Research Article
Association of TNF-857C>T, TNFRSF1A36A>G, and TNFRSF1B676T>G Polymorphisms with Ischemic Stroke in a Greek Population

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Background. The role of genetic factors in the predisposition to develop ischemic stroke has been assessed by previous studies. The main goal of the current study was to determine any possible role of TNF-857C>T, TNFRSF1A36A>G, and TNFRSF1B676T>G polymorphisms in risk for stroke.

Materials and Methods. One hundred seventy-three patients with first ever ischemic stroke of solely atherosclerotic etiology in Northwest Greece and a control group of 179 healthy unrelated subjects were evaluated.

Results. TNFα-857TT, TNFR136AA, and TNFR2676TT genotypes were significantly increased in the patient group compared to controls (P = .008, OR = 2.47 (1.26–4.84), P = .005, OR = 1.97 (1.22–3.17), and P = .003, OR = 2.2 (1.43–3.37), resp.). In addition, the TNFR136A and the TNFR2676T alleles were found significantly increased in patients compared to controls (P = .009, OR = 1.48 (1.1–2) and P = .001, OR = 1.75 (1.25–2.46), resp.).

Conclusion. The high incidence of these genotypes and alleles in patient group suggests that they are potentially predisposing factors for stroke in the Greek population studied. Large-scale multicenter controlled studies are needed to verify these polymorphisms effects on stroke susceptibility.

1. Introduction
Ischemic stroke is a multifactorial disease, caused by the interactions of genetic and environmental factors, based on atherosclerosis and arterial thrombogenesis [1]. Central nervous system (CNS) can mount a well-defined inflammatory response to a variety of insults including ischemia and atherosclerosis [2]. Atherosclerosis has many inflammatory mediators contributing to atheroma formation, atheroma-tous injury, rupture of the plaque, and hence to intraluminal thrombosis [2, 3]. Tumor necrosis factor-(TNF-) alpha is involved in every step of inflammation, from initiation to downregulation, and elevated levels of TNF and other cytokines have been demonstrated in the cerebrospinal fluid (CSF) and the plasma of acute stroke and subarachnoid hemorrhage patients [4–6]. TNF exerts its biological effects via the two cell surface receptors that act as physiological attenuators, TNFRSF1A and TNFRSF1B.

Functional polymorphisms inside the TNF gene promoter and polymorphisms that could influence the function or the expression of TNFRSF1 and TNFRSF1B have been investigated in various immune, inflammatory, and neurodegenerative conditions [7–14] but only few genetic studies have focused on the association of these polymorphisms with stroke [15, 16].

Under the rationale that individuals with genetic variants might possibly be at greater risk for ischemic stroke, we analyzed the role of the TNF-857C>T, TNFRSF1A36A>G, and TNFRSF1B676T>G polymorphisms in ischemic stroke patients in Northwestern Greece, a rather restricted area with limited recent immigration.
2. Materials and Methods

2.1. Study Population. Cases eligible for recruitment were 173 patients hospitalized with first ever ischemic stroke in the Stroke Reference Center of Northwestern Greece in a period of 18 months, from May 2008 to November 2009, defined according to the WHO definition, and confirmed by brain imaging showing a recent brain infarct corresponding to the clinical presentation. As a control group, 179 age-matched unrelated subjects were recruited from the same medical center, among nonfamily visitors. Controls had no history of previous ischemic cerebrovascular event, established by the Questionnaire for Verifying Stroke-Free Status (QVSFS) [17] and normal neurological examination. The study protocol was in compliance with the Helsinki Declaration, approved by the Institutional Ethics Committee. An informed consent was signed by all eligible patients and controls.

In all subjects a detailed medical history was obtained and a thorough physical examination was performed. Demographic data, biochemical profile, and established risk factors for stroke were recorded. Arterial hypertension was documented when systolic blood pressure (BP) was >140 mmHg or diastolic BP ≥ 90 mmHg or when individuals were receiving antihypertensive drugs for previously established hypertension. Smoking, active or ceased within the last 3 months, was considered as current. Diabetes mellitus was considered as present if fasting glucose was >126 mg/dl or the individual was treated with antidiabetic medication. Dyslipidemia was defined as fasting cholesterol >220 mg/dl or the individual was on current treatment with specific antilipemic agents. Further evaluation of the patients included triplex ultrasound imaging or CT angiography of extracranial or and intracranial arteries, electrocardiogram (ECG), echocardiography (transthoracic or transesophageal), and clinical/laboratory assessment to rule out systemic thrombotic and autoimmune disorders.

Recruitment in the study was performed according to TOAST definitions and included patients with lacunars (small, subcortical, hypodense lesions with a diameter less than 15 mm and corresponding to lesion clinical lacunar syndrome) and patients with large artery atherosclerosis (hypodense lesions with a diameter >15 mm and >50% stenosis in the appropriate intracranial artery) [18].

Cardioembolic strokes were excluded since they could result from different etiology (e.g., atrial fibrillation). Strokes occurred in the course of systemic conditions such as coagulopathies or immunological disorders or of undetermined etiology were also excluded in order to assure the solely atherosclerotic etiology of the stroke.

2.2. Genetic Analysis. Genomic DNA was extracted from peripheral blood lymphocytes according to the standard salt extraction procedure. Polymorphisms TNF-857C>T (rs1799724), TNFRSF1A36A>G (rs767455), and TNFRSF1B-676T>G (rs1061622) were amplified using the following primer pairs: 36F: 5′-GAG CCC AAA TGG GGG AGT -GAG AGG-3′, 36R: 5′-ACC AGG CCC GGG CAG GA-G AG-3′, 676F: 5′-ACT CTC TCA TCC TGC CTG CTG-3′, 676R: 5′-TTC TGG AGT TGG CTG CGT GT-3′, and 857F: 5′-AAG TCG AGT ATG GGG ACC CCC CGT TA-A-3′ 857R: 5′-CCC CAG TGT GTG GCC ATA TCT TCT -T-3′. Subsequently, restriction assays were employed using the restriction endonucleasesMspA1 I, Nla III, and Hinc II, respectively. All samples were run in duplicates with positive and negative for each genotype samples as controls and blanks.

2.3. Statistical Analysis. The Chi-square test was used to test the agreement of genotype frequencies with Hardy-Weinberg equilibrium expectations. Binary data were described as percentages, while continuous data were expressed as the mean ± standard deviation (SD). We calculated odds ratios and 95% confidence intervals (CIs) to compare allele and genotype distributions. All tests were two-sided with 95% significance level (P < .05). The statistical analysis was performed with the statistical package StatXact 3.0 (Cytel Inc., Cambridge, Mass, USA). Furthermore, a power analysis showed that at least 302 subjects should be recruited in each group (cases and controls) for TNF-857C>T: 272 subjects for TNFRSF1A36A>G, and 85 subjects in each group for TNFRSF1B676T>G polymorphism, if the study power required was 80 per cent with a significance level of 0.05.

3. Results

Of 173 patients, 113 (65%) were men and 60 (35%) were women with a mean age of 58.6 (SD ± 7.2 years). Of 179 controls, 123 (69%) were men and 56 (31%) were women and mean age was 57.1 (SD ± 5.9 years). Patients and controls demographics are shown in Table 1. In terms of stroke subtype, 84 patients had lacunar strokes, whereas 89 patients had large artery strokes.

The genotype frequencies for all three polymorphisms were in Hardy-Weinberg equilibrium in healthy controls. The genotype frequencies for TNFRSF1A and TNFRSF1B polymorphisms were in Hardy-Weinberg equilibrium in patients. Regarding the TNF-857C>T polymorphism distribution, a high prevalence of TT genotype in patients (P = .008, OR = 2.47 (1.26–4.84)) was revealed, implying a TT selection in patients causing Hardy-Weinberg disequilibrium, despite equilibrium in the controls. No statistically significant difference was observed in the allele frequencies, to further establish the hypothesis of the TT selection in patients.

The distribution and frequencies of genotypes and alleles for TNF, TNFRSF1A, and TNFRSF1B are summarized in Table 2. Regarding TNFRSF1A36A>G, a statistically significant difference was observed between patients carrying the AA genotype and controls (P = .005, OR = 1.97 (1.22–3.17)). Significant differences were also noted in the allele frequencies, namely, A and G with 58.1% in patients versus 48.3% in controls for A allele and 41.9% in patients versus 51.7% in controls for G allele (P = .009, OR = 1.48 (1.1–2)). For the TNFRSF1B676T>G, the TT genotype was more frequent in the stroke group than in controls, with 61.9% versus 42.5%, respectively (P = .003, OR = 2.2 (1.43–3.37)). Similarly, statistically significant differences between
the allele frequencies with 78.3% in patients versus 67.3% in controls for T allele and conversely 21.7% in patients versus 32.7% in controls for G allele (P = .001, OR = 1.75 (1.25–2.46)) (Table 2) were revealed.

In terms of stroke subtype, subgroup analysis did not disclose any statistically significant result when genotypes and alleles distribution in the lacunar stroke group was compared to the large artery atherosclerosis stroke group (data not shown).

4. Discussion

In the present study, we investigated the association of TNF-857C>T, TNFRSF1A36A>G, and TNFRSF1B676T>G polymorphisms with ischemic stroke, under the rationale that, as the proinflammatory cytokines play an important role in cerebral ischemia [4, 5, 19, 20], certain TNF and TNF receptors polymorphisms may be implicated in stroke occurrence.

A gene located on chromosome 6p21 encodes TNF-α. The TNF-857C>T polymorphism is a functional polymorphism through binding to the transcription factor octamer transcription factor-1 (OCT-1) [7]. No research group, working on stroke, has focused on this polymorphism. Most groups have worked on the TNF-308G>A polymorphism with Pereira et al. suggesting that the TNF-308G>A polymorphism may play a role in ischemic stroke [21] showing that young subjects of European ancestry carrying the A allele (AA + GA versus GG) were associated with a statistically significant increase in the risk of stroke compared with individuals homozygous for the G allele (OR 2.04, P = .004). In a cohort of young Italian stroke patients, the A allele of −308G>A polymorphism exerted an independent effect on predisposition to ischemic stroke in young stroke patients with AA + GA genotypes frequency being 25.2% + 1.7%, respectively, in the stroke group versus 15% + 0.6 5% in the control group [15]. Dissimilar findings suggest that this polymorphism is clinically important in stroke in sickle cell anemia children [22] but shows protective effects of TNF(-308)A allele (OR = 0.39, P = .006) with 18% carriers of the A allele in stroke group versus 38% in the control group.

In our healthy population in Northwest Greece, as recorded in a recent study conducted by our group [10], the TNF-308G>A polymorphism is not in Hardy-Weinberg equilibrium, and our study was designed on the specific characteristics of our population to exclude this polymorphism and focus on the TNF-857C>T polymorphism. Our study exerted that this other functional polymorphism plays a role in stroke development with the recessive genotype being more frequent in ischemic stroke patients.

TNFRSF1A is encoded by a gene located on chromosome 12p13.2. The TNFRSF1A36A>G polymorphism is a silent mutation in codon 12, and it although has no obvious functional influence on protein structure, many studies have tried to find any association of this polymorphism with specific inflammatory disease manifestations and response to treatment, such as Multiple Sclerosis (MS), Crohn’s disease, and Rheumatoid Arthritis (RA) [10, 11, 14].

TNFRSF1B is encoded by a gene located on chromosome 1p36.3 with a higher affinity for TNF than TNFRSF1, and polymorphisms in this receptor affect the binding of TNF and the pathway involved in inflammation; associations of TNFRSF1B polymorphisms with chronic inflammatory diseases such as RA and systemic lupus erythematosus have been reported [10, 12].

Table 1: Clinical characteristics of patients and controls.

| Data          | Cases N = 173 | Controls N = 179 | P values |
|---------------|---------------|------------------|----------|
| Male          | 113 (65)      | 123 (69)         | .498     |
| Female        | 60 (35)       | 56 (31)          | .498     |
| Age, mean     | 58.6 (7.2)    | 57.1 (5.9)       | .167     |
| Hypertension  | 137 (79.1)    | 129 (72.0)       | .121     |
| Smoking       | 74 (42.8)     | 70 (39.1)        | .484     |
| Diabetes mellitus | 40 (23.1)   | 31 (17.3)        | .176     |
| Dyslipidemia  | 109 (63.0)    | 99 (55.3)        | .142     |

Numbers in parentheses for nominal data indicate percentages and for continuous data SD.

Table 2: Genotypes and alleles in patients and control.

| Polymorphisms | TNF-857C>T | TNFRSF1A36A>G | TNFRSF1B676T>G |
|---------------|------------|---------------|---------------|
| Genotypes     | TT         | CT + CC       | AA            |
| Patients, n (%) n = 173 | 30 (17.3)   | 43 + 100 (24.9 + 57.8) | 60 (34.7) |
| Controls, n (%) n = 179 | 14 (7.8)    | 63 + 102 (35.2 + 57.0) | 38 (21.2) |
| P values, OR (95% CI) | P = .008, 2.47 (1.26–4.84) | P = .005, 1.97 (1.22–3.17) | P = .003, 2.2 (1.43–3.37) |

| Alleles | C | T | A | G | T | G |
|---------|---|---|---|---|---|---|
| Patients, n (%) n = 173 | 243 (70.2) | 103 (29.8) | 201 (58.1) | 145 (41.9) | 271 (78.3) | 75 (21.7) |
| Controls, n (%) n = 179 | 267 (74.6) | 91 (25.4) | 173 (48.3) | 185 (51.7) | 241 (67.3) | 117 (32.7) |
| P values, OR (95% CI) | P = .196, 0.8 (0.57–1.12) | P = .009, 1.48 (1.1–2) | P = .001, 1.75 (1.25–2.46) |
Our results indicate that the most studied, for many inflammatory diseases, polymorphisms of TNFRSF1A and TNFRSF1B are also associated with stroke, with the AA genotype of TNFRSF1A and the TT of TNFRSF1B being more frequent in stroke patients. The same applies for the A allele of TNFSF1A and the T allele of TNFSF1B.

Limitations of our study include the relatively small number of patients and the TNF-857TT Hardy-Weinberg disequilibrium in our cases. As a consequence of the small number of the cases the power of our study is limited regarding the TNF-857C>T and the TNFRSF1A36A>G polymorphism. Regarding the TNFRSF1B676T>G polymorphism, power analysis confirmed a sound power of this result. The strengths of the study were the solely atherosclerotic etiology of subjects and the prospective design with all ischemic stroke patients under certain inclusion criteria and the significant result for TNFRSF1B676T>G polymorphism.

5. Conclusions

Conclusively, our results indicate a possible association of –857C>T polymorphism of TNF with stroke and are in favor of a direct, contributory effect of the most studied polymorphisms of TNFRSF1A and TNFRSF1B on ischemic stroke predisposition, with TNFRSF1B676T>G polymorphism showing the closest association. Our assumption is that specific polymorphisms are involved in the process of inflammation, and thus they promote extra- and intracranial atherosclerosis and subsequently strokes. The genetically homogenous population allows the extractions of assumptions concerning our population, but no further conclusions should be reached without large-scale multicenter studies and a wider range of results. Any role for TNF and TNF receptor genes on stroke is far from being established with these genes being excellent candidates for further research on the genetics of stroke.

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