

# Tumour necrosis factor gene polymorphisms and migraine in Greek children

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## Abstract

**Introduction:** Migraine is considered to be a multifactorial, complex disease. Various genetic and environmental factors contribute to the manifestation of this disease. The aim of this study was to determine whether polymorphisms in the tumour necrosis factor (TNF) region are associated with the risk of migraine. We examined the association between 6 single nucleotide polymorphisms in the coding regions of TNF- $\alpha$  and TNF- $\beta$  genes and migraine.

**Material and methods:** The study included two groups of children (group A and group B). Group A consisted of 103 unrelated children with typical migraine without aura 5-14 years of age. Group B (control group) consisted of 178 unrelated healthy children. The diagnosis of migraine was, in all patients, made according to the International Classification of Headache Disorders (ICHD II).

**Results:** According to our results positive family history was present in 62.2% of patients of group A. No significant differences were found in the frequencies of genotypes or alleles between patients and controls. The non-parametric analyses of variance showed no significant differences in the age at onset between genotype groups of the TNF- $\alpha$  and TNF- $\beta$  gene polymorphisms. Comparison of genotype frequencies between boys and girls in affected patients and control individuals were not significantly different ( $p = 0.089$ ,  $p = 0.073$  respectively). The distribution of TNF polymorphisms was not associated with the presence of family history of migraine in patients.

**Conclusions:** Our data indicate that TNF- $\alpha$  and TNF- $\beta$  gene polymorphisms are not a significant risk factor for migraine without aura in Greek children.

**Key words:** migraine, tumour necrosis factor  $\alpha$ , tumour necrosis factor  $\beta$ , polymorphism, children.

## Introduction

Migraine is a multifactorial, complex disease. The aetiology of a migraine attack is only partly understood [1]. It is considered that various genetic and environmental factors contribute to the manifestation of this disease [2, 3]. The mode of transmission of migraine in families is still unclear since migraine does not fit a simple Mendelian pattern.

The type and the number of genes which link to migraine are still unclear [4]. An increased knowledge of the genetic risk factors as well as an understanding of the underlying pathogenesis is expected to enable clinicians to identify children at a high risk and to treat childhood migraine more effectively [4].

Many of the genetic factors, such as the TNF gene, the calcium channel gene CACNA1A, and the EDNRB gene, which have to date been shown to be linked to adult migraine susceptibility have also been investigated in children [5, 6]. Polymorphisms in several candidate genes have been proposed either as susceptibility markers or as useful tools for the dissection of the related phenotypes [5-7]. The pathophysiology of migraine is still unknown, although “sterile inflammation” or “neurogenic inflammation” seems to play a key role [8].

Tumour necrosis factor (TNF) is a cytokine implicated in inflammatory reactions and endothelial function. There is considerable evidence for a major role of TNF in initiating inflammatory hyperalgesia [10-12].

Tumour necrosis factor can promote powerful hyperalgesia by causing prostanoïd release, increasing the expression of bradykinin receptors or by modulation of activity within sympathetic fibres. Increased levels of TNF- $\alpha$  in migraineurs have been documented; therefore, it may be involved in migraine [13]. Also, TNF- $\alpha$  is a possible pain mediator in neurovascular inflammation. Therefore it might be a potential mechanism for the generation of migraine pain. Rainero *et al.* [14] have reported that homozygosity for the G allele in TNF- $\alpha$ -308 was associated with an increased risk of migraine in an Italian population. However, previous studies concerning the role of TNF- $\alpha$  in migraine have provided conflicting results and there has been no confirmation of the role of TNF- $\alpha$ -308 markers in migraine patients [10, 14-16].

The aim of this study was to determine whether polymorphisms in the TNF region are associated with the risk of migraine. Therefore we examined the association between 6 single nucleotide polymorphisms in the coding regions of TNF- $\alpha$  and TNF- $\beta$  genes and migraine in Greek children.

## Material and methods

The study included two groups of children (group A and group B). Group A consisted of 103 unrelated children with typical migraine without aura 5-14 years of age (mean age  $\pm$  SE = 10.5  $\pm$  0.7), 46 boys (44.7%) and 57 girls (55.3%). All children of group A were attending the paediatric headache outpatient clinic of the 2<sup>nd</sup> Department of Paediatrics of the Medical School of Aristotle University of Thessaloniki. The diagnosis of migraine was, in all patients, made according to the International Classification of Headache Disorders (ICHD II) by a neurologist specialized in paediatric headaches, while a family history concerning the presence of migraine attacks in parents and siblings was obtained from the parents themselves, through an interview by specialized personnel [17]. The patient underwent

an extensive physical and neurological examination. Positive family history was present in 82% of patients of group A.

Group B (control group) consisted of 178 unrelated healthy children, 75 boys (42.1%) and 103 girls (57.9%), the age range (mean age  $\pm$  SE = 12.2  $\pm$  1.7). The healthy children (used as controls) consisted of individuals whose parents were interviewed and indicated in a questionnaire that they had never suffered from migraine or any similar condition and that the same was true of their first and second degree relatives. Patients and controls originated from the same geographical region (northern Greece) and were recruited in parallel, at a similar time and geographical location as the case group, to avoid the potential bias of population stratification. The mean age of the control group was, in an attempt to exclude subjects with late-onset migraine, purposely higher than the age of the patients. Written informed consent was obtained from all parents of the participants. This research was approved by the Local Ethics Committee.

## Genetic analysis

Genomic DNA was isolated from whole peripheral blood according to the protocol of the manufacturer (Pure gene, DNA purification system, Gentra). The analysis of DNA samples was performed by the PCR-RFLPs method. Six single nucleotide polymorphisms (SNPs) in TNF genes (TNF- $\alpha$ -238 G/A, TNF- $\alpha$ -308G/A, TNF- $\alpha$ -1031T/C, TNF- $\alpha$ -857C/T, TNF- $\alpha$ -376G/A, and TNF- $\beta$ -252 A/G) were genotyped by the PCR restriction fragment length polymorphism (RFLP) method.

## Genotyping

The forward primer sequence and the reverse sequence of all 6 SNPs are shown in Table I. In Table II are shown the restriction enzyme, the PCR product and digest fragment (bp) after the action of the restriction enzyme of all 6 polymorphisms.

## Statistical analysis

Hardy-Weinberg equilibrium (HWE) of alleles at individual *loci* and comparison of genotype frequencies between cases and controls were assessed by  $\chi^2$  statistics.

Estimation of allele frequencies and haplotypes was performed with the Gene Hunter program. Genotypes were tested for HWE using the exact test. The significance of differences in the allele and genotype frequencies between the groups was determined by the  $\chi^2$  test. One way analysis of variance performed via the Mann-Whitney test was used to evaluate the possible influence of TNF polymorphisms on the age at onset. The samples

**Table I.** Primer sequences of all TNF polymorphisms

SNPs	Primer name		Primers
TNF- $\alpha$ : -238G/A	TNF_238F	F	5'-AGAAGACCCCTCGGAACC-3'
	TNF_238_376R	R	5'-GCTGGTCTCTGCTGCCTTG-3'
TNF- $\alpha$ : -308G/A	TNF- $\alpha$ -308F	F	5'-AGGCAATAGGTTTTGAGGGCCAT-3'
	TNF- $\alpha$ -308R	R	5'-TCCTCCCTGCTCCGATTCCG-3'
TNF- $\alpha$ : -376A/G	TNF_376F	F	5'-CAACCCGTTTTCTCTCCCTC-3'
	TNF_238_376R	R	5'-GCTGGTCTCTGCTGCCTTG-3'
TNF- $\alpha$ : -857C/T	TNF_1031_857_F	F	5'-CAGGGGAAGCAAAGGAGAAG-3'
	TNF_1031_857_R	R	5'-CCCTCTACATGGCCCTGTCTAC-3'
TNF- $\alpha$ : -1031T/C	TNF_1031_857_F	F	5'-CAGGGGAAGCAAAGGAGAAG-3'
	TNF_1031_857_R	R	5'-CCCTCTACATGGCCCTGTCTAC-3'
TNF- $\beta$	TNF-B_F	F	5'-GGTTTCCTTCTGTCTCTGACTCTCC-3'
	TNF-B_R	R	5'-GAGAGAGATCGACAGAGAAGGGGAC-3'

**Table II.** The restriction enzyme, the PCR product and digest fragment (bp) after the action of the restriction enzyme of all 6 polymorphisms

SNPs	Restriction enzyme	PCR product [bp]	Restriction enzyme digest fragment [bp]	
			Normal	Mutant
TNF- $\alpha$ : -238G/A	MspI	299	280 $\pm$ 19	299
TNF- $\alpha$ : -308G/A	NcoI	107	87 $\pm$ 20	107
TNF- $\alpha$ : -376A/G	Tsp509I	504	504	418 $\pm$ 86
TNF- $\alpha$ : -857C/T	HpyCH <sub>4</sub> IV	226	203 $\pm$ 23	226
TNF- $\alpha$ : -1031T/C	BbsI	226	226	194 $\pm$ 32
TNF- $\beta$	Nco I	173	102 $\pm$ 71	173

were stratified for genotypes at each locus. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 11.0). Value of  $p < 0.05$  was considered significant.

## Results

In control and case populations, at the polymorphic *loci* considered, the genotype counts were in Hardy-Weinberg equilibrium, with non-significant  $\chi^2$  values. The genotype distribution and allele frequencies of TNF gene polymorphisms are shown in Table III. No significant differences were found in the frequency of the genotype or allele between patients and controls.

We assessed pairwise linkage disequilibrium (LD) among SNPs in control subjects and we observed strong LD among the polymorphisms TNF-857 and TNF-1031, TNF-857 and TNF- $\alpha$ -308, TNF-857 and TNF- $\beta$ , TNF-1031 and TNF- $\beta$ , TNF- $\alpha$ -308 and TNF- $\beta$ , TNF-1031 and TNF-238, TNF-376 and TNF-238 ( $r^2 = 0.1-0.28$ ,  $D' = 0.84-1$ , Table IV).

The genotypes for the TNF resulted in five possible haplotypes, the frequencies of which are

given in Table V. The haplotype distribution did not differ between group A and group B. Further haplotype analysis therefore did not give any additional information to the analysis of the individual SNPs. (The likelihood ratio test (LRT) for the haplotype-phenotype association is not statistically significant [ $\chi^2 = 0.72$ ,  $df = 4$ ,  $p = 0.949$ ]).

The non-parametric analyses of variance performed via Mann-Whitney test showed no significant differences in the age at onset between genotype groups and the six polymorphisms (Table VI).

Comparison of genotype frequencies between boys and girls in affected patients and control individuals were not significantly different (Table VII).

The presence of family history of migraine in patients is shown in Figure 1. The distribution of TNF polymorphisms was not associated with the presence of family history of migraine in patients (Table VIII).

## Discussion

Our study demonstrates no significant differences in the genotype distributions and allele frequencies of TNF gene polymorphisms between

**Table III.** Genotype distribution and allele frequencies of TNF gene polymorphisms in groups A and B

TNF	Genotypes		Alleles		
	Group A	Group B		Group A	Group B
	n (%)	n (%)		n (%)	n (%)
TNF- $\alpha$ -238					
GG	96 (93.2)	170 (95.5)	All 1 (G)	199 (96.6)	348 (97.8)
GA	7 (6.8)	8 (4.5)	All 2 (A)	7 (3.4)	8 (2.2)
AA				$p = 0.419$	
	$p = 0.546$ Exp(B) = 0.707 95% CI 0.230-2.180				
TNF- $\alpha$ -308					
GG	89 (86.4)	145 (81.5)	All 1 (G)	192 (93.2)	321 (90.2)
GA	14 (13.6)	31 (17.4)	All 2 (A)	14 (6.8)	35 (9.8)
AA	0 (0)	2 (1.1)		$p = 0.281$	
	$p = 0.370$ Exp(B) = 1.388 95% CI 0.678-2.840				
TNF- $\alpha$ -376					
GG	102 (99)	178 (100)	All 1 (G)	205 (99.5)	356 (100)
GA	1 (1)	0 (0)	All 2 (A)	1 (0.5)	
AA				$p = 0.364$	
	$p = 1.000$ Exp(B) = 0.000				
TNF- $\alpha$ -857					
CC	64 (62.1)	113 (63.5)	All 1 (C)	159 (77.2)	280 (78.7)
CT	31 (30.1)	54 (30.3)	All 2 (T)	47 (22.8)	76 (21.3)
TT	8 (7.8)	11 (6.2)		$p = 0.754$	
	$p = 0.758$ Exp(B) = 1.091 95% CI 0.627-1.900				
TNF- $\alpha$ -1031					
TT	66 (64.1)	110 (61.8)	All 1 (T)	165 (80.1)	284 (79.8)
TC	33 (32)	64 (36)	All 2 (C)	41 (19.9)	72 (20.2)
CC	4 (3.9)	4 (2.2)		$p = 1.000$	
	$p = 0.560$ Exp(B) = 1.180 95% CI 0.677-2.057				
TNF- $\beta$					
GG	4 (3.9)	10 (5.6)	All 1 (G)	39 (18.9)	77 (21.6)
GA	31 (30.1)	57 (32)	All 2 (A)	167 (81.1)	279 (78.4)
AA	68 (66)	111 (62.4)		$p = 0.508$	
	$p = 0.640$ Exp(B) = 0.741 95% CI 0.211-2.603				

patients with migraine without aura and healthy controls. We report for the first time the distributions of TNF alleles and genotypes in Greek children affected by migraine without aura. Also, we report for the first time the molecular analysis of TNF- $\alpha$ -238, TNF- $\alpha$ -376, TNF- $\alpha$ -857 and TNF- $\alpha$ -1031 alleles in children affected by migraine.

Previous studies concerning the role of TNF gene polymorphisms in migraine have provided conflicting results and there has been no confirmation of the role of TNF gene polymorphisms as markers in migraine patients [10, 14, 16].

Our data for TNF-308G/A are in accordance with the results of Trabace *et al.*, as well as Asuni *et al.*

**Table IV.** Linkage disequilibrium coefficients ( $|D'|$  and  $|r^2|$ ) among TNF polymorphisms

	TNF-238	TNF-308	TNF-376	TNF-857	TNF-1031	TNFB
TNF-238	1	1.00	0.02	1.00	0.95	1.00
TNF-308	0.00	1	0.11	1.00	0.59	0.84
TNF-376	0.00	0.00	1	0.27	0.25	0.28
TNF-857	0.01	0.03	0.00	1	0.91	1.00
TNF-1031	0.08	0.01	0.00	0.06	1	1.00
TNF- $\alpha$	0.01	0.28	0.00	0.07	0.07	1

**Table V.** Association between different TNF haplotypes and migraine

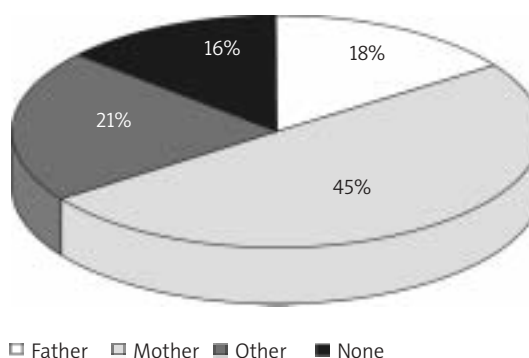
Haplotypes	TNF-238 G/A	TNF-308 G/A	TNF-376 G/A	TNF-857 C/T	TNF-1031 T/C	TNF- $\beta$ G/A	Frequencies		OR (95% CI)	Value of p
							Group A	Group B		
Ht1	G	G	G	C	T	A	0.371	0.367		
Ht2	G	G	G	T	T	A	0.222	0.209	1.041 (0.658-1.645)	0.864
Ht3	G	G	G	C	C	A	0.177	0.168	1.054 (0.626-1.776)	0.843
Ht4	G	G	G	C	T	G	0.126	0.130	0.965 (0.550-1.691)	0.900
Ht5	G	A	G	C	T	G	0.068	0.086	0.776 (0.370-1.627)	0.502

**Table VI.** Correlation between genotypes and the age of onset of the disease

SNPs	Genotypes	Age of onset of disease (group A)
TNF-238	GG	9.10 $\pm$ 0.318
	GA	10.00 $\pm$ 1.528
		$p = 0.463$
TNF-308	GG	9.31 $\pm$ 0.342
	GA + AA	8.33 $\pm$ 0.667
		$p = 0.278$
TNF-376	GG	9.17 $\pm$ 0.318
	GA	
TNF-857	CC	9.08 $\pm$ 0.392
	CT + TT	9.31 $\pm$ 0.522
		$p = 0.748$
TNF-1031	TT	9.44 $\pm$ 0.375
	TC + CC	8.67 $\pm$ 0.540
		$p = 0.234$
TNF- $\beta$	GG	
	GA + AA	9.15 $\pm$ 0.317

[10, 12], but the same data are in discordance with those of Rainero *et al.* and Mazaheri *et al.* [14, 16]. These two recent studies showed an increased risk of migraine without aura associated with the TNF- $\alpha$  G308A polymorphism (Table IX).

Our data for TNF- $\beta$  are in discordance with those of Trabace *et al.*, as well as Asuni *et al.*, and



**Figure 1.** Family history of migraine in group A

Martelletti *et al.* [10, 14, 15]. These three recent studies showed an increased risk of migraine without aura associated with the TNF- $\alpha$  G308A polymorphism (Table IX).

These discordant results may be explained considering the significant differences among the allele frequencies of TNF gene variants in populations from different ethnic groups and hypothesizing the same linkage disequilibrium with the susceptibility genes of migraine without aura. In particular, the TNF- $\alpha$ -308A allele is very rare in Asians (A/G 16/84%) and Japanese (A/G 2/98%) [18]. Additionally, gene-gene, as well as gene-environment interactions are likely, since migraine does not fit a simple Mendelian pattern but is a “multifactorial disease”. This could in part explain why investigations of candidate susceptibility genes in case control group studies as well as linkage analyses

**Table VII.** Correlation between genotypes and sex

SNPs	Genotypes	Group A		Group B	
		Boys	Girls	Boys	Girls
TNF-238	GG	43 (39.2)	53 (53.8)	111 (62.4)	59 (33.1)
	GA + AA	4 (4.1)	3 (2.9)	7 (3.9)	1 (0.6)
<i>p</i> = 0.171 Exp(B) = 0.395 95% CI 0.105-1.494					
TNF-308	GG	41 (39.8)	48 (46.6)	97 (54.5)	48 (27.0)
	GA + AA	5 (4.8)	9 (8.7)	21 (11.8)	12 (6.7)
<i>p</i> = 0.278 Exp(B) = 1.457 95% CI 0.738-2.875					
TNF-376	GG	46 (44.7)	56 (54.4)	118 (66.3)	60 (33.7)
	GA	0 (0.0)	1 (0.9)		
<i>p</i> = 1.000 Exp(B) 6.7E+09					
TNF-857	CC	29 (28)	35 (36.1)	74 (41.6)	40 (22.5)
	CT + TT	16 (15.2)	21 (21.5)	44 (24.7)	20 (11.2)
<i>p</i> = 0.729 Exp(B) = 0.908 95% CI 0.528-1.563					
TNF-1031	TT	26 (24.8)	38 (38.8)	69 (38.8)	41 (23.0)
	TC + CC	19 (18.5)	18 (17.9)	49 (27.5)	19 (10.7)
<i>p</i> = 0.128 Exp(B) = 0.656 95% CI 0.382-1.129					
TNF-β	GG	3 (2.9)	1 (0.9)	7 (3.9)	3 (1.7)
	GA + AA	42 (41.8)	55 (54.4)	111 (62.4)	57 (32.0)
<i>p</i> = 0.093 Exp(B) = 2.943 95% CI 0.835-10.371					

**Table VIII.** The distribution of TNF polymorphisms with the presence of family history of migraine in patients

SNPs	Genotypes	Positive family history of migraine		
		Father (%)	Mother (%)	Brother-sister (%)
TNF-238	GG	8.2	28.4	3
	GA + AA	0.7	1.5	0
NS				
TNF-308	GG	8.2	25.4	2.2
	GA + AA	0.7	4.5	0.7
NS				
TNF-376	GG	9	29.9	3.0
	GA	0	0	0
NS				
TNF-857	CC	7.5	14.9	0
	CT + TT	1.5	14.9	3.0
NS				
TNF-1031	TT	6.7	22.4	3
	TC + CC	2.2	7.5	0
NS				
TNF-β	GG	10	3.0	0.7
	GA + AA	8.9	26.9	2.2
NS				

Table IX. Results of different studies

Study	SNP	Origin	Results
<b>Tumour necrosis factor (TNF)-<math>\alpha</math></b>			
Rainero <i>et al.</i> (2004)	-G308A	Italian (299/306)	Significant association $p \leq 0.001$ for MO
Mazaheri <i>et al.</i> (2006)	-G308A	Iranian (221/183)	Significant association $p \leq 0.001$ for MO
Trabace <i>et al.</i> (2002)	-G308A	Italian (79/101)	No association $p \geq 0.05$ MA
Asuni <i>et al.</i> (2009)	-G308A	Sardinian	No association $p \geq 0.05$
Our study	-238 G/A	Greek (103/178)	No association $p \geq 0.05$
	-308 G/A	Greek (103/178)	No association $p \geq 0.05$
	376A/G	Greek (103/178)	No association $p \geq 0.05$
	-857C/T	Greek (103/178)	No association $p \geq 0.05$
	-1031T/C	Greek (103/178)	No association $p \geq 0.05$
<b>Tumour necrosis factor (TNF)-<math>\beta</math></b>			
Trabace <i>et al.</i> (2002)	TNF- $\beta$	Italian (79/101)	Significant association $p \leq 0.05$ for MO
Asuni <i>et al.</i> (2009)	TNF- $\beta$	Sardinian (219/278)	Significant association $p \leq 0.05$
Martelletti <i>et al.</i> (2000)	TNF- $\beta$	Italian (77/1010)	Significant association $p \leq 0.05$
Lee KA	LTA-294T/C	Korean (439/382)	Significant association $p \leq 0.05$
Our study	TNF- $\beta$	Greek (103/178)	No association $p \geq 0.05$

have at times shown variable results and attempts at independent replication have failed [14, 18].

Additional analyses could focus on clinical profiles, including symptoms, triggers, and successful treatments, thus providing a more comprehensive depiction of interactions between the various components of the disorder [10, 14, 16]. The severity of migraine symptoms, such as the recurrence and duration of attacks and the age at onset of disease, are variable among patients, thus rendering difficult the selection of the best population in which to investigate the genetic load.

Complicating the issue is the likelihood that many genetic variants may provide a modest yet significant contribution to an individual's migraine susceptibility. Moreover, there might be a genetic linkage between TNF polymorphisms and other genes involved in the immune response that increases the susceptibility of patients to development of migraine without aura.

Nevertheless, due to the complex polygenic nature of migraine, the search for migraine susceptibility genes will remain challenging. Also, the effective treatment options for migraine sufferers are limited. Thus, with greater knowledge of the genes and multiple gene profiles involved in migraine susceptibility, future applications may include individual genetic susceptibility profiling and personally tailored pharmacogenetics in order to abort attacks and control the disorder.

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