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# Tumor Necrosis Factor- $\alpha$ -308 Gene Polymorphism in Croatian and Slovenian Multiple Sclerosis Patients

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## Key Words

Multiple sclerosis · Tumor necrosis factor- $\alpha$  polymorphism · Association studies

## Abstract

Previous findings regarding the role of TNF- $\alpha$ -308 gene polymorphism in multiple sclerosis (MS) are contradictory. The aim of this study was to investigate the possible influence of TNF- $\alpha$ -308 polymorphism on MS susceptibility and the MS disease process in a Croatian and Slovenian population. Genotyping was performed in 338 patients and 460 healthy controls. The TNF2 allele was present in 123 (26.8%) healthy controls vs. 67 (19.9%) MS patients ( $p = 0.023$ , odds ratio = 0.68, 95% confidence interval = 0.48–0.95), suggesting that carriage of the TNF2 allele might decrease MS risk. The difference in TNF2 allele carrier frequency between patients and controls was identified in the relapsing-remitting MS group. There was no association between TNF2 allele carrier status and age at disease onset or disease progression. Our results suggest that, in the study populations, the TNF- $\alpha$ -308 polymorphism may play a role in MS susceptibility.

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## Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. Its etiology is clearly complex, and genetic influences as well as exogenous factors are likely to play a role in disease pathogenesis. The autoimmune nature of MS logically suggests cytokine genes as candidate genetic loci determining disease susceptibility and/or influencing disease behavior.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a proinflammatory cytokine, is believed to play an important role in the pathogenesis of several immune-mediated diseases, including MS. Therefore, several groups have investigated the relevance of the TNF- $\alpha$ -308 gene polymorphism to MS, but with conflicting results [1–10].

The TNF- $\alpha$  gene has a restriction fragment polymorphism in the promoter region: the TNF1 allele contains a guanine (G), and the TNF2 allele contains an adenosine (A). This polymorphism has been shown to have functional implications. The less common TNF2 allele is associated with increased transcription and a high level of TNF- $\alpha$  in blood [11].

A number of previous studies demonstrated a lack of association between the TNF2 allele and susceptibility to

**Table 2.** Genotype and allele frequencies of the TNF- $\alpha$ -308 gene polymorphism in MS patients and controls

TNF- $\alpha$ gene	MS patients				controls (n = 460)
	PPMS (n = 25)	SPMS (n = 127)	RRMS (n = 173)	total (n = 338)	
Genotype					
GG	21 (84.0)	99 (77.9)	140 (80.9)	271 (80.2)	337 (73.3)
AG	4 (16.0)	25 (19.7)	27 (15.6)	58 (17.2)	114 (24.8)
AA	0	3 (2.4)	6 (3.5)	9 (2.7)	9 (2.0)
Allele, %					
G (TNF1)	92.0	87.8	88.7	88.8	85.7
A (TNF2)	8.0	12.2	11.3	11.2	14.3

PPMS = Primary progressive MS; SPMS = secondary progressive MS; RRMS = relapsing-remitting MS. Figures in parentheses are percentages.

to each other. However, the number of primary progressive MS patients was too small (n = 25) to permit firm conclusions.

We also investigated whether TNF- $\alpha$ -308 polymorphism influenced disease behavior. There was no association between genotype or carrier status of the studied polymorphism and age at disease onset, disease duration or EDSS (table 3).

## Discussion

Strong evidence supports the hypothesis that MS is determined by genetic and environmental factors, but these factors remain largely undefined. A large body of data indicates that variations in the genes coding for or regulating the cytokine expression may play a role in MS susceptibility. There are several studies addressing the possible association between TNF- $\alpha$ -308 polymorphism and MS (table 4). Most of these have suggested that, although presence of the less common TNF2 allele may increase TNF- $\alpha$  expression in vitro, the polymorphism does not contribute to genetic susceptibility [1–6]. On the other hand, 4 studies have reported findings suggestive of an association between MS and the TNF2 allele. Three studies (the first comprised Swedish patients, the second Norwegians and the third Caucasian patients from the State of Minnesota, USA) showed a trend towards greater prevalence of the TNF2 allele in controls [7–9]. More recently, Drulovic et al. [10] investigated 143 unrelated patients and 123 ethnically matched controls from a Serbian population. They demonstrated that the frequency of the TNF2 allele was significantly decreased in MS patients

**Table 3.** Clinical characteristics according to TNF- $\alpha$ -308 genotype status

Genotype	Age of onset years	Duration years	EDSS	PI
GG (n = 271)	29.14 $\pm$ 9.13	13.68 $\pm$ 11.01	4.19 $\pm$ 2.52	0.36 $\pm$ 0.26
GA (n = 58)	31.16 $\pm$ 8.52	13.32 $\pm$ 9.02	4.35 $\pm$ 2.21	0.38 $\pm$ 0.22
AA (n = 9)	28.22 $\pm$ 5.43	11.89 $\pm$ 6.86	3.19 $\pm$ 2.22	0.21 $\pm$ 0.13

Values are means  $\pm$  SD.

(14%) in comparison to the frequency in controls (24%, p = 0.044), suggesting that TNF2 may be an MS protective factor in the HLA region.

An apparent inconsistency in the results of previous studies may be due to differences in MS etiology for patients of different ethnic origin. Indeed, the variable frequency of the TNF2 allele in different populations of both MS patients and healthy individuals may indicate that it is expressed differentially within populations with heterogeneous genetic background. For this reason, we specifically chose a Croatian and a Slovenian population for investigation of the contribution of the TNF- $\alpha$ -308 polymorphism to MS susceptibility and/or disease behavior. Historical data suggest that these countries are not only geographically close to Serbia but also have a similar Slavic origin.

In our study, genotype frequencies showed that the TNF2 allele was found in 26.8% of healthy controls compared with 19.9% of MS patients. These results are in agreement with those of Drulovic et al. [10], which also

**Table 4.** Results of published studies of the association between the TNF- $\alpha$ -308 polymorphism and susceptibility to MS

Reference	Population studied	Study group	TNF2 allele/ carrier frequency, %	p	OR (95% CI)	Comment
Mihailova et al. [1], 2005	Bulgarian	MS: 55 control: 86	20.0/30.9 13.9/26.7	0.593	1.22 (0.58–2.58)	cases: RRMS
De Jong et al. [2], 2002	Dutch	MS: 159 control: 273	31.0–37.0/ 27.0/–	–	–	cases: RRMS
Luccote et al. [3], 2000	French	MS: 74 control: 75	12.0/– 14.0/–	–	–	
Maurer et al. [4], 1999	German	MS: 283 control 1: 72 control 2: 66	16.8/30.0 11.8/23.6 18.9/36.4	0.268 0.335	1.40 (0.77–2.56) 0.76 (0.43–1.33)	control 1: ALS patients control 2: stroke patients
	Cypriot (Greek)	MS: 60 control: 20	–/15.0	–	–	
Fernandez-Arquero et al. [5], 1999	Spanish	MS: 238 control: 324	12.4/23.5 12.8/24.4	0.815	0.95 (0.04–1.41)	
Mycko et al. [6], 1998	Polish	MS: 53 control: 81	14.15/24.3 8.6/16.05	0.014	1.88 (0.80–4.40)	cases: RRMS and SPMS
He et al. [7], 1995	Swedish	MS: 93 control: 95	10.2/17.2 17.4/30.5	0.088	0.56 (0.28–1.1)	
Fernandes Filho et al. [8], 2002	Norwegian	MS: 133 control: 148	16.2/29.3 22.0/35.8	0.247	0.74 (0.45–1.23)	
Wingerchuck et al. [9], 1997	Caucasian from Minnesota, USA	MS: 110 control: 110	14.1/27.3 17.3/31.8	0.556	0.80 (0.45–1.43)	
Drulovic et al. [10], 2003	Serbian	MS: 143 control: 123	7.0/14.0 12.2/23.6	0.044	0.53 (0.28–0.99)	

Dutch, French, Cypriot: genotypes not specified. p value and OR: AA plus AG versus GG. RRMS = Relapsing-remitting MS; ALS = amyotrophic lateral sclerosis; SPMS = secondary progressive MS.

suggested that heterozygosity for the rare TNF2 allele, that is, the A/G genotype, confers protection against MS.

Another possible reason for the discrepancy between the findings of previous studies is a limited power to detect small differences in genotype frequencies between cases and controls. Most of the previous studies sampled smaller numbers of patients than did our study, which had an 80% power to detect a 1.45-fold increase in the frequency of the TNF2 carriers.

In conclusion, our data indicate that the TNF2 allele might be regarded as a protective factor with respect to MS development in our study population. Regarding the

lack of agreement among the results obtained in previous studies, larger samples of patients from different populations should be examined to clarify the possible role of the TNF- $\alpha$ -308 polymorphism in MS susceptibility.

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**Table 1.** Clinical profiles of MS patients (n = 338)

Female/male	2.5:1
Mean age at onset $\pm$ SD, years	29.47 $\pm$ 8.9
Course	
PPMS	26 (7.7%)
SPMS	127 (37.6%)
RRMS	185 (54.7%)
Mean duration $\pm$ SD, years	13.6 $\pm$ 10.6
Mean EDSS $\pm$ SD	4.20 $\pm$ 2.5
Mean PI $\pm$ SD	0.36 $\pm$ 0.25

PPMS = Primary progressive MS; SPMS = secondary progressive MS; RRMS = relapsing-remitting MS.

MS [1–6]. In contrast, 3 studies observed the TNF2 allele to be present less frequently among MS patients than among controls, although the differences were not statistically significant [7–9]. Drulovic et al. [10] (2003) provided the first report of an association between the TNF2 allele and MS, based on data from a Serbian population. In an effort to clarify the relationship between the TNF2 allele and MS, we investigated whether the TNF- $\alpha$ -308 polymorphism contributes to MS susceptibility and/or modifies the disease behavior in a Croatian and Slovenian population, another 2 populations of Slavic origin.

### Patients and Methods

A total of 338 patients (242 female; 96 male) with clinically definite MS, according to the criteria of Poser et al. [12], were recruited at collaborating genetic centers in Croatia (n = 175 patients) and Slovenia (n = 163 patients). The patients' clinical characteristics are summarized in table 1. Disability was evaluated using the Kurtzke Expanded Disability Status Scale (EDSS). Disease duration was calculated as the time between disease onset and the most recent examination at the clinic. The EDSS/disease duration (years) served as an index of disease progression (PI) and was calculated if disease duration was  $\geq$  5 years. The control group consisted of 460 unrelated healthy blood donors, matched for ethnicity, age and gender, whose families had no history of MS, or any other inflammatory-demyelinating disease. The study protocol was approved by the ethics committees of both centers and informed consent was obtained from each subject.

Genomic DNA was extracted from whole blood using a QIAmp blood kit (Qiagen, Hildren, Germany), as described by the manufacturer. The TNF-308 polymorphism was analyzed using the forward primer 5'-AGGCAATAGGTTTTGATTTTCAT-3' and the reverse primer 5'-TCCTCCCTGCTCCGATTCCG-3' to create a restriction site for the NcoI enzyme. The standard PCR reaction conditions have been described elsewhere [13]. PCR products were digested overnight at 37°C and analyzed by

running on 3% agarose gel and staining with ethidium bromide. The polymorphism was designed as TNF1 (-308G), which yielded 2 fragments of 87 and 20 bp, and TNF2 (-308A), which yielded a single 107-bp fragment.

Group differences in genotype and allele distributions were analyzed for statistical significance using the  $\chi^2$  test or Fisher's exact test as appropriate. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated in order to evaluate the effects of different genotypes/alleles. One-way ANOVA was employed for comparison of clinical characteristics between patients who were carriers (genotype A/G or A/A) and noncarriers (genotype G/G) of the TNF2 allele. A p value < 0.05 was considered statistically significant. The statistical power analysis revealed that the overall sample had 80% power to detect a 1.45-fold increase in frequency of TNF2 carriers (carrier frequency in patients = 19.9%).

### Results

Analysis of genotype and allele frequencies of the TNF- $\alpha$ -308 polymorphism in MS patients and controls is shown in table 2. The genotype distributions in both study groups were compatible with Hardy-Weinberg expectations (p = 0.960 in patients and p = 0.270 in controls). No significant differences in allele frequencies between MS patients overall and controls were found, although there was a trend toward an increased frequency of the TNF2 allele in controls (14.3%) compared to patients (11.2%; p = 0.12).

Due to the low frequency of patients and controls homozygous for the TNF2 allele (2.7 and 2%, respectively), all carriers of the TNF2 (A) allele, both homozygous and heterozygous, were grouped for further analysis. The genotype frequencies showed that the TNF2 allele was found in 123 (26.8%) healthy controls compared with 67 (19.9%) MS patients (p = 0.023, OR = 0.68, 95% CI = 0.48–0.95). Also, when the genotype frequencies were analyzed taking into account the origin of the samples, we were unable to find statistically significant differences between Croatian [cases: GG = 143 (81.8%), AG = 27 (15.4%), AA = 5 (2.8%); controls: GG = 217 (74.8%), AG = 67 (23.1%), AA = 6 (2.1%)] and Slovenian populations [cases: GG = 128 (78.5%), AG = 31 (19.0%), AA = 4 (2.5%); controls: GG = 120 (70.6%), AG = 47 (27.6%), AA = 3 (1.8%)].

As MS comprises different disease subtypes, it is important to evaluate whether MS patients with certain genotypes have an altered MS disease course. The statistical difference of TNF2 allele carrier frequency between patients and controls was identified in the relapsing-remitting MS group (p = 0.046). No significant differences were observed when the 3 MS subtypes were compared