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Lack of Evidence for Contribution of eNOS, ACE and AT1R Gene Polymorphisms with Development of Ischemic Stroke in Turkish Subjects in Trakya Region

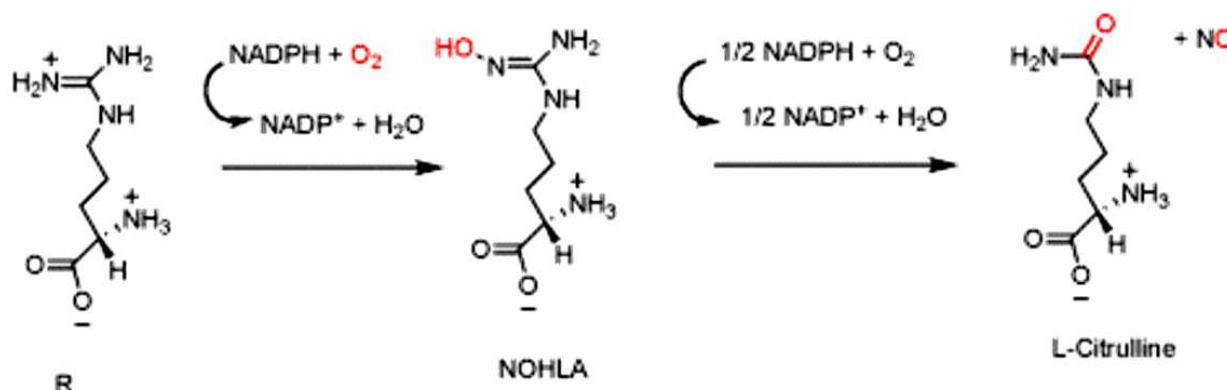
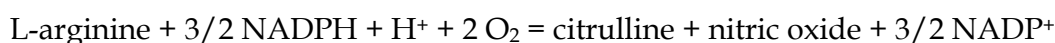
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1. Introduction

Nitric oxide (NO) is produced in the endothelial cells, neurons, glia, and macrophages by the nitric oxide synthase (NOS) isoenzymes. Endothelial nitric oxide synthase (eNOS) is a subgroup of this family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine and oxygen, which causes vascular relaxation (1) by activates guanylate cyclase, which induces smooth muscle relaxation.

The reaction catalyzed by eNOS is:



NO can also promote vasorelaxation indirectly by inhibiting the release of renin which converts angiotensinogen to angiotensin I. This is in turn cleaved to form active angiotensin II by Angiotensin-converting enzyme (ACE), the key component of the physiological control of blood pressure in human. Angiotensin II exerts its effects by binding to angiotensin II type 1, 2, 3, and 4 receptors (AT1R, AT2R, AT3R, AT4R). AT1R is the major mediator of physiological effects of angiotensin II. AT1R mediates its action by association with G proteins and followed by vasoconstriction. The activated receptor in turn couples to G proteins and thus activates phospholipases, increases the cytosolic

Ca²⁺ concentrations, which triggers cellular responses such as stimulation of protein kinases. Activated receptor also inhibits adenylyl cyclases and activates various tyrosine kinases (2).

Ischemic stroke, caused either by thrombosis or embolism, is the most frequent disease leading to disability and/or to death (3). The genetic differentiations varying with ethnic properties may be related to the arrangement of the classic and non-classic risk factors for ischemic stroke (4).

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 stroke disease (5, 6). The RAS gene system comprises the angiotensinogen (AGT), renin, angiotensin I, angiotensin I-converting enzyme (ACE), angiotensin II, and angiotensin II receptors (7).

The ACE is a key component of both the RAS and the kinin-kallikrein system. ACE cleaves the carboxy-terminal dipeptide of angiotensin I, releasing the physiologically active octapeptide angiotensin II (8). Angiotensin II is a potent vasoconstrictive molecule that plays a key role in modulating vascular tone. Angiotensin II exerts its effects by binding to the major mediator AT1R. Human AT1R is present predominantly in vascular cells and in both kidney and adrenal gland mediating physiological actions of angiotensin II. 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 (9).

Our study aimed to assess the distribution of gene polymorphisms of ACE, AT1R and eNOS gene polymorphisms in ischemic stroke patients compared to healthy controls in the subjects from Trakya region.

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 (10, 11).

The AT1R gene maps to chromosome 3 (3q21q25), spans 45.123 kb, and comprises 5 exons and 4 introns (12). 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 (13).

The eNOS gene is located on chromosome 7q35-36 and comprises 26 exons spanning 21 kb (14). Three classes of genetic polymorphisms in eNOS have been identified: those in intron regions, those in the promoter, and those in exon regions (15).

The variable number of tandem repeat (27 VNTR) polymorphism in intron 4 of the eNOS gene (eNOS 4 a/b), and Guanine (G) to Thymine (T) conversion at nucleotide position 894 in exon 7 causing Glutamic acid (Glu) to Aspartic acid (Asp) change at 298 are two of the most encountered polymorphisms. This polymorphism was shown to affect the response of vascular endothelium and the NO levels of plasma (16, 17).

In view of the aging population stroke is becoming a major problem, it is the most frequent disease leading to disability (3) and estimates forecast a continuing increase in the incidence, prevalence, and mortality of stroke in the next decades.

2. Material and methods

The study included 341 subjects; 197 stroke patients and 144 controls (**Table 1**). All participants gave informed consent that was approved by the local ethics committee. DNA was isolated from peripheral blood, collected into tubes containing ethylenediamine-tetraacetic acid (EDTA) by eZNA (EaZy Nucleic Acid Isolation) blood DNA kits (Omega Bio-tek, Doraville, USA). eNOS (4 a/b) and ACE (I/D) gene polymorphisms were identified using a polymerase chain reaction (PCR) technique (5, 18). The AT1R (A1166C) and eNOS (Glu298Asp) gene polymorphisms were identified using PCR technique and restriction fragment length polymorphism (RFLP) assay (5, 19).

	Control Group	Stroke Group	p
Hypertension (%)	61.3	83.1	<0.001
Current smoker (%)	3.6	28.3	<0.001
Diabetes mellitus (%)	17.6	33.8	0.001
Family history of stroke (%)	17.6	33.0	0.002
Age (years)	63.0 (17.0)	69.0 (14.0)	<0.001
SBP (mmHg)	120.0 (20.0)	140.0 (40.0)	<0.001
DBP (mmHg)	70.0 (10.0)	80.0 (20.0)	<0.001
FBG (mg/dl)	89.5 (18.3)	105.5 (41.0)	<0.001
TG (mg/dl)	117.5 (93.5)	145.0 (105.0)	0.008
TC (mg/dl)	189.0 (44.0)	190.0 (52.0)	NS
HDL-C (mg/dl)	39.0 (19.5)	38.5 (14.0)	NS
LDL-C (mg/dl)	120.5 (35.0)	124.0 (41.5)	NS

SBP/DBP; Systolic/Diastolic blood pressure, FBG; Fasting blood glucose, TG; Triglycerides, TC; Total cholesterol, HDL-C/LDL-C; High/Low density lipoprotein cholesterol, NS: Non-significant.

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

PCR technique, developed in 1983 by Kary Mullis, is an in vitro indispensable scientific technique used in medical genetics and hereditary disorders researches to amplify a single (or a few copies) of a piece of DNA to generating millions of copies of a particular DNA sequence (20).

The method relies on thermal cycling, consisting of steps of thermal cycling which can be accomplished automatically with the DNA thermal cycler. First step is DNA denaturation. DNA denaturation is necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. The four bases found in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T). A base on one strand normally binds only to T on the other strand, and C base on one strand normally binds only to G on the other strand. The two types of base pairs form different numbers of hydrogen bonds, AT forming two hydrogen bonds, and GC forming three hydrogen bonds. To separate the two strands of DNA, typical strand separation temperatures (T_{ss}) are 95°C for 30 seconds, or 97°C for 15 seconds (21). For G and C rich region higher temperature may be appropriate (21). The second step is primer annealing. Primers contain sequences complementary to the target region of the DNA template. Primer annealing is required for initiation of DNA synthesis at a lower temperature. A temperature of 55°C is a starting degree for 20 base primers with equal GC/AT content (22). Annealing temperatures in the range of 55°C to 72°C generally yield the best results and occurs in a few seconds (21). The third step is primer extension. Primer extension depends upon the length of the target sequence. Extension at 72°C for fragments shorter than 500 base takes only 20 seconds, and fragments up to 1.2 kilo base 40 seconds is sufficient (23).

In the PCR a thermostable Taq DNA polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*, are used. The half life of Taq DNA polymerase activity is larger than 2 hours at 92.5°C, 40 minutes at 95°C, and 5 minutes at 97.5°C (21). This DNA polymerase enzymatically assembles a new DNA strand from deoxynucleotide triphosphates (dNTPs), by using separated single-stranded DNA as a template and DNA primers. Because the primer extension products synthesized in one cycle can serve as a template in the next, the DNA template is exponentially amplified. Thus, 20 cycles of PCR yields about a million - fold (2^{20}) amplification (22). Since strand dissociation temperatures, primer annealing, product specificity, and Taq DNA polymerase activity affected by magnesium concentration, the magnesium ion concentration was optimized for all gene amplifications in the study. Also, a recommended buffer for PCR is 10 to 50 mM Tris-HCl pH 8.3, up to 50 mM KCl, and up to 0.1% detergents such as Tween 20 must be included. The PCR products of a particular segment of DNA in an ethidium bromide stained agarose gel visualized by UV transillumination. The minimum amount which can be detected by UV transillumination is larger than 10 ng DNA.

Restriction endonucleases are a set of enzymes expressed in bacteria against foreign DNA. Restriction enzymes cut or cleave double stranded DNA at specific recognition base sequences. In 1970 Smith H. et al identified the first restriction enzyme Hind II. Over 3000 of restriction enzymes have been isolated from different bacterial species (24, 25). Restriction enzymes can be used to distinguish single base changes in DNA (26). This method can be used to genotype a DNA sample without the need for expensive gene sequencing. The sample is first digested with the restriction enzyme to generate DNA fragments, and then the different sized fragments separated by gel electrophoresis. The choice of a restriction enzyme for PCR product is dictated by the product itself. All restriction enzymes require Mg^{2+} ions as a cofactor and 37°C is optimal for most of them to work. The recommended units and digestion buffer for 100% digestion with restriction enzymes is 10-20 units for 0.1

to 0.5 g of PCR products and 10 mM Tris-HCl (pH 7.5 to 8.5), 10 mM MgCl₂, 100 mM KCl and 0.1 mg/mL BSA.

A genomic DNA were amplified by PCR technique in a total 25 L PCR mixture containing 200 ng of DNA, deoxynucleotide triphosphates (0.2 mM of each), 0.5 nmol of sense and anti-sense oligonucleotide primers, 1X Taq buffer and 1.25 U of Taq DNA polymerase. eNOS (4a/b), ACE (I/D) and eNOS (Glu298Asp) gene polymorphism reactions were contained 2.5 mM MgCl₂ whereas AT1R (A1166C) gene polymorphism reaction were contained 1.5 mM MgCl₂. All reagents for PCR amplification and gel electrophoresis were purchased from Fermentas Life Sciences (ELİPS, Istanbul, Turkey). All other chemicals were from Sigma and Merck (BO&GA, Istanbul, Turkey) and of the highest purity available. DNA amplifications were performed with a Techne (TechGene) DNA Thermal Cycler.

3. ACE I/D gene polymorphism (rs 4646994)

The PCR primers with the sequences reported by Rigat B. et al. (27) were used. Sense and anti-sense primers were; 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGTGGCCATCACATTTCGTCAGAT-3', respectively. Normally the sense primer in Rigat et al. didn't contain (G), so our PCR products also didn't contain (G), and the anti-sense primer in Rigat et al. didn't contain (G); which is normally must be included, but it was instead of (G) contained (A). Figure 1 shows the sequencing of the region which contains ACE (D) polymorphism.

PCR Conditions

94°C	5 min	
94°C	1 min	} 30 Cycles
58°C	1 min	
72°C	1 min	
72°C	7 min	

The expected insertion (I) and deletion (D) alleles were visualized after electrophoresis on a 2% agarose gel and ethidium bromide staining under UV light transillumination (Fig. 2). Preferential amplification of the D allele in the heterozygotes has led to their mistyping as DD homozygotes (28). To exclude this possibility, all DD homozygotes were retyped using I

***CTGGAGA(G)CCACTCCCATCCTTTCTCCCATTTCTCTAGACCTGCTGCCTATACAG
TCACTTTTATGTGGTTTCGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCC
TTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCA
GATCTGACGAATGTGATGGCCAC(G→A)TC***

Fig. 1. The sequencing of the region which contains ACE (D) polymorphism. Italic and bold letters were used for the primer sequences.

allele specific sense primer 5'-TTTGAGACGGAGTCTCGCTC-3' and anti-sense primer, also reported by Rigat B et al. (27) were 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.

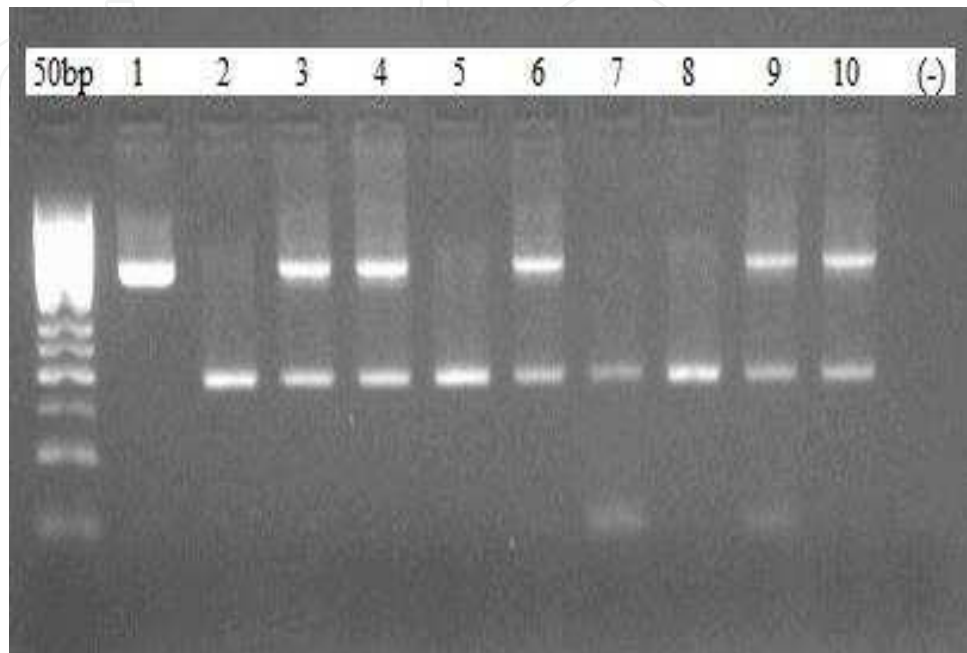


Fig. 2. PCR products of ACE gene I/D polymorphism. The DD (190 bp; lane 2, 5, 7, and 8), the ID (190 bp, and 490 bp, lane 3, 4, 6, 9, and 10) and the II (490 bp, lane 1), 50 bp is a size marker, (-) is a negatif control.

4. eNOS 4 a/b (27 VNTRs) gene polymorphism

The PCR primers with the sequences reported by Wang et al. (29) were used. Sense and anti-sense primers were; 5'-AGGCCCTATGGTAGTGCCTT-3' and 5'-TCTCTTAGTGCTGTGGTCAC-3', respectively. Figure 3 shows the sequencing of the region which contains eNOS 4 a/b (27 VNTRs) polymorphism.

PCR Conditions

94°C	1 min	
95°C	25 sec	} 38 Cycles
56°C	35 sec	
72°C	40 sec	
72°C	5 min	

The PCR products were electrophorized on 2.5% agarose gels, stained with ethidium bromide, and checked under UV light transillumination (Fig. 4).

AGGCCCTATGGTAGTGCCTTGGCTGGAGGAGGGGAAAGAAGTCTAGACCTGCTG
CAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGCTG
 CAGGGGTGAGGAAGTCTAGACCTGCTGCGGGGGT**GAGGAAGTCTAGACCTGCTG**
 CGGGGGT**GAGGACAGCTGAGCGGAGCTTCCCTGGGCGGTGCTGT**CAGTAGCAGG
 AGCAGCCTCCTGGAAAAGCCCTGGCTGCTGCTTCTCCCCAAGAGAGAAGGCTTC
 TCCCGCCAGGCCAGTCCAGTGCAGCCCCTCACCCACACCCACTGCTACCCAGTT
 CCCCTGCTTCGGCCCGCACCCCTCCCTCACACCCAGCCCACAGACTCGGGGCTGG
 CCTTAGTTACTGGAACGCCTGTGACCACAGCACTAAGAGA

Fig. 3. The sequencing of the region which contains eNOS 4 a/b (27 VNTRs) polymorphism. Italic letters were used for the primer sequences and bold letters were used for 27-bp repeats which are deleted in the VNTR 4a polymorphism.

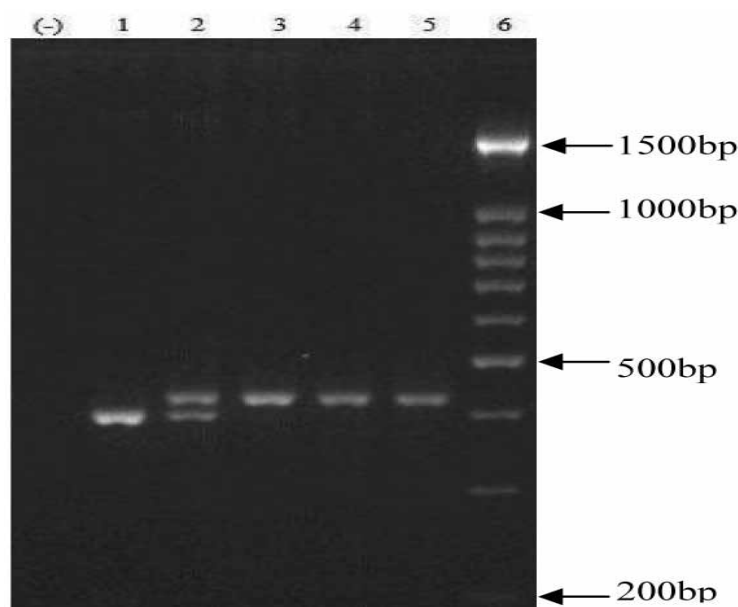


Fig. 4. PCR products of eNOS VNTR gene polymorphism. The aa genotype (394 bp; lane 1), the ab genotype (394 bp and 421 bp; lane 2), and the bb genotype (421 bp; samples 3, 4, and 5). Lane (-) is a negative control, and 6 is a size marker (O'RangeRuler 100bp DNA Ladder).

5. AT1R A1166C gene polymorphism (rs 5186)

AT1R A1166C gene polymorphism was identified with PCR technique followed by RFLP with the restriction enzyme HaeIII (30).

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. Figure 5 shows the sequencing of the region which contains AT1R A1166C gene polymorphism. The sense primer contains one mismatch (A→G) which was required for restriction site.

PCR Conditions

94°C	5 min	
94°C	1 min	} 35 Cycles
55°C	1 min	
72°C	1 min	
72°C	7 min	

The PCR products were electrophorized on 2% agarose gels, stained with ethidium bromide, and checked under UV light transillumination.

GCAGCACTTCACTACCAAATG(A→G)GCAATTAGCTACTTTTCAGAATTGAAGGAGA
 AAATGCATTATGTGGACTGAACCGACTTTTCTAAAGCTCTGAACAAAAGCTTTTC
 TTTCTTTTGCAACAAGACAAAGCAAAGCCACATTTTGCATTAGACAGATGACGG
 CTGCTCGAAGAACAATGTCAGAAACTCGATGAATGTGTTGATTTGAGAAATTTTA
 CTGACAGAAATGCAAT***TCTCCCTAGCCTGCTTTTGTCTG***

Fig. 5. The sequencing of the region which contains AT1R A1166C polymorphism. Italic and bold letters were used for the primer sequences. The underlined and bold letters represent the restriction site for HaeIII (5'-GG↓CC-3').

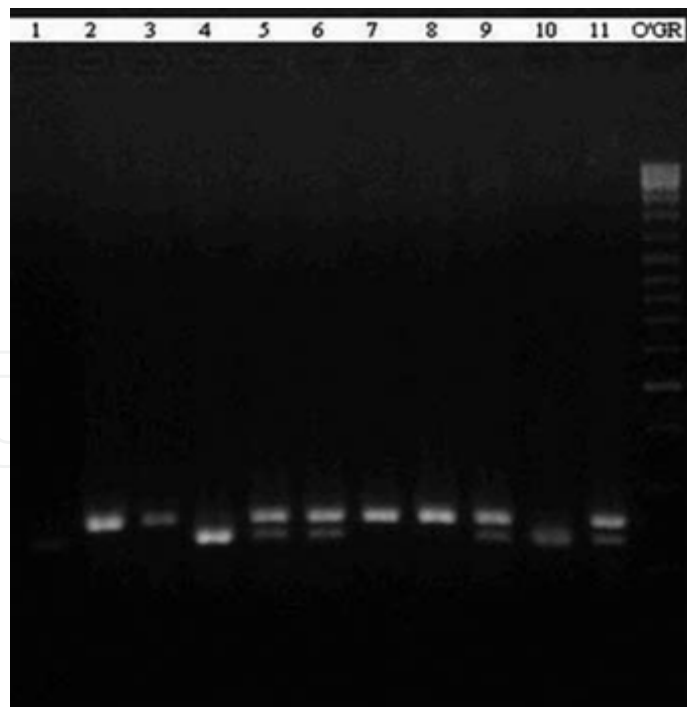


Fig. 6. EtBr stained gel of HaeIII digested PCR products of AT1R A1166C shows the AA genotype (255 bp; lane 2, 3, 7, and 8), the AC genotype (255 bp, 231 bp, and 24 bp; lane 5, 6, 9, and 11), the CC genotype (231 bp, and 24bp; lane 1, 4, and 10), lane O'GR is a size marker (100bp DNA Ladder).

Ten microliters of PCR product were digested with 5 units of the restriction enzyme HaeIII (Takara Bio Inc, Japan) in 1 X M buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM Dithiothreitol and 50 mM NaCl) for 2 hours at 37°C. When mutant allele (cytosine), digested with HaeIII that yield two fragments, whereas a wild allele (adenine) at nucleotide position 1166, had no cutting site for HaeIII, so that the PCR product was not cleaved into two fragments. The restriction digest products were visualized after electrophoresis on a 2.5% agarose gel and ethidium bromide staining (Fig. 6).

6. eNOS Glu298Asp (rs 1799983) gene polymorphism

Glu298Asp polymorphism of eNOS was identified with PCR technique followed by RFLP with the restriction enzyme BanII (19, 31).

PCR primers were generated to amplify the 248 bp fragment encompassing the eNOS Glu298Asp variant primers 5'-AAGGCAGGAGACAGTGGATGGA-3' (sense) and 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' (anti-sense). Figure 7 shows the sequencing of the region which contains eNOS Glu298Asp gene polymorphism.

PCR conditions

eNOS Glu298Asp;

95°C	5 min	
94°C	1 min	} 38 Cycles
59°C	1 min	
72°C	1 min	
72°C	5 min	

The PCR products were electrophorized on 2% agarose gels, stained with ethidium bromide, and checked under UV light transillumination.

AAGGCAGGAGACAGTGGATGGAGGGGTCCCTGAGGAGGGCATGAGGCTCAGCCC
CAGAACCCCTCTGGCCCACTCCCCACAGCTCTGCATTCAGCACGGCTGGACCCC
*AGGAAACGGTCGCTTCGACGTGCTGCCCCTGCTGCTGCAGGCCCCAGAT***GATCCC**
CCAGAACTCTTCCTTCTGCCCCCGAGCTGGTCCTTGAGGTGCCCTGGAGCACCC
CACGTGAGCACCAAAGGGATTGACTGGG

Fig. 7. The sequencing of the region which contains eNOS Glu298Asp polymorphism. Italic and bold letters were used for the primer sequences. The underlined and bold letters represent the restriction site for Ban II (5'-G(A/G)GC(T/C)↓C-3').

Ten microliters of PCR product were digested with the restriction enzyme BanII to digest wild allele (guanine). When a guanine is at nucleotide position 894, resulting in a glutamic acid at amino acid position 298, BanII restriction enzyme produces two fragments of 163 and

85 bp. In contrast, when a thymine is at nucleotide position 894 (mutant allele), resulting in an aspartic acid in the amino acid sequence, the Asp298 variant had no cutting site for BanII, so that the 248 bp PCR product was not cleaved into 163 and 85 bp fragments. The restriction digest products were analyzed through electrophoresis on 2.5% agarose gel and ethidium bromide staining (Fig. 8).

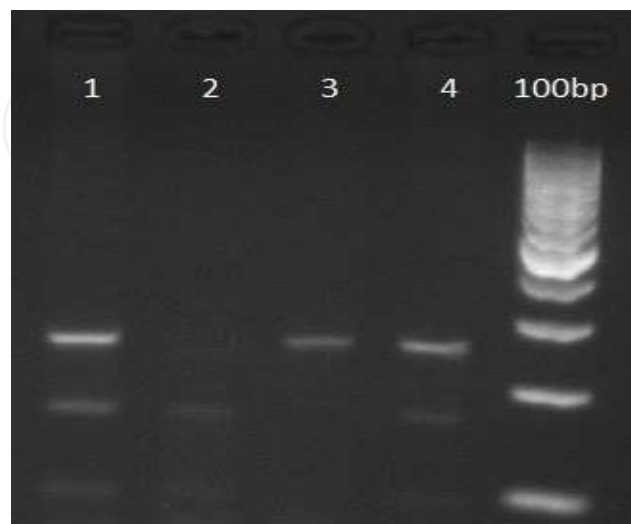


Fig. 8. EtBr stained gel of BanII digested products of eNOS gene Glu298Asp polymorphism. Line 1 and 4; GT alleles (85, 163, and 248 bp), line 2; GG alleles (85 and 163 bp), line 3; TT alleles (248 bp) and line 100bp; Gene Ruler 100 bp DNA Ladder.

7. Results and discussion

Table 2, 3, 4, and 5 shows the distributions of ACE I/D, eNOS (4 a/b), AT1R (A1166C), and eNOS Glu298Asp genotypes, respectively.

Statistical analyses were performed with the SPSS 15.0 software and STATA program. Genotypic distributions were in accordance with Hardy-Weinberg equilibrium in the stroke group as well as in the control group. Several studies have shown differences in the genotypic distributions of these genes while, others have shown no differences between the controls and patients. Our results didn't show any significant difference between the ischemic stroke patients and the controls ($p > 0.05$) and suggested the lack of an association between the 4 gene polymorphisms and ischemic stroke (Table 2, 3, 4, and 5). So the 4 gene polymorphisms did not enhance the predictability of stroke.

	DD	ID	II
Controls (%)	34.3	49.7	16.1
Stroke Patients (%)	34.0	50.0	16.0
	Non-Significant	Non-Significant	Non-Significant

Table 2. Distribution of ACE (I/D) genotype frequency in the controls and stroke patients

	aa	ab	bb
Controls (%)	2.8	29.8	67.4
Stroke Patients (%)	2.0	35.0	63.0
	Non-Significant	Non-Significant	Non-Significant

Table 3. Distribution of eNOS (4 a/b) genotype frequency in the controls and stroke patients

	AA	AC	CC
Controls (%)	60.1	35.7	4.2
Stroke Patients (%)	58.0	34.6	7.4
	Non-Significant	Non-Significant	Non-Significant

Table 4. Distribution of AT1R (A1166C) genotype frequency in the controls and stroke patients

	GG	GT	TT
Controls (%)	49.3	45.8	4.9
Stroke Patients (%)	56.3	40.6	3.1
	Non-Significant	Non-Significant	Non-Significant

Table 5. Distribution of eNOS (Glu298Asp) genotype frequency in the controls and stroke patients

In our previous study about potential angiotensinogen (AGT) gene that predispose to hypertension, we failed to detect any relation between T174M and M235T gene polymorphisms of the AGT gene in the RAS and the development of hypertension (32).

Now, we are working on the AGT gene to clarify the role of T174M and M235T gene polymorphisms of the AGT gene in the stroke Turkish patients from Trakya region.

8. Conclusions

In addition to demographic and clinical characteristics, which are important in the developing of ischemic stroke, our data does not suggest that ACE (I/D), AT1R (A1166C), eNOS (4 a/b) and eNOS (Glu298Asp) gene polymorphisms, in contrast to other studies which shows a positive association between this gene polymorphisms and ischemic stroke, are a common cause of ischemic stroke in Turkish patients from Trakya region.

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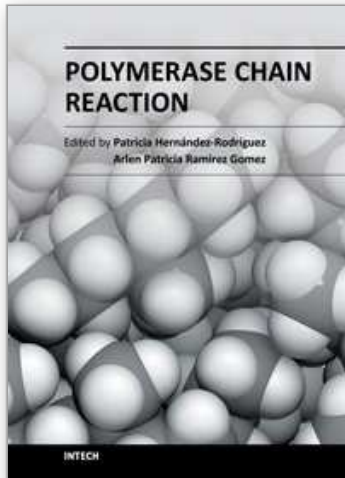
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