Toll-like receptor 4 (Tlr4) gene polymorphisms in giant cell arteritis

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ABSTRACT

Objective. *To investigate potential associations between toll-like receptor 4 (TLR4) gene polymorphisms and susceptibility to, and clinical features of giant cell arteritis (GCA).*

Methods. *A total of 155 patients with biopsy-proven GCA who were residents of Reggio Emilia, Italy, and 210 population-based controls from the same geographical area were genotyped for two coding single nucleotide polymorphisms of TLR4 (Asp299Gly and Thr399Ile) by molecular methods. The patients were subgrouped according to the presence or absence of polymyalgia rheumatica and severe ischemic complications (visual loss and/or cerebrovascular accidents).*

Results. *The distribution of allele and genotype frequencies did not differ significantly between GCA patients and healthy controls. Carriers of the -299 G allele (G/A+ G/G) [odds ratio (OR) 1.78, 95% confidence intervals (CI) 0.90-3.50)] were more frequent among GCA patients than among the controls, but the difference was not statistically significant.*

No significant associations were found when GCA patients with and without PMR or with and without severe ischemic complications were compared.

Conclusion. *Our data suggest that the TLR4 gene polymorphisms are not associated with susceptibility to, and clinical expression of, GCA in Italian patients.*

Introduction

Giant cell arteritis (GCA) is the most common vasculitis in Western countries in individuals 50 years and older (1, 2). Its incidence increases with the age and peaks in subjects older than 70 years (3, 4). GCA is a systemic vasculitis in which T cells and macrophages infiltrate the wall of medium-sized and large arteries. GCA is considered a T-cell-driven disease, in particular CD4+ T cells are thought to play a central role in inducing and maintaining the vasculitic process. CD4 T cells are recruited to the adventitia, where they undergo local activation, suggesting antigen-driven stimulation. Tissue destruction in GCA largely depends on macrophage effector functions, with T-cell derived cytokines controlling the activity/differentiation of such macrophages (5).

Krupa *et al*. (6, 7) have found that mature, highly activated dendritic cells (DCs) are present in temporal artery inflammatory lesions in GCA and that they are located at the adventitia-media junction. These adventitial DCs produce chemokines, recruit and locally activate T cells. Toll-like receptors (TLR) are expressed on DCs. Ligands of TLR4 are able to induce the activation and maturation of adventitial DCs, which initiate the cascade of events that leads to the activation of T cells in the arterial adventitia and subsequent vessel wall inflammation.

GCA is a polygenic disease and several studies have shown the implication of genetic variants in key components of immune and inflammatory pathways in GCA susceptibility (8). Recently, two co-segregating single nucleotide polymorphisms (SNPs), Asp299Gly and Thr399Ile, within the gene encoding TLR4 have been characterized (9) and studied in different inflammatory and infectious conditions (10-18). These polymorphisms have been shown to impair the efficacy of lipopolysaccharide signalling and the capacity to elicit inflammation (9).

The aim of this study was to examine the relationship between Asp299Gly and Thr399Ile polymorphisms and the susceptibility to and clinical expression of GCA in a population-based cohort of Italian patients with biopsy-proven disease.

Patients and methods

Study population

We reviewed the computerized registry of the Pathology Laboratory at Arcispedale Santa Maria Nuova, which keeps records of the results of all temporal artery biopsies performed in Reggio Emilia, Italy, between 1986 and 2005. GCA-positive specimens were reviewed by a pathologist. A total of 184 GCA patients residing in the Reggio Emilia area were identified. Their median age was 74 years (range 56-90 years). Of these, it was possible to contact 155 patients, all of whom were willing to participate in this study.

Patients were diagnosed as having biopsy-proven GCA if histologic examination of the temporal artery biopsy specimen showed disruption of the internal elastic lamina, with infiltration of mononuclear cells into the arterial wall, with or without giant cells. Temporal artery biopsy procedures in Reggio Emilia have been described in detail elsewhere (19, 20). Temporal artery biopsy was routinely performed in all patients with clinical manifestations of GCA. Segments longer than 2 cm were generally obtained.

The clinical findings at diagnosis and during follow-up, the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) values at diagnosis, as well as the initial prednisone dosage, were ascertained through interviews with the patients and by reviewing the patients' medical records. The patients were subgrouped according to the presence or absence of polymyalgia rheumatica (PMR) (marked aching and early morning stiffness bilaterally, without any other apparent cause, in at least 2 of the 3 following regions: neck, shoulder girdle, or hip girdle) and the presence or absence of severe ischemic complications (vision loss and/or cerebrovascular accidents).

Severe ischemic complications were attributed to GCA if they occurred within the time between the onset of GCA symptoms/signs and one month after the onset of corticosteroid therapy.

Severe ischemic complications developing later were considered GCA-related only when associated with at least one of the other GCA signs/symptoms and elevation of ESR and/or CRP.

Controls were randomly recruited from the lists of patients who were under the care of the medical practitioners of the same public health service. Control patients had no evidence of GCA and/or PMR. Stratification by the random-number method according to age and sex was used to approximately match the controls with the patients according to their age and sex distribution. At the end of this selection process, 210 control subjects were identified. The median age of the controls was 68 years (range 51-82 years).

All study subjects were white, of Italian descent, and had been residents of Italy for at least 1 generation. No ethnic differences were found between the patients and the controls. None of the study participants were of Jewish ancestry.

The study was approved by the Ethics Committee of Reggio Emilia Hospital. Informed consent was obtained from all patients or their relatives.

DNA extraction and genotyping

DNA was obtained from whole blood using phenol/chloroform method, according to standard procedures (21). PCR was performed to amplify two small regions of TLR4 gene (22), a fragment of 248 bp containing rs4986790 Ex4+636A>G Asp299Gly polymorphism (forward primer 5'GATTAGCATACTTAGACTACTACCTC<u>C</u>ATG3' and reverse primer 5'GATCAACTTCTGAAAAAGCATTCCCAC3') and a fragment of 405 bp containing rs4986791 Ex4+936C>T Ile399Thr polymorphism (forward primer 5'GGTTGCTGTTCTCAAAGTGATTTTGGGA<u>G</u>AA3' and reverse primer 5'CCTGAAGACTGGAGAGTGAGTTAAATGCT3'). Both forward primers have an altered nucleotide (underlined bases) that is useful to create a restriction site for the endonucleases Nco I and Hinf I respectively. PCR amplifications was performed in 25 ul reaction containing 100 uM of each dNTP, 20 pmol each primer, 1 units Taq polymerase. Amplification profile for two amplicons was as follows:

- initial denaturation 95 °C for 2 min 35 cycles of: 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec final extension at 72 °C for 3 min for 248 bp amplicon;
- initial denaturation 95 °C for 2 min 35 cycles of: 94 °C for 30 sec, 66 °C for 30 sec, 72 °C for 30 sec final extension at 72 °C for 3 min for 405 bp amplicon.

Digestion was performed on 10 ul PCR products of two amplicons using Nco I and Hinf I restriction endonucleases. These enzymes can reveal the alleles because Nco I cuts the amplicon only if A allele is replaced by G and Hinf I cuts the 405 bp amplicon if C nucleotide is replaced by T. Different genotypes were shown by electrophoresis analysis of digested PCR products in 2.5% agarose gel stained with ethidium bromide (0.5 ug/ml).

Statistical analysis

Statistical analysis was done using SPSS statistical package (SPSS Inc., Chicago, IL, USA). Student's t-test and Mann-Whitney test were computed to compare means for parametrically and non-parametrically distributed data, respectively. The frequencies of the alleles and genotypes among the case patients and controls were compared by chi-square test. Odds ratios (OR) were calculated together with their 95% confidence intervals (95% CI). We performed power calculation for an unmatched case-control study and estimated relative risk using Power and Sample Size calculation version 2.1.31 software.

Results

Table I shows the clinical and demographic characteristics of the 155 patients with GCA. Permanent partial or total visual loss and/or cerebrovascular accidents were diagnosed in 33 patients. Anterior ischemic optic neuropathy was seen in 24 patients, and central retinal artery occlusion in 6 patients. 4 patients had a vertebro-basilar stroke.

The allele and genotype frequencies of the TLR4 Asp299Gly polymorphism in GCA patients and in the control group are shown in Table II. Of the 155 GCA patients, 21 were heterozygous for the Asp299Gly TLR4 allele, while of 210 controls, 17 were heterozygous. No homozygous subjects were found in either group. In these 21 GCA patients

Table I. Demographic and clinical features of the 155 patients with biopsy proven giant cell arteritis*.

% Males/% Females	33/155 (21.3) / 122/155 (78.7)
Age at onset of disease, years, mean ± SD	74 ± 7
Headache	123/155 (79.4%)
Abnormalities of temporal arteries**	103/154 (66.9%)
Scalp tenderness	60/152 (39.5%)
Jaw claudication	75/155 (48.4%)
Visual loss	30/155 (19.4%)
Severe ischemic complications***	33/155 (21.3%)
Systemic symptoms and/or signs§	113/155 (72.9%)
Polymyalgia rheumatica	70/155 (45.2%)
Duration of therapy months, mean ± SD	19 ± 15
Duration of follow-up months, mean ± SD	25 ± 20
ESR at diagnosis mm/hour, mean ± SD	90 ± 30
CRP at diagnosis mg/dl, mean ± SD	9.2 ± 6.4

*Except where indicated otherwise, values are the number (%) of patients; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.
**Artery tenderness and/or decreased or absent temporal artery pulsation
***Severe ischemic complications comprised visual loss and/or cerebrovascular accidents.
§Presence of at least one of the following: anorexia, weight loss of at least 4 kg, or fever.

Table II. Frequencies of alleles, genotypes and carriage rates of Toll-like receptor (TLR) 4 polymorphism Asp299Gly in patients with giant cell arteritis and controls*.

	GCA patients (155)	Controls (210)	*p*-value	OR (95% CI)
Allele				
G	21/310 (6.8)	17/420 (4.0)		
A	289/310 (93.2)	403/420 (96.0)	0.13	1.72 (0.89-3.32)
Genotypes				
G/G	0/155 (0.0)	0/210 (0.0)		
G/A	21/155 (13.5)	17/210 (8.1).	NS	
A/A	134/155 (86.5)	193/210 (91.9)		
Carriage rate				
G/A+ G/G	21/ 155 (13.5)	17/210 (8.1).	0.11	1.78 (0.90-3.50)
AA	134/155 (86.5)	193/210 (91.9)		

*Values are the number/total number examined (%). GCA: giant cell arteritis.
OR: odds ratio; 95% CI: 95% confidence interval.

and in these 17 controls, co-segregation of the Thr399Ile polymorphism was observed, while no GCA patients or controls had an isolated Asp299Gly polymorphism. The distribution of the TLR4 Asp299Gly did not differ significantly between GCA patients and controls, although the G/A genotype was more frequent in GCA patients as compared with the controls (13.5% *vs*. 8.1%, respectively).

Allele G frequency (6.8% *vs*. 4.0%) and carriers of the G allele (G/A+ G/G) (OR 1.78, 95% CI 0.90-3.50) were more frequent in GCA patients than in controls. However, these differences were not statistically significant.

Given the sample size (155 patients with GCA and 210 controls) and the allele frequencies of the polymorphism examined, we can conclude with 80% certainty that there is a genetic relative risk of 2.9 at rs4986790, Ex4+636A>G, (Asp299Gly) TLR-4 polymorphism.

The association between the TLR4 Asp299Gly polymorphism and the clinical features of GCA was evaluated by comparing the 70 GCA patients who had PMR with the 85 patients who did not have PMR. We also compared the 33 GCA patients who had severe ischemic complications (visual loss and/or cerebrovascular accidents) with the 122 patients who did not have ischemic complications. No significant associations were found (data not shown).

Discussion

TLR-4 is an important pathogen recognition receptor, which plays a major role in the innate and adaptative immune responses by binding to pathogens, microbial toxins, or endogen ligands such as heat-shock proteins, modified lipids, fibrinogen, fibronectin, hyaluronic acid, all of which are abundantly present in the inflamed tissues (23, 24). Activation of TLRs results in the release of antimicrobial peptides, inflammatory cytokines, and costimulatory molecules that initiate adaptive immunity.

Weyand *et al*. have hypothesized that the initial events in GCA are mediated by the transition of immature adventitial DCs to the mature state (6, 7). DCs in arteries of GCA patients are highly activated, as indicated by the expression of CD83 and CD86, and can provide the necessary costimulatory signals to activate T cells. In noninflamed temporal arteries immature and quiescent DCs are located at the adventitia-media border. Transcripts for TLRs 2 and 4 were detected in all normal temporal arteries, and immunohistochemical staining confirmed expression of TLR2 and 4 on DCs in the adventitia. DCs are induced to mature by TLR activation. Weyand *et al*. using a model of human temporal arteries engrafted into severe combined immunodeficiency (SCID) mice demonstrated that blood-borne TLR ligands can initiate the transition of immature arterial DCs to mature DCs (6). In particular, triggering of TLR 4 by lipopolysaccharide (LPS) determined full DC activation, highlighting the key role of TLR4 in the induction of inflammatory process in GCA.

Accumulating evidence indicates that TLRs play a role in many disease processes, including bacterial sepsis, atherosclerosis, allergic and autoimmune diseases (24-27). In particular, inappropriate activation of TLR pathways by endogenous or exogenous ligands may lead to the initiation and/or perpetuation of autoimmune responses.

In 2002, Arbour *et al*. described two cosegregating polymorphisms of the human TLR4 gene, Asp299Gly and Thr399Ile (9). These two single nucleotide polymorphisms (SNPs) are constituted by an A/G transition causing

an aspartic acid/glycine substitution at amino acid location Asp299Gly (rs4986790), and a C/T transition causing a threonine/isoleucine switch at amino acid location Thr399Ile (rs4986791).

Both polymorphisms occur with an allelic frequency of approximately 3% to 6% in the Caucasian populations (28). In our control population we found an allelic frequency (6.8%) similar to that reported in a previous Italian study (7.2%) (29). Furthermore, consistent with the very rare occurrence of homozygous mutations, we did not find homozygous in our patients and controls.

Arbour *et al*. were the first to investigate the functional consequence of TLR4 polymorphisms (9). They reported that individuals with either the Asp299Gly and/or Thr399Ile polymorphisms had a blunted response to inhaled LPS. Furthermore, transfected cells with TLR4 polymorphisms have a decreased NF-κB activity compared with wild-type TLR4, leading to reduced cytokine production.

Since their identification, a series of studies have examined the impact of these polymorphisms on the incidence and course of infectious diseases with conflicting results (18). TLR4 polymorphism have also been examined in atherosclerosis, myocardial infarction and in different chronic inflammatory diseases. Although several studies have reported a reduced risk of carotid atherosclerosis and myocardial infarction in individuals with these mutations, these results could not be reproduced in larger studies (26, 30). Association of these polymorphisms with Crohn's disease is also controversial. However, a recent meta-analysis using datasets from published study showed a significantly higher frequency of Asp299Gly mutant allele in Crohn's patients as compared to healthy controls (10). A significant association was also found between the chronic course of sarcoidosis and TLR4 mutations (16). The TLR4 polymorphisms have also been studied in some rheumatic conditions. There was no evidence for a role of these mutations in susceptibility to ankylosing spondylitis (AS) in 4 studies, while only a modest association was observed in a Canadian study (11, 12, 31-33). Discordant data have also been reported regarding the association of these polymorphisms with susceptibility to RA and response to treatment (14, 15, 34). However, these polymorphisms have never been evaluated in patients with vasculitides.

Our study is the first to examine TLR4 polymorphisms in GCA. We evaluated these polymorphisms in an ethnically homogeneous and large inceptional cohort of Italian patients with GCA. There was a trend for increased frequency of the G allele carrier status in GCA patients, but this did not reach statistical significance. GCA is a granulomatous vasculitis that affects large and medium-sized arteries. Interestingly, TLR4 mutations have been implicated in the risk of developing Crohn's disease and chronic sarcoidosis (10, 16), that are two inflammatory granulomatous disorders, suggesting that a genetically determined defective signaling through the TLR4 receptor may predispose to an exaggerated inflammatory granulomatous response.

A second aim of this study was to determine whether these TLR4 polymorphisms might be associated with the presence of severe ischemic complications (vision loss and/or cerebrovascular accidents) or with PMR. However, when patients with and those without these manifestations were compared, no associations were found.

Our study has several strengths such as the population-based design and the use of temporal artery biopsy for identifying GCA patients, however it is limited by the low sample size and requires validation.

In conclusion, we did not find any association between Asp299Gly and Thr399Ile TLR4 polymorphisms and susceptibility to, and clinical expression of, GCA. These results do no support the hypothesis that a genetically determined regulation of TLR4 signaling may predispose to the development and clinical expression of this vasculitis in Italian patients. Further, larger studies are required to confirm our findings in other populations.

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