Association of a Functional Inducible Nitric Oxide Synthase Promoter Variant with Susceptibility to Biopsy-Proven Giant Cell Arteritis

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ABSTRACT. ***Objective.*** To assess the contribution of 2 polymorphisms within the inducible nitric oxide (*NOS2A*) promoter region to susceptibility to giant cell arteritis (GCA).

Methods. One hundred three patients with biopsy-proven GCA and 198 ethnically matched controls from the Lugo region (Northwest Spain) were studied. Patients and controls were genotyped using polymerase chain reaction techniques for a multiallelic $(CCTTT)_n$ and for the TAAA repeat polymorphism in the promoter region of the *NOS2A* gene.

Results. No significant differences in allele or genotype frequencies for the $(CCTTT)_n$ repeat polymorphism in the *NOS2A* gene between patients with GCA and controls were observed. However, significant differences for the TAAA repeat polymorphism between patients and controls were found. The overall distribution of *NOS2A* TAAA genotypes in patients with biopsy-proven GCA was significantly different than controls (p = 0.026). Patients with GCA had an increased frequency of the *NOS2A* TAAA+ allele (16.5%) compared with controls (9.1%) (p = 0.007; OR 1.98; 95% CI 1.20–3.27). This was due to an increased frequency of both heterozygotes (27.2%) and homozygotes (2.9%) for *NOS2A* TAAA+ observed in patients compared to controls (15.2% and 1.5%, respectively) (p = 0.007; OR 2.15; 95% CI 1.23–3.78).

Conclusion. Our results suggest a potential implication for *NOS2A* TAAA gene polymorphism in GCA susceptibility. (J Rheumatol 2005;32:2178–82)

Key Indexing Terms:
GIANT CELL (TEMPORAL) ARTERITIS TEMPORAL ARTERY BIOPSY
DISEASE SUSCEPTIBILITY NITRIC OXIDE NOS2A POLYMORPHISMS

Giant cell arteritis (GCA) involves large and medium-size blood vessels with a predisposition to the involvement of cranial arteries[1]. It is the most common systemic vasculitis in people over the age of 50 years in Western countries[2], in particular in those with Northern European ancestry[2-4]. The main manifestations of GCA are due to vascular involvement.

Reports of well documented families of first-degree relatives with GCA support a genetic component in the pathogenesis of this vasculitis[5]. Associations between GCA and genes that lie within the HLA class II region have been described[6]. However, GCA appears to be a polygenic disease and different genes may influence the phenotype and the outcome of this condition[7].

Nitric oxide (NO) is the product of conversion of L-arginine to L-citrulline by a class of enzymes called NO synthases (NOS). It plays a role in both prevention and development of atherosclerosis. NO is produced constitutively by endothelial (eNOS or NOS3) or neuronal (nNOS or NOS1) synthases or in higher concentrations by iNOS (or NOS2) after stimulation of a variety of proinflammatory cytokines[8].

Several functionally relevant polymorphisms in the *NOS2A* and *NOS3* genes have been identified. Association of these polymorphisms with different vascular[9], autoimmune[10], and infectious diseases[11] has been reported. Haplotype association between 3 polymorphisms of the *NOS3* gene was found in patients with GCA from Northwest Spain[12]. Also, an association with a Glu/Asp(298) polymorphism in exon 7 of the *NOS3* gene has recently been reported in patients from Italy with GCA[13].

NOS2A is located on chromosome 17q11.2-12, and 2 *NOS2A* microsatellites, the CCTTT repeat sequence in position –2662 to –2608, and the TAAA repeat polymorphism in

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position –754 to –739, have been described (Genebank accession number X97821).

A highly polymorphic pentanucleotide $(CCTTT)_n$ repeat located at the *NOS2A* promoter region has been shown to be functionally important in the regulation of *NOS2A* transcription[10]. A trend toward association of $(CCTTT)_n$ repeat variations with rheumatoid arthritis (RA) has been reported[14]. Of note, significant differences in this *NOS2A* promoter polymorphism genotype frequency between patients with RA from Northwest Spain and controls have been observed[15]. Also, a functional polymorphism in the proximal promoter involving the insertion or deletion of one unit of a TAAA repeat[16,17] has proved to be associated with increased risk of renal abnormalities and other complications of type 2 diabetes[18].

We assessed the contribution of these 2 polymorphisms within the *NOS2A* promoter region to susceptibility to GCA in a series of patients with biopsy-proven GCA.

MATERIALS AND METHODS

Study population. We examined iNOS polymorphisms in a series of 103 consecutive patients diagnosed with biopsy-proven GCA in the Division of Rheumatology of the Hospital Xeral-Calde (Lugo, Spain) who gave informed consent for immunogenetic studies. All patients fulfilled the 1990 American College of Rheumatology criteria for the classification of GCA[19]. The mean age ± standard deviation (SD) of the patients was 74.5 ± 6.0 years, and the ratio of women/men was 1.3/1.0. Age (± 3 yrs) and sex matched controls were also studied. Since all patients assessed in this study were from the Lugo region in Northwest Spain, all controls (n = 198) were recruited from the same area. They were required to be healthy volunteers living in and around the city of Lugo, and we could trace their ancestry in the Lugo region for at least 3 generations. The main characteristics of the Lugo population have been reported[20]. Only patients with GCA who 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 were included in this study.

Patients with GCA were considered to have an associated polymyalgia rheumatica (PMR) if they had severe bilateral aches and pains involving the neck, the shoulder, and/or the pelvic girdles, associated with morning stiffness[21,22]. As reported[23], patients were considered to have severe ischemic manifestations if they suffered visual manifestations (transient visual loss including amaurosis fugax, permanent visual loss, or diplopia), cerebrovascular accidents (stroke and/or transient ischemic attacks), jaw claudication, or limb claudication of recent onset. With regard to smoking, as reported, we established 2 categories: (1) heavy smokers who still smoked at the time of disease diagnosis or who had smoked within 10 years before onset of GCA symptoms; and (2) patients who never smoked or stopped smoking at least 10 years before disease onset[24,25]. Corrections were made for age and sex within the GCA groups with or without severe ischemic manifestations or PMR and for sex and history of heavy smoking.

Patients and controls gave written informed consent prior to participation in the genetic studies, which was approved by the local institutional committee.

TAAAn and $(CCTTT)_n$ genotyping. DNA was isolated from anticoagulated peripheral blood mononuclear cells using standard methods. We determined the $TAAA_n$ and $(CCTTT)_n$ genotypes by a polymerase chain reaction (PCR) method as described[26,27]. Forward and reverse primers were 5' TGC CAC TCC GCT CCAG 3' and 5' GGC CTC TGA GAT GTT GGT CTT 3' for $TAAA_n$, and 5' ACC CCT GGA AGC CTA CAA CTG CAT 3' and 5' GCC ACT GCA CCC TAG CCT GTC TCA 3' for $(CCTTT)_n$. The forward primers were 5' labeled with 6-FAM fluorescent dye. PCR aliquots of 0.5 μl were added to 3 μl of formamide and 0.5 μl of internal size standard. Samples were analyzed in denaturing gels (6% acrylamide/7 M urea) and sized using Genescan 672 software (Applied Biosystems, Foster City, CA, USA). With respect to $TAAA_n$ repeats, sequencing revealed that the longer PCR fragment, 224 bp in length, contained 5 TAAA repeats (denoted *NOS2A*+ allele) whereas the shorter one, 220 bp, contained 4 repeats (denoted *NOS2A*– allele). Concerning $(CCTTT)_n$ repeats, the size of the PCR products ranged from 171 bp to 216 bp, depending on the number of pentanucleotide repeat units.

Statistical analysis. Strength of association between patient groups and controls and alleles or genotypes of *NOS2A* polymorphisms was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher's exact analysis. Statistical significance was defined as $p \leq 0.05$. Calculations were performed with the Stata statistical package, V6. We used the UNPHASED software created for case-control analysis of haplotypes. The power of the study to detect an effect of a polymorphism in disease susceptibility was estimated using Quanto 0.5 software (Department of Preventive Medicine, University of Southern California, Los Angeles, CA, USA). The sample size used had 66% power to detect the effect of the TAAA polymorphism, conferring an OR of 1.9 at the 5% significance level.

RESULTS

Clinical characteristics of the GCA patients. The group of patients with biopsy-proven GCA comprised 59 women and 44 men (median age at disease diagnosis was 75 yrs, range 60–92 yrs). From onset of GCA symptoms until 1 month after onset of steroid therapy, 87 (84%) had headache, 77 (75%) abnormal temporal artery on physical examination, 41 (40%) PMR, and 53 (51%) developed severe ischemic manifestations. Among them, 41 (40%) had jaw claudication, 23 (22%) visual ischemic manifestations, and 1 (1%) stroke. Other clinical features of the 103 patients with biopsy-proven GCA are summarized in Table 1.

NOS2A promoter CCTTT repeat microsatellite polymor-

Table 1. Main clinical features of 103 patients with biopsy-proven GCA from Lugo (Northwest Spain). Numbers in brackets represent percentages.

Variable	
Age, yrs	
Mean ± SD	74.5 ± 6.0
Median	75
Range	60–92
Men: women	44:59
Women, %	57
Headache	87 (84)
Abnormal temporal artery on physical examination	77 (75)
PMR	41 (40)
Jaw claudication	41 (40)
Visual manifestations*	23 (22)
Stroke	1 (1)
Arm claudication due to ischemia of the humeral artery	1 (1)
Severe ischemic manifestations**	53 (51)
ESR > 40mm/h	103 (100)

* Transient visual loss including amaurosis fugax, permanent visual loss, or diplopia. ** Visual manifestations, cerebrovascular accidents (stroke and/or transient ischemic attacks), jaw claudication, or limb claudication of recent onset.

phism in GCA. $(CCTTT)_n$ allele and genotype frequencies were examined in patients with biopsy-proven GCA and controls. No evidence of departure from Hardy-Weinberg equilibrium was observed (p = 0.80) in controls. Table 2 shows the allelic frequencies in patients and controls. Overall $(CCTTT)_n$ allelic distribution did not show statistically significant differences between patients with GCA and controls (p = 0.31) or any significant differences in allele or genotype frequencies for the $(CCTTT)_n$ repeat polymorphism in *NOS2A* (Table 2). No significant differences in allele or genotype distribution were found when patients with GCA were stratified according to presence of PMR, severe ischemic manifestations, smoking history, or sex (data not shown).

NOS2A promoter TAAA repeat polymorphism in GCA. When overall genotype distribution of patients with GCA was compared to controls, a significant difference was observed (p = 0.026, by chi-square test from 3 × 2 contingency table; Table 3). This was due to an increase in both heterozygotes +/– and homozygotes +/+ for *NOS2A* genotypes observed in patients with biopsy-proven GCA compared to controls (p = 0.007, OR 2.15, 95% CI 1.23–3.78). In addition, a significantly increased frequency of the *NOS2A* TAAA+ allele was observed in patients with biopsy-proven GCA compared with controls (p = 0.007, OR 1.98, 95% CI 1.20–3.27; Table 3). However, no allele or genotype differences were observed when patients with GCA were stratified according to presence of PMR, severe ischemic manifestations, smoking history, or sex (Table 4). It was also the case when patients with GCA were corrected by age, sex, and heavy smoking history.

NOS2A promoter CCTTT– TAAA haplotypes. The relationship between the 2 microsatellites repeats in the *NOS2A* promoter was investigated in our population. For the 2 TAAA alleles, there was a strikingly wide distribution of CCTTT microsatellite alleles, reflecting lack of linkage disequilibrium (LD) between the 2 markers analyzed (global Δ 0.27).

DISCUSSION

Our study is the first to assess the influence of *NOS2A* polymorphism in the development of GCA in a large series of patients whose diagnosis was confirmed by temporal artery

Table 2. Allele frequencies of *NOS2A* $CCTTT_n$ gene polymorphism in patients with biopsy-proven giant cell arteritis (GCA) and controls. * The overall allelic distribution for the NOS2A $CCTTT_n$ gene polymorphism did not show statistically significant differences between patients and controls.

Repeat Number	Size, Base Pair	Patients, n = 206 (%)	Controls, n = 396 (%)	OR	95% CI	p
7	171	1 (0.5)	0 (0)	—	—	0.20
8	176	2 (1.0)	5 (1.3)	0.78	0.14–4.14	0.77
9	181	9 (4.3)	23 (5.8)	0.77	0.34–1.74	0.52
10	186	22 (10.7)	38 (9.6)	1.13	0.62–2.06	0.68
11	191	30 (14.6)	72 (18.1)	0.82	0.49–1.36	0.44
12	196	69 (33.5)	135 (34)	1	(reference)	—
13	201	43 (20.9)	64 (16.1)	1.31	0.81–2.13	0.27
14	206	15 (7.2)	42 (10.6)	0.70	0.36–1.35	0.28
15	211	7 (3.4)	11 (2.8)	1.25	0.46–3.35	0.66
16	216	8 (3.9)	6 (1.5)	2.60	0.87–7.82	0.08

Table 3. Allele frequencies and genotype distribution of *NOS2A* TAAA polymorphism in patients with GCA and controls.

	GCA, n = 103	Controls, n = 198	OR	95% CI	p
Allele (2N) (%)					
–	172 (83.5)	360 (90.9)	1	(reference)	—
+	34 (16.5)	36 (9.1)	1.98	1.20–3.27	0.007[a]
Genotype[b] (%)					
–/–	72 (69.9)	165 (83.3)	1	(reference)	—
–/+	28 (27.2)	30 (15.2)	2.14	1.19–3.84	0.001
+/+	3 (2.9)	3 (1.5)	2.29	0.45–11.63	0.30
–/+ plus +/+	31 (30.1)	33 (16.7)	2.15	1.23–3.78	0.007[c]

[a] Allele + was increased in patients compared to controls (p = 0.007, OR 1.98, 95% CI 1.20–3.27). [b] Genotype distribution showed statistically significant differences between patients and controls (p = 0.026). [c] Patients showed an increased frequency of –/+ and +/+ genotypes compared to controls (p = 0.007, OR 2.15, 95% CI 1.23–3.78).

Table 4. Clinical features according to *NOS2A* TAAA polymorphism in patients with GCA. No statistically significant differences among the different groups were observed.

	GCA with PMR		GCA with Severe Ischemic Manifestation		Heavy Smoking			
	Yes	No	Yes	No	Yes	No	Female	Male
Allele (2N)								
–	70 (85.4)	102 (82.3)	88 (83)	84 (84)	24 (92.3)	148 (82.2)	97 (82.2)	75 (85.2)
+	12 (14.6)	22 (17.7)	18 (17)	16 (16)	2 (7.7)	32 (17.8)	21 (17.8)	13 (14.8)
Genotypes								
–/–	30 (73.2)	42 (67.7)	36 (67.9)	36 (72)	11 (84.6)	61 (67.8)	40 (67.8)	32 (72.7)
–/+	10 (24.4)	18 (29)	16 (30.2)	12 (24)	2 (15.4)	26 (28.9)	17 (28.8)	11 (25)
+/+	1 (2.4)	2 (3.3)	1 (1.9)	2 (4)	0 (0)	3 (3.3)	2 (3.4)	1 (2.3)

PMR: polyarthralgia rheumatica.

biopsy. No association with the polymorphic pentanucleotide $(CCTTT)_n$ repeat in the promoter region of *NOS2A* was found. However, significant differences were observed when a biallelic TAAA – repeat located 0.75 kb upstream of the gene were assessed. Unlike healthy controls, those with biopsy-proven GCA exhibited an increased frequency of *NOS2A* TAAA+ allele. The relative absence of LD between CCTTT and TAAA markers indicates that the association of TAAA repeats with GCA is independent of the CCTTT repeats. Of interest, a recent study has shown a lack of LD between the CCTTT microsatellites and dinucleotide repeats in the *NOS2A* promoter region[28]. Also, the *NOS2A* TAAA association observed in our study was independent of the previous NOS3 gene haplotype association found in patients with GCA[12].

Our results reinforce the potential implication of *NOS2A* TAAA polymorphism in GCA susceptibility. *NOS2A* TAAA+ allele has been also associated with an elevated plasma glucose level and unstable angina[29]. In addition, a recent study has proposed a role of the *NOS2A* TAAA repeat in more pronounced coronary artery disease[30].

Immunochemistry and *in situ* hybridization techniques have shown that macrophages and vascular smooth muscle cells express iNOS in both early and advanced atherosclerotic lesions[31-33]. Production of NO by increased expression of iNOS may have a double role in the development of atherosclerotic lesions. As a pro-oxidant, NO may also promote the process of atherogenesis by increasing platelet adherence, cell death, and necrosis[34]. Interestingly, patients with GCA present with clinical manifestations that are the result of vascular involvement. Since it is often difficult to differentiate healed arteritis from arteriosclerosis on morphological grounds, some investigators have suggested that atherosclerosis and GCA may have a common pathway[35,36]. Machado, *et al*, in a retrospective case-control study of 88 patients with biopsy-proven GCA, reported an association between smoking and disease development[37]. Duhaut, *et al*, in a prospective multicenter case-control study of 207 patients with biopsy-proven GCA, described a strong association between smoking and previous atheromatous disease in women and GCA[38]. As a result of atherosclerosis and subclinical inflammation, as described in Rochester, USA, where GCA incidence is high[39], we also observed the development of aortic aneurysmal disease in followup of patients with GCA from Northwest Spain[24]. Thus, abnormalities in *NOS2A* may play a potential role in the development of this vasculitis.

Since in transient transfection assays the presence of *NOS2A* TAAA+ allele has been associated with increased *NOS2A* promoter activity[18], our results suggest that the higher production of NO by iNOS due to an increased frequency of *NOS2A* TAAA+ allele in patients with biopsy-proven GCA may be detrimental rather than beneficial to individuals for the development of this vasculitis. Additional studies are required to ascertain the mechanism behind the association between *NOS2A* TAAA polymorphism and GCA. In this regard, studies still are needed to evaluate whether the *NOS2A* 4 bp insertion/deletion affects iNOS expression in other cell lines or in temporal artery biopsy specimens. In addition, replication studies in other populations would confirm the validity of our observations.

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