Other clinical features are shown in Table 1. Further, 678 controls were included in the analysis.

Influence of TLR-(+896 A/G) gene polymorphism in susceptibility to GCA. The case:control ratio was 1:3.2. The estimated power of our study for an estimated OR between 1.5 and 2.0 was 73.1-99.6% for a type I error rate of 0.05.

No evidence of departure from Hardy-Weinberg equilibrium was observed in controls.

Table 2 shows the allele and genotype frequencies of the *TLR4 (+896 A/G)* gene polymorphism in patients with biopsy-proven GCA and controls. When this biallelic polymorphism was assessed, we found that the G allele of the *TLR4* SNP was significantly increased in the patients compared to controls (p = 0.01; OR 1.65; 95% CI 1.08-2.52). The increase was due to a significantly increased frequency of heterozygosity for the *TLR4* –896 A/G genotype in the group of patients with biopsy-proven GCA compared to controls (*TLR4* –896 A/G heterozygous in patients 18.1% vs

Table 1. Main clinical features of 210 patients with biopsy-proven giant cell arteritis.

	Variable
Age, yrs, at time of disease diagnosis median, [range]	74 [52–93]
Women:men	140:70
Headache	168 (80)
Abnormal temporal artery on examination	133 (63)
Polymyalgia rheumatica	96 (46)
Jaw claudication	84 (40)
Visual manifestations*	52 (25)
Permanent visual loss	20 (10)
Stroke	10 (5)
Severe ischemic manifestations**	112 (53)
ESR greater than 40 mm/h	206 (98)

Data in parentheses are %. * Transient visual loss including amaurosis fugax, permanent visual loss, or diplopia. ** At least one of the following: visual manifestations, cerebrovascular accidents (stroke and/or transient ischemic attacks), jaw claudication, or limb claudication of recent onset.

Table 2. Genotypic and allelic frequencies of TLR4 rs4986790 polymorphism in patients with biopsy-proven giant cell arteritis (GCA) and controls.

TLR4	GCA patients n (%)	Controls n (%)	p	OR (95% CI)
rs4986790	n = 210	n = 678		
Genotype				
A/A	172 (81.9)	601 (88.6)	0.01	0.58 (0.37–0.91)
A/G	38 (18.1)	77 (11.4)	0.01	1.72 (1.10–2.69)
G/G	0 (0)	0 (0)	—	—
Allele				
A	382 (91.0)	1279 (94.3)	0.01	0.61 (0.40–0.93)
G	38 (9.0)	77 (5.7)	0.01	1.65 (1.08–2.52)

OR: odds ratio; CI: confidence interval.

11.4% in controls: p = 0.01; OR 1.72; 95% CI 1.10-2.69) (Table 2). In this regard, although none of the patients or controls from our study was found to be homozygous for the *TLR4* –896 G/G genotype, patients with biopsy-proven GCA exhibited a significantly reduced frequency of *TLR4* –896 A/A homozygous compared to matched controls (p = 0.01; OR 0.58; 95% CI 0.37-0.91) (Table 2). Moreover, the genotype distribution for the *TLR4-(+896 A/G)* polymorphism showed significant differences between patients with GCA and controls (p = 0.01).

We further stratified patients with GCA according to sex, presence of PMR, visual ischemic complications, and severe ischemic manifestations. However, no significant differences were observed when patients with GCA were compared according to the presence or absence of these specific clinical features of the disease. This was also the case when we compared patients who presented specific features of the disease (PMR, visual ischemic complications, and severe ischemic manifestations) with controls (data not shown).

DISCUSSION

Our study constitutes the first attempt to establish the potential influence of the functional *TLR4-(+896 A/G)* gene polymorphism in the susceptibility to biopsy-proven GCA. Our data show an increased frequency of the mutant *TLR4*-allele G in patients with biopsy-proven GCA compared to controls. However, no association with specific features of the disease was found.

Population-based studies have disclosed a specific geographical distribution of the *TLR4-(+896 A/G)* gene polymorphism[7]. This polymorphism is very common in African and very rare in Asian populations[7]. The frequency of the mutant allele G in our population was 5.7%. It was similar to that found in the Netherlands and Belgium (5.3% and 5.0%, respectively) and slightly higher than that observed in Germany and Greece (4.0% and 3.0%, respectively)[30]. Also, in accordance with the very rare occurrence of homozygous mutations in Caucasian individuals, we did not find this among the patients with biopsy-proven GCA and controls.

In a geo-epidemiology based study, Lee, *et al*[41] confirmed that incidence of GCA is decreased in the African population[2]. This finding might be slightly in conflict with our data due to the frequency of this polymorphism in African people. Nevertheless, it could be explained by the lower life expectancy found in Africans, as many African individuals do not reach the age at which GCA would be manifested.

The *TLR4-(+896 A/G)* gene polymorphism has been assessed in patients with rheumatic diseases. However, conflicting results were observed in patients with RA[31-33], and a modest or negative association of this gene polymorphism was also found in patients with ankylosing spondylitis[42-44].

GCA is a granulomatous vasculitis and, in keeping with our observations, *TLR4* gene mutations have also been reported in patients with other granulomatous diseases such as Crohn's disease and chronic sarcoidosis[45,46]. These findings may support the presence of a genetically determined defective signaling through the TL4 receptor leading to abnormal inflammatory granulomatous response.

Infectious agents have been postulated to be involved in the modulation of the innate immune system, and low-grade chronic or recurrent infections and several infectious agents have been proposed to play a role in autoimmunity, cardiovascular disease, and the pathogenesis of GCA[2]. Interestingly, *TLR4* (+896 A/G) has also been found to be positively correlated with several infectious diseases and susceptibility to septic shock by a defective TLR4 response[47,48].

Functional studies in human primary cells from *TLR4* –896 *A/G* heterozygous individuals disclosed significantly higher levels of tumor necrosis factor-α (TNF-α), interleukin 10 (IL-10), and monocyte chemoattractant proteins (MCP) such as MIP-1α and MCP-1, which in turn may modify the chemoattraction of different cellular types, than those from donors homozygous for *TLR4* –896 *GG*[49,50]. These data are in keeping with the described association of TNF-α gene microsatellite polymorphisms with biopsy-proven GCA[11] and the higher TNF-α production found in these patients[51]. However, *TLR4 +896 G* allele has also been reported to be associated with lower NF-κB activity[52] and lower levels of IL-1ß, IL-6, and IL-8[50], which are elevated in GCA patients. Thus, the influence of *TLR 4* gene polymorphisms in the cytokine profile of GCA needs further elucidation.

Inflammatory lesions in GCA are composed of activated $CD4^+$ T cells and macrophages. Selected $CD4^+$ T cells undergo expansion in the artery, strongly suggesting antigen-driven responses[4]. Activation of adventitial DC is an early and critical event in GCA. These cells also express a series of TLR. Ligands of TLR are able to start maturation of adventitial DC, which fail to leave the peripheral tissue site. Instead, these adventitial DC produce chemokines, recruit T cells, and support their local activation. Systemic administration of ligands for TLR2 or TLR-4 in human artery-SCID chimeras drives differentiation of adventitial DC into chemokine-producing effector cells with high-level expression of both CD83 and CD86 and mediates T cell regulatory function through release of IL-18[4]. These data identify tissue-residing DC as gatekeepers in vasculitis and support a model in which TLR ligands may function as instigators of vessel wall inflammation.

Taken together, all these observations and our present data suggest that *TLR* genes may be involved in genetic predisposition of GCA. Nevertheless, further studies are needed to clearly establish the implication of TLR and its relevance in the cytokine profiling in the pathogenesis of GCA.

Our study shows, for the first time, an association of the *TLR4-(+896 A/G)* gene polymorphism with susceptibility to

biopsy-proven GCA. Further studies are required to confirm if this association is also present in populations with different genetic backgrounds.

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