

The Association of Gene Polymorphisms of the Angiotensin-Converting Enzyme and Angiotensin II Receptor Type 1 with Ischemic Stroke in Turkish Subjects of Trakya Region

Anjiyotensin Dönüşürücü Enzim ve Anjiyotensin II Tip 1 Reseptörü Gen Polimorfizmlerinin
Trakya Bölgesindeki Türk Hastalarda Görülen İskemik İnme ile İlişkisi

Tammam SİPAHİ¹, Babürhan GÜLDİKEN², Sibel GÜLDİKEN³, Sedat ÜSTÜNDAĞ³, Nilda TURGUT²,
Metin BUDAK¹, Suat CAKİNA¹, Hülya ÖZKAN⁴, Seralp ŞENER¹

Departments of ¹Biophysics, ²Neurology, ³Internal Medicine, Medical Faculty of Trakya University, Edirne;

⁴Department of Neurology, Social Security Hospital, Edirne

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Objectives: The aim of this study was to investigate the frequency of ACE insertion/deletion (I/D) and AT1R (A1166C) gene polymorphisms in ischemic stroke patients in Trakya region and the relation between these gene polymorphisms and stroke subtypes and vascular risk factors.

Patients and Methods: The study involved 162 patients with ischemic stroke and 146 control subjects. Ischemic stroke patients were divided into large and small vessel disease subgroups according to ORG 10172 in Acute Stroke Treatment TOAST criteria. The ACE I/D polymorphism was investigated using polymerase chain reaction (PCR), and the AT1R (A1166C) polymorphism was identified using PCR and restriction fragment length polymorphism (RFLP) assay.

Results: The ACE I/D genotype distribution in patients (DD=34.0%, ID=50.0%, II=16.0%) did not differ from those in controls (DD=34.3%, ID=49.7%, II=16.1%). The AT1R A1166C genotype distribution in patients (AA=58.0%, CA=34.6%, CC=7.4%) did not significantly differ from those in controls (AA=60.1%, CA=35.7%, CC=4.2%). There was also no difference among the stroke subgroups regarding the distribution of ACE I/D and AT1R (A1166C) polymorphisms.

Conclusion: Our results show that ACE I/D and AT1R (A1166C) gene polymorphisms were not genetic risk factors for ischemic stroke in subjects in Trakya region.

Key words: ACE I/D gene polymorphism; AT1R (A1166C) gene polymorphism; ischemic stroke; renin-angiotensin system.

Amaç: Bu çalışmanın amacı, Trakya bölgesinde yaşayan iskemik inme geçirmiş hastalarda ACE insersyon/delesyon (I/D) ve AT1R (A1166C) gen polimorfizmlerinin sıklığını, vasküler risk faktörleri ve inme alt-grupları ile ilişkisini araştırmaktır.

Hastalar ve Yöntemler: Çalışmaya 162 iskemik inme geçirmiş hasta ile 146 sağlıklı olgu alındı. İskemik inme hastaları, ORG 10172 Akut Inme Tedavisi (TOAST) kriterlerine göre büyük ve küçük damar hastalığı olarak inme alt gruplarına ayrıldı. ACE I/D polimorfizmi polimeraz zincir reaksiyonu (PZR), AT1R (A1166C) gen polimorfizmi ise PZR ve restriksiyon fragment uzunluk polimorfizmi (RFLP) yöntemleri kullanılarak yapıldı.

Bulgular: Hasta grubundaki ACE I/D genotip dağılımı (DD=34.0%, ID=50.0%, II=16.0%), kontrol grubu ile karşılaştırıldığında (DD=34.3%, ID=49.7%, II=16.1%) fark bulunmadı. Ayrıca hasta grubundaki AT1R (A1166C) genotip dağılımları ile (AA=58.0%, CA=34.6% ve CC=7.4%) kontrol grubu ile karşılaştırıldığında (AA=60.1%, CA=35.7% ve CC=4.2%) anlamlı fark saptanmadı. Her iki inme alt grubu arasında ACE I/D ve AT1R (A1166C) polimorfizmlerinin dağılımı açısından farklılık bulunmadı.

Sonuç: Çalışmamızda Trakya bölgesinde yaşayan insanlarda ACE I/D ve AT1R (A1166C) gen polimorfizmlerinin iskemik inme gelişmesinde genetik risk faktörleri olmadıkları belirlendi.

Anahtar sözcükler: İskemik inme; ACE I/D gen polimorfizmi; AT1R (A1166C) gen polimorfizmi; renin-anjiyotensin sistemi.

During the last two decades, there has been an increasing interest in the study of the different polymorphisms of genes of the renin-angiotensin system (RAS) and its association with the pathogenesis of essential hypertension,^[1] ischemic heart disease,^[2] coronary artery disease,^[3] myocardial infarction,^[4] stroke,^[5-7] and progressive renal disease.^[8] The RAS plays a central role in the regulation of blood pressure, sodium metabolism and renal hemodynamics.^[9,10] The RAS gene system comprises the angiotensinogen (AGT), renin, angiotensin I, angiotensin I-converting enzyme (ACE), angiotensin II, and angiotensin II receptor types 1, 2, 3 and 4 (AT1R, AT2R, AT3R and AT4R) genes.^[11]

The ACE is a key component of both the RAS and the kinin-kallikrein system (Fig. 1). ACE cleaves the carboxy-terminal dipeptide of angiotensin I, releasing the physiologically active octapeptide angiotensin II.^[12] Angiotensin II is a potent vasoconstrictive molecule that plays a key role in modulating vascular tone. Angiotensin II exerts its effects by binding to AT1R, AT2R, AT3R and AT4R. The AT1R is the major mediator of physiological effects of angiotensin II. Human AT1R is present predominantly in vascular cells and in both kidney and adrenal gland mediating physiological actions of angiotensin II as shown in Figure 1. The AT1R mediates its action by association with G proteins that activate a phosphatidylinositol-calcium second messenger system, followed by vasoconstriction, hypertrophy, or catecholamine liberation at sympathetic nerve endings.^[13-19]

The ACE gene maps to chromosome 17 (17q23.3), spans 21 kb, and comprises 26 exons and 25 introns, and is characterized by a polymorphism resulting from the presence (insertion) or absence (deletion) of a 287 base pairs fragment of a repeated Alu sequence at intron 16 hence, the corresponding designation of insertion (I) or deletion (D) of the two resulting alleles.^[20, 21]

The AT1R gene maps to chromosome 3 (3q21-q25), spans 45.123 kb, and comprises 5 exons and 4 introns.^[22] AT1R entire coding region

harbored only on exon 5, and is characterized by a polymorphism resulting from an A/C (adenine/cytosine) transversion located at position 1166 (A1166C polymorphism) in 3' untranslated region.^[23]

Since AT1R (A1166C) and ACE I/D polymorphisms have been reported,^[23,24] they have been widely examined in association with various diseases.^[2-6]

In view of the aging population stroke is becoming a major problem, it is the most frequent disease leading to disability,^[25] and estimates forecast a continuing increase in the incidence, prevalence, and mortality of stroke in the next decades. Evidence is accumulating that genetic factors contribute to the risk of stroke.^[26,27] Some investigators have suggested that genetic factors, which appear insignificant when occurring alone, may increase susceptibility to stroke in the presence of other risk factors as hypertension, diabetes mellitus, smoking, and alcohol consumption.^[28,29] Among potential candidate genes that predispose to stroke, ACE I/D, and AT1R (A1166C) polymorphisms have been implicated in the pathophysiology of stroke.^[5,30] Coexisting risk factors, such as hypertension and high cholesterol levels, seem to increase the effects of these polymorphisms, although the available evidence remains inconclusive. Risk factors, underlying mechanisms and pathophysiologic responses to stroke vary according to the subtypes of stroke.^[31] And this prompted us to initiate to assess the influence of the ACE I/D, and AT1R (A1166C) polymorphisms, and their interaction with coexisting risk factors, on the risk of ischemic stroke subtypes.

PATIENTS AND METHODS

The study included 308 Turkish individuals from Trakya region, who were informed of the study protocol, comprising 162 patients with ischemic stroke (85 males, 77 females; mean age 67±11 years) which were categorized according to the trial of ORG 10172 in Acute Stroke Treatment (TOAST) into large and small vessel stroke subtypes, and 146 healthy individuals as a control group (37 males, 109 females; mean age

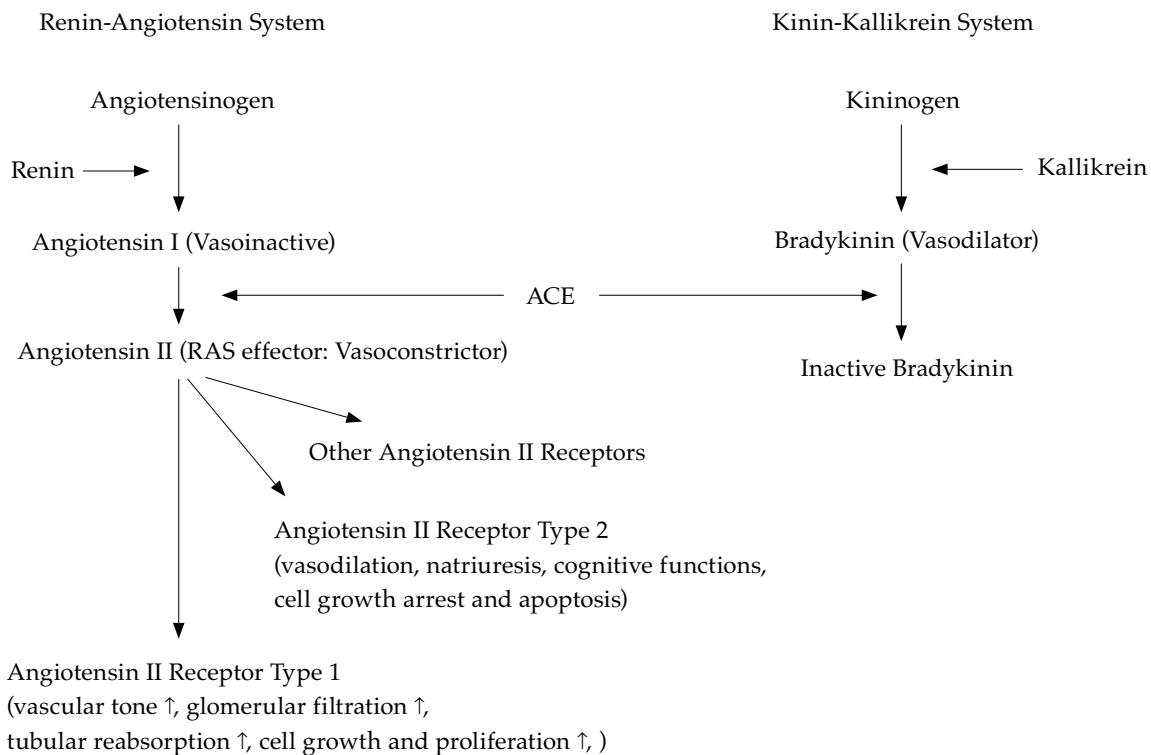


Fig. 1. Renin-angiotensin and Kinin-Kallikrein systems.

62±11 years) for the distribution of the polymorphisms. Approval for the study was obtained from the Ethics Committee of Trakya University School of Medicine.

For DNA isolation; e.Z.N.A. blood DNA kits were purchased from Omega Bio-tek (BOGA), Istanbul, Turkey. All reagents for PCR, RFLP and gel electrophoresis were purchased from Fermentas Life Sciences (ELIPS), Istanbul, Turkey.

DNA isolation

Peripheral blood (2 ml) was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. DNA was isolated from peripheral blood, containing EDTA, by e.Z.N.A. (EaZy Nucleic Acid Isolation) blood DNA kits (Omega Bio-tek Doraville USA).

DNA purity and quantity were assessed by absorbance values in spectrophotometer and checked by 0.8% agarose gel electrophoresis.

Analysis of polymorphisms of ACE and AT1R genes

Genotyping of ACE gene polymorphism

To determine the ACE gene I/D polymorphism of the patients and control groups, a genomic DNA fragments on intron 16 of the ACE gene was amplified by PCR in a 15 µl PCR reaction mixture containing 100 ng of DNA, dNTP (0.2 mM of each), upstream and downstream oligonucleotide primers (300 pmol), 1X Taq Buffer with $(\text{NH}_4)_2\text{SO}_4$ (75 mM Tris-HCl pH 8.8, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% Tween 20), 2.5 mM MgCl₂ and 0.75 U of Taq DNA polymerase (Fermentas Life Sciences). Amplification were performed with a Techne (TechGene) DNA Thermal Cycler with 5 min of denaturation at 94°C, followed by 30 cycles with denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C.^[32]

The PCR primers with the sequences reported by Rigat B et al.^[32] were used (sense and anti-sense primers were; 5'-CTGGAGACCACTCCCATCCTTCT-3' and 5'-GATGTGCCATCACATTCTCAGAT-3', respectively). The expected I and D alleles, 490 and 190 base pairs in size, were visualized after electrophoresis on a 2% agarose gel and ethidium bromide staining (Fig. 2). Preferential amplification of the D allele in the heterozygotes has led to their mistyping as DD homozygotes.^[33,34] To exclude this possibility, all DD homozygotes were retyped using I allele specific sense primer 5'-TTTGAGACGGAGTCTCGCTC-3' and anti-sense primer reported by Rigat B et al.^[32] was used. Amplification was performed with a DNA Thermal Cycler with 3 min of denaturation at 93°C, followed by 30 cycles with 1 min of denaturation at 93°C, annealing for 1.5 min at 68°C, and extension for 2 min at 72°C, followed by 3 min of extension at 72°C. When a DD sample amplified using the I-specific primer, it was retyped ID.

Genotyping of the AT1R A1166C gene polymorphism

A1166C polymorphism of AGTR₁ was identified with PCR followed by RFLP with the restriction enzyme *Hae*III. PCR primers were generated to amplify the 255 bp fragment encompassing the A1166C variant (sense and anti-sense primers were 5'-GCAGCACTTCACTACCAAATGGGC-3' and 5'-CAGGACAAAAGCAGGCTAGGGAGA-3', respectively) in a 25 μl PCR mixture con-

taining 200 ng of DNA, dNTP (0.2 mM of each), sense and antisense primers (500 pmol of each), 1X Taq buffer with (NH₄)₂SO₄ (75 mM Tris-HCl (Ph 8.8), 20 mM (NH₄)₂SO₄, 0.01% Tween 20), 1.5 mM MgCl₂ and 1.25 U of Taq DNA polymerase. Amplification were performed with a Techne (TechGene) DNA Thermal Cycler with 5 min of denaturation at 94°C, followed by 35 cycles with denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, followed by 7 min of extension at 72°C. Ten microliters of PCR product were digested with 5 unite of the restriction enzyme *Hae*III (Takara Bio Inc, Japan) in 1 X M buffer (10mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM Dithiothreitol and 50mM NaCl) for 2 hours at 37°C. When mutant allele (cytosine), digested with *Hae*III that yield tow fragments 231 bp and 24 bp, whereas a wild allele (adenine) at nucleotide position 1166, had no cutting site for *Hae*III, so that the 255 bp PCR product was not cleaved into 231 bp and 24 bp fragments.^[35,36] The restriction digest products were visualized after electrophoresis on a 2% agarose gel and ethidium bromide staining (Fig. 3).

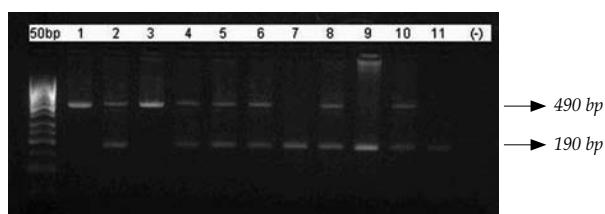


Fig. 2. Ethidium bromide-stained 2% agarose gel of representative PCR products of ACE gene I/D polymorphism shows the D allele (190 bp, lane 7, 9, and 11), the ID genotype (190 bp, and 490 bp, lane 2, 4, 5, 6, 8, and 10) and the I allele (490 bp, lane 1, and 3); lane 50 bp is a size marker (GeneRuler 50bp DNA Ladder-Fermentas Life Sciences).

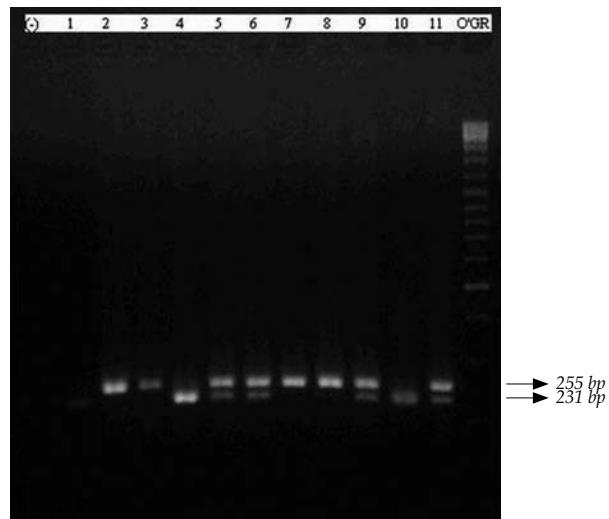


Fig. 3. Ethidium bromide-stained gel of representative PCR products of AGTR1 gene A1166C polymorphism shows the AA genotype (255 bp, lane 2, 3, 7, and 8), the AC genotype (255 bp, 231 bp, and 24 bp (not detectable in agarose gel) lane 5, 6, 9, and 11), and the CC genotype (231 bp, lane 1, 4, and 10); lane O'GR is a size marker (O'GeneRuler 100bp DNA Ladder-Fermentas Life Sciences).

In all PCR experiments, for ACE and AGTR₁ genes, several reactions containing no DNA were included to control the possibility of contamination.

Statistical analysis

Statistical analyses were performed with the SPSS 15.0 software and STATA program, version 8. Normality of distribution was checked by using the Kolmogorov-Smirnov test. If the test distribution was found normal, Independent-Samples T test was used to evaluate differences in continuous variables between controls and patients with large vessel disease and patients with small vessel disease. In this condition, the data are presented as mean ± standard deviation (SD). As variables did not have normal distribution, Mann-Whitney U test was used to evaluate differences in continuous variables between controls and patients with large vessel disease and patients with small vessel disease. In this condition, the data are presented as median (25-75 percentiles). Chi-square test was performed to determine the differences in dichotomized variables between control and patient group. Allele frequencies were calculated from the genotypes of all subjects. Hardy-Weinberg equilibrium was assessed by chi-square analysis. Allele and genotype frequencies were compared by standard contingency table analysis using chi-square test. Significance was defined as p<0.05.

RESULTS

The control group was younger than the patient group, and had lower systolic blood pressures, diastolic blood pressures, fasting blood glucose

Table 1. Demographic and clinical characteristics of the control and stroke groups

	Control group (n=146)	p	Stroke group (n=162)
Gender (M/F)	37/109	<0.001	85/77
Hypertension (%)	62.1	<0.001	85.6
Current smoker (%)	3.7	<0.001	28.2
Diabetes mellitus	17.9	0.008	31.3
Family history of stroke (%)	17.9	0.007	34.1
Age (years)	62 (53-72)	0.001	70 (60-74)
SBP (mmHg)	120 (110-130)	<0.001	140 (120-160)
DBP (mmHg)	70 (70-80)	<0.001	80 (70-90)
FBG (mg/dl)	89.5 (82-105.5)	<0.001	102 (90-128)
TG (mg/dl)	116.0 (81.8-174.5)	NS	136.5 (90.8-136.5)
TC (mg/dl)	189 (169-214.8)	NS	190.5 (166-219)
HDL-C (mg/dl)	39 (32-50.5)	NS	38 (31-46)
LDL-C (mg/dl)	120±33	NS	125±38

Values are given as mean ± SD. SBP and DBP: Systolic and diastolic blood pressures; FBG: Fasting blood glucose; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; NS: Non-significant.

levels than the patients (p<0.001). The controls were predominantly female (74.7%), whereas the patients were very similar between the male and the female subjects (p<0.001). The patient group had a higher prevalence of hypertension, smoking, diabetes mellitus and family history of stroke compared with the controls (p<0.001, p<0.001, p<0.01, and p<0.01, respectively). Demographic and clinical characteristics of the control and stroke patient subjects are given in Table 1.

No interaction was found between the studied polymorphisms and biochemical parameters and vascular risk factors.

Table 2. Distributions of ACE genotype and allele frequencies in the controls and stroke patient subjects

	ACE genotype frequency*			ACE allele frequency*	
	DD	ID	II	D	I
Control group (%)	34.3	16.1	49.7	59	41
Stroke group (%)	34.0	16.0	50.0	59	41

*Non-significant between groups.

Table 3. Distributions of AGTR₁ genotype and allele frequencies in the controls and stroke patient subjects

	AGTR ₁ genotype frequency*			AGTR ₁ allele frequency*	
	AA	AC	CC	A	C
Control group (%)	60.1	4.2	35.7	78	22
Stroke group (%)	58.0	7.4	34.6	75.3	24.7

*Non-significant between groups.

Table 4. Demographic and clinical characteristics of the stroke subtypes classified according to TOAST criteria

	Large vessel disease	p	Small vessel disease
Gender (% F)	50	NS	39
Hypertension (%)	85	NS	85.1
Current smoker (%)	27.5	NS	27.7
Diabetes mellitus	45	NS	28.4
Family history of stroke (%)	36.8	NS	38.4
Age (years)	66±11	NS	67±10
SBP (mmHg)	138±21	NS	145±27
DBP (mmHg)	80 (80-90)	NS	80 (80-92.5)
FBG (mg/dl)	109.5 (94.8-137.8)	0.021	102 (92-121)
TG (mg/dl)	114 (86.3-162.8)	NS	167.5 (93.5-239)
TC (mg/dl)	199±42	NS	192±51
HDL-C (mg/dl)	40±12	NS	37±12
LDL-C (mg/dl)	129±39	NS	123±42

Values are given as mean ± SD. SBP and DBP: Systolic and diastolic blood pressures; FBG: Fasting blood glucose; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; NS: Non-significant.

Table 2 shows the distributions of ACE genotype and allele frequencies in the controls and stroke patient subjects. The distributions of AGTR₁ genotype and allele frequencies in the controls and stroke patient subjects are shown in Table 3.

Table 4 shows the demographic and clinical characteristics of the stroke subtypes classified according to TOAST criteria. The difference within the ischemic stroke subgroups was also

not significant for ACE I/D and AT1R A1166C genotype distribution and allelic frequency Table 5, 6.

DISCUSSION

Several studies have shown a high prevalence of the D allele of ACE gene and C1166 allele of AT1R among patients with stroke disease.^[6,37-39] On the other hand, researches have shown no differences in the allele frequencies and genotype distributions of ACE and AT1R gene polymorphisms between the healthy and stroke disease groups.^[40,41] Studies analyzing the gene-gene and gene-environment interactions in stroke have revealed much of useful information. The increasing knowledge has proved to be very consistent. The apparent contradictions can be solved after careful examination. The diverse data only reflect the diverse aspects of the complex interactions. The differences in the results can also stem from distinctions in ethnicity.

In our study, the overall frequencies of the genotypes DD, ID, and II in patient group were 34.0%, 50.0% and 16.0% did not differ from genotype distribution in healthy group 34.3%, 49.7% and 16.1%. No differences were detected in alleles (D or I) frequency between the healthy and ischemic stroke groups. Additionally, our results showed no significant difference in either the genotypic distribution or allelic frequency among the stroke subtypes. Our study supports the study which was done in Istanbul region by Tuncer et al.^[7] They reported that the ACE I/D polymorphisms were not associated with ischemic stroke and its subtypes. On the other hand,

Table 5. Distributions of ACE genotype and allele frequencies in the stroke patients classified according to TOAST criteria

	ACE genotype frequency*			ACE allele frequency*	
	DD	ID	II	D	I
Large vessel disease (%)	37.5	15.0	47.5	38.7	61.3
Small vessel disease (%)	37.3	16.4	46.3	39.5	60.5

*Non-significant between groups.

Table 6. Distributions of AGTR₁ genotype and allele frequencies in the stroke patients classified according to TOAST criteria

	AGTR ₁ genotype frequency*			AGTR ₁ allele frequency*	
	AA	AC	CC	A	C
Large vessel disease (%)	57.5	10.0	32.5	73.8	26.2
Small vessel disease (%)	52.2	7.5	40.3	72.4	27.6

*Non-significant between groups.

the overall frequencies of the genotypes AA, CA, and CC in patient group 58.0%, 34.6% and 7.4% did not differ from genotype distribution in healthy group 60.1%, 35.7% and 4.2%, respectively. No statistical differences were detected in alleles (A or C) frequency between the healthy and ischemic stroke groups. No interactions between the studied polymorphisms and biochemical parameters were found.

The observed genotype frequencies are in agreement with frequencies predicted by Hardy-Weinberg equilibrium. Statistical analysis have shown no statistically significant difference between the frequencies of the genotype of the two groups, and have not verified a relationship between ACE I/D and AT1R A1166C genotype polymorphisms and the development of ischemic stroke in Turkish subjects of Trakya region.

Two-thirds of strokes occur in people over the age of 65. In our study, the mean age of the patients with ischemic stroke was 67±11 years. But, patients with young-onset stroke are thought to possess a stronger genetic background than those who have stroke relatively late in life. The present study has limitation regarding the small sample of young-onset stroke. So, further studies including large samples of young-onset stroke are needed to clarify the role of ACE I/D and AT1R A1166C gene polymorphisms in ischemic stroke in Turkish patients of Trakya region.

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