Tumour necrosis factor gene polymorphisms and migraine in Greek children

Styliani Pappa, Maria Hatzistilianou, Anastasia Kouvatsi, Chrysa Pantartzí, Afroditi Sakellaropoulou, Evangelos Pavlou, Ioannis Mavromichales, Fanni Athanassiadou

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-α and TNF-β 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-α and TNF-β 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-α and TNF-β gene polymorphisms are not a significant risk factor for migraine without aura in Greek children.

Key words: migraine, tumour necrosis factor α, tumour necrosis factor β, 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 CACNAIA, and the ednra 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 prostanoid release, increasing the expression of bradykinin receptors or by modulation of activity within sympathetic fibres. Increased levels of TNF-α in migraineurs have been documented; therefore, it may be involved in migraine [13]. Also, TNF-α 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-α -308 was associated with an increased risk of migraine in an Italian population. However, previous studies concerning the role of TNF-α in migraine have provided conflicting results and there has been no confirmation of the role of TNF-α -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-α and TNF-β 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 ± SE = 10.5 ±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 2nd 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 ± SE = 12.2 ±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-α -238 G/A, TNF-α -308G/A, TNF-α -1031T/C, TNF-α -857C/T, TNF-α -376G/A, and TNF-β -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
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-α-308, TNF-857 and TNF-β, TNF-1031 and TNF-β, TNF-α-308 and TNF-β, 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

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Table I. Primer sequences of all TNF polymorphisms

| SNPs       | Primer name             | Primers                      |
|------------|-------------------------|------------------------------|
| TNF-α: -238G/A | TNF_238F               | F S'AGAGAAGACCCCCCTCGGAACC-3' |
|            | TNF_238_376R            | R S'GCTGGTCCTCTGCTCTTG-3'    |
| TNF-α: -308G/A | TNF-α-308F             | F S'AGGGAATAGGTTTTAGGGGACAT-3' |
|            | TNF-α-308R              | R S'TCCTCCTCTCGGATCCTTG-3'   |
| TNF-α: -376A/G | TNF_376F               | F S'CAACCCGGTTTTCTCTCCCTC-3'  |
|            | TNF_238_376R            | R S'GCTGTCTCTCTGCTCTTG-3'    |
| TNF-α: -857C/T | TNF_1031_857_F         | F S'CAGGGAAGCAAGGAGAG-3'     |
|            | TNF_1031_857_R          | R S'CCCTCTACATGCCTGTCATAC-3' |
| TNF-α: -1031T/C | TNF_1031_857_F         | F S'CAGGGAAGCAAGGAGAG-3'     |
|            | TNF_1031_857_R          | R S'CCCTCTACATGCCTGTCATAC-3' |
| TNF-β:     | TNF-B_F                 | F S'GGTTTCCTCTCTCTGCTGACTCTCC-3' |
|            | TNF-B_R                 | R S'GAGAGAGATCGACAGAGAAGGAC-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-α: -238G/A | MspI              | 299              | 280 ±19     | 299          |
| TNF-α: -308G/A | Ncol              | 107              | 87 ±20      | 107          |
| TNF-α: -376A/G | Tsp509I           | 504              | 504         | 418 ±86      |
| TNF-α: -857C/T | HpyCH4V           | 226              | 203 ±23     | 226          |
| TNF-α: -1031T/C | BbsI              | 226              | 226         | 194 ±32      |
| TNF-β:     | Nco I              | 173              | 102 ±71     | 173          |
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-α-238, TNF-α-376, TNF-α-857 and TNF-α-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 III. Genotype distribution and allele frequencies of TNF gene polymorphisms in groups A and B

| TNF          | Genotypes | Alleles |
|--------------|-----------|---------|
|              | Group A   | Group B |
| n (%)        | n (%)     | All 1   |
| TNF-α-238    | GG 96 (93.2) 170 (95.5) All 1 (G) 199 (96.6) 348 (97.8) |
| TG           | 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-α-308    | GG 89 (86.4) 145 (81.5) All 1 (G) 192 (93.2) 321 (90.2) |
| TG           | GA 14 (13.6) 31 (17.4) All 2 (A) 14 (6.8) 35 (9.8) |
|              | AA p = 0.281 |
| p = 0.370 Exp(B) = 1.388 95% CI 0.678-2.840 |
| TNF-α-376    | GG 102 (99) 178 (100) All 1 (G) 205 (99.5) 356 (100) |
| TG           | GA 1 (1) 0 (0) All 2 (A) 1 (0.5) |
|              | AA p = 0.364 |
| p = 1.000 Exp(B) = 0.000 |
| TNF-α-857    | CC 64 (62.1) 113 (63.5) All 1 (C) 159 (77.2) 280 (78.7) |
| TG           | 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-α-1031   | TT 66 (64.1) 110 (61.8) All 1 (T) 165 (80.1) 284 (79.8) |
| TG           | 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-β        | GG 4 (3.9) 10 (5.6) All 1 (G) 39 (18.9) 77 (21.6) |
| TG           | 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 |
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-α G308A polymorphism (Table IX).

Our data for TNF-β are in discordance with those of Trabace et al., as well as Asuni et al., and Martelleti et al. [10, 14, 15]. These three recent studies showed an increased risk of migraine without aura associated with the TNF-α 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-α -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 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 | 100 | 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-α | 0.01 | 0.28 | 0.00 | 0.07 | 0.07 | 1 |

| Table V. Association between different TNF haplotypes and migraine |
|---------------------------------|-----------|-----------|-----------|-----------|
| Haplotypes | TNF-238 | TNF-308 | TNF-376 | TNF-857 | TNF-1031 |
| 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 |
|---------------------------------|-----------|-----------|-----------|-----------|
| SNP | Genotypes | Age of onset of disease (group A) | p |
| TNF-238 | GG | 9.10 ±0.318 | 0.463 |
| | GA | 10.00 ±1.528 |
| TNF-308 | GG | 9.31 ±0.342 | 0.278 |
| | GA + AA | 8.33 ±0.667 |
| TNF-376 | GG | 9.17 ±0.318 | 0.748 |
| | GA |
| TNF-857 | CC | 9.08 ±0.392 | 0.944 ±0.375 |
| | CT + TT | 9.31 ±0.522 | 8.67 ±0.540 | 0.234 |
| TNF-1031 | TT | 9.44 ±0.375 | 8.67 ±0.540 | p = 0.234 |
| | TC + CC | 8.67 ±0.540 |
| TNF-β | GG | 9.15 ±0.317 | 16% | 18% | 45% |

Figure 1. Family history of migraine in group A.
### Table VII. Correlation between genotypes and sex

| SNPs   | Genotypes | Group A | Group B |
|--------|-----------|---------|---------|
|        |           | Boys    | Girls   |
|        |           |         |         |
|        |           | 96 (64) | 37 (53) |
|        |           | 2 (1.3) | 1 (1.4) |
|        | p = 0.075 | 1.736   | 1.084   |
|        | Exp(B)    | 0.438   | 1.000   |
|        | 95% CI    | 0.186-5|
|        |           | 1-9.403 |
|        | 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)   |
|        | p         | 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) |
|        | p         | 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)   |
|        | p         | 1.000   | Exp(B)    | 6.7E+09   |
|        |            | 95% CI   | 1-1.97E+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) |
|        | p         | 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) |
|        | p         | 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) |
|        | p         | 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 (%) |
|        |           | 8.2 | 28.4 | 3 |
|        |           | 0.7 | 1.5 | 0 |
|        | TNF-238   | GG     | 8.2 | 25.4 | 2.2 |
|        | GA + AA   | 0.7 | 4.5 | 0.7 |
|        | TNF-308   | GG     | 7.5 | 14.9 | 0 |
|        | GA + AA   | 1.5 | 14.9 | 3.0 |
|        | TNF-376   | GG     | 6.7 | 22.4 | 3 |
|        | GA        | 2.2 | 7.5 | 0 |
|        | TNF-857   | CC     | 10 | 3.0 | 0.7 |
|        | CT + TT   | 8.9 | 26.9 | 2.2 |
|        | TNF-1031  | TT     | 8.9 | 26.9 | 2.2 |
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.

Table IX. Results of different studies

| Study               | SNP       | Origin     | Results                      |
|---------------------|-----------|------------|------------------------------|
| Tumour necrosis factor (TNF)-α |           |            |                              |
| Rainero et al. (2004) | –G308A   | Italian (299/306) | Significant association \( p \leq 0.001 \) for MO |
| Mazaheri et al. (2006) | –G308A   | Iranian (221/183) | Significant association \( p \leq 0.001 \) for MO |
| Trabace et al. (2002) | –G308A   | Italian (79/101)  | No association \( p \geq 0.05 \) MA |
| Asuni et al. (2009)  | –G308A   | Sardinian   | No association \( p \geq 0.05 \) |
| This 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 \( p \geq 0.05 \) |
| Tumour necrosis factor (TNF)-β  |           |            |                              |
| Trabace et al. (2002) | TNF-β    | Italian (79/101)  | Significant association \( p \leq 0.05 \) for MO |
| Asuni et al. (2009)  | TNF-β    | Sardinian (219/278) | Significant association \( p \leq 0.05 \) |
| Martelletti et al. (2000) | TNF-β | Italian (77/1010) | Significant association \( p \leq 0.05 \) |
| Lee KA               | LTA-294T/C | Korean (439/382) | Significant association \( p \leq 0.05 \) |
| This study           | TNF-β    | Greek (103/178) | No association \( p \geq 0.05 \) |

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