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Investigation of eNOS gene intron 4 A/B VNTR and intron 23 polymorphisms in patients with essential hypertension

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Aim: Nitric oxide (NO) plays a major role in the regulation of vascular tone. Production of NO can be influenced by polymorphisms of the endothelial NO synthase (eNOS) gene, which may be associated with the pathogenesis of essential hypertension (EHT). Therefore, eNOS gene intron 4 a/b variable number of tandem repeats (VNTR) and intron 23 polymorphisms were investigated in patients with EHT living in a central area of Turkey.

Materials and methods: The study was performed in 91 patients (34 M, 57 F) with EHT, aged 38-76 years, and 75 age- and sex-matched healthy controls (35 M, 40 F). eNOS gene polymorphisms were detected by polymerase chain reaction method.

Results: There was no significant difference between the G-allele frequency of the G10-T polymorphism in intron 23 and intron 4 a/b VNTR polymorphism of the eNOS gene in EHT patients and in the controls.

Conclusion: eNOS gene intron 4 a/b VNTR and intron 23 gene polymorphisms were not associated with EHT patients living in a central area of Turkey. Further studies are needed to investigate whether these 2 polymorphisms of the eNOS gene could represent useful genetic markers for indentifying individuals at risk of developing EHT.

Key words: Essential hypertension, eNOS gene polymorphisms, Intron 4 a/b VNTR, Intron 23

Esansiyel hipertansiyon hastalarında eNOS gen intron 4 a/b VNTR ve intron 23 polimorfizimlerinin araştırılması

Amaç: Nitrik oksit (NO), vasküler tonusun düzenlenmesinde önemli bir rol oynar. NO'in üretimi esansiyel hipertansiyon (EHT)'un patogenezi ile ilişkili olan endotelyal NO sentaz (eNOS) gen polimorfizimlerinden etkileniyor olabilir. Bundan dolayı, çalışmamızda Türkiye'nin orta bölgesinde yaşayan EHT hastalarında eNOS gen intron 4 a/b değişken sayıdaki ardışık tekrarlar (VNTR) ve intron 23 gen polimorfizimleri araştırıldı.

Yöntem ve gereç: Çalışma 38-76 yaşları arasındaki 91 EHT hastası (34 E, 57 K) ile uygun yaş ve cinsiyette 75 sağlıklı kontrol (35 E, 40 K) vakası üzerinde gerçekleştirildi. eNOS gen polimorfizmleri polimeraz zincir reaksiyon metodu ile belirlendi.

Bulgular: EHT hastaları ile kontrollerin eNOS gen intron 23 G10-T polimorfizminin G-allel sıklığı ile intron 4 a/b VNTR polimorfizimleri arasında önemli bir fark bulunamadı.

Sonuç: eNOS geni intron 4 a/b VNTR ve intron 23 polimorfizimleri Türkiye'nin orta bölgesinde yaşayan EHT hastaları ile ilişkili değildir. EHT gelişme riski olan kişilerin tanınmasında eNOS genindeki bu iki polimorfizmin faydalı birer genetik markır olup olmayacağının anlaşılması için daha ileri çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Esansiyel hipertansiyon, eNOS gen polimorfizmi, İntron 4 a/b VNTR, İntron 23

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Introduction

Hypertension is currently defined as a usual blood pressure (BP) of 140/90 mm Hg or higher. In 90%-95% of hypertensive patients, a single reversible cause of the elevated BP cannot be identified: hence the term essential or primary hypertension (EHT) (1). Approximately 30% of the variance in BP was reported to be attributable to genetic heritability (2,3). Therefore, study of the candidate genes that may contribute to the pathogenesis of HT may be a good approach for understanding the etiology of EHT.

Nitric oxide (NO), derived from vascular endothelium and various cells, is a simple and short-lived gas that plays a major role in the regulation of vascular tone (4,5). It is synthesized from L-arginine by the enzyme NO synthase (NOS) (6,7). It has key functions in the relaxation of vascular smooth muscle, inhibits adhesion of platelets and leukocytes, and limits the oxidation of atherogenic low-density lipoproteins (8).

The synthesis of NO by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of BP (9). Therefore, reduced NO synthesis may be associated with the pathogenesis of hypertension.

NO production can be influenced by polymorphisms of the endothelial NOS (eNOS) gene. Several investigations have been performed to show the association between various eNOS gene polymorphisms and coronary artery disease (CAD) (6-8,10,11). However, studies on the association between genetic polymorphisms of the eNOS gene and EHT have been few and the findings are conflicting (12,13).

The gene that encodes eNOS is located on chromosome 7q35-36. It comprises 26 exons spanning 21 kilobases, showing a 27 bp repeat polymorphism in intron 4 of the gene intron 4 a/b variable number of tandem repeats (VNTR) (10,14,15).

The eNOS gene has 2 common alleles containing 4 repeats (a) and 5 repeats (b) which produce 2 homozygous (aa and bb) and 1 heterozygous (ab) genotypes (15). In recent studies, the eNOS intron 4 a/b VNTR polymorphism has been found to be associated with altered plasma NO levels and responsible for variations in the genetic control of plasma nitrite and nitrate levels and enzyme production (16-18).

In the present study, we investigated eNOS gene intron 4 a/b VNTR and intron 23 polymorphisms in patients with EHT and normotensive healthy controls living in a central area (Konya) of Turkey.

Materials and methods

Subjects

Ninety-one patients with EHT (34 M and 57 F), aged 38-76 (56.23 ± 9.0) years, and 75 healthy controls (35 M, 40 F), aged 41-73 (55.43 ± 8.7) years, were enrolled in the study. Subjects with BP equal to or greater than 140 mmHg systolic and 90 mmHg diastolic were defined as having HT. Informed consent was obtained from all the subjects prior to their enrollment in the study. Smoking, alcohol consumption, HT, diabetes mellitus, and the family history of all cases were recorded. HT patients with any other risk factors were excluded from the study. All diagnostic criteria were based on WHO guidelines. The study was approved by the ethics committee of the University of Selçuk's Meram Medical School.

Clinical and laboratory features of the subjects are given in Table 1.

Laboratory analysis

Ten milliliters of venous blood samples were obtained from all subjects into EDTA tubes. DNA samples were separated immediately and stored at -80 °C until the time of analyses.

Determination of eNOS 4 a/b VNTR polymorphism

Genomic DNA from leukocytes was purified according to the method of Miller et al. (19). The eNOS gene intron 4, 27 bp VNTR polymorphism was detected by polymerase chain reaction (PCR) according to the method described by Wang et al. (16) and Channon et al. (20). The template DNA (0.5 μg per sample) was amplified using the following primers: (forward) 5'-AGG CCC TAT GGT AGT GCC TTT-3' and 5'-TCT CTT AGT GCT GTG CTC AC-3' (reverse). These primers (10 pmol of each) were

Table 1. Clinical and laboratory features of the controls and the hypertensive patients.

Features	Control (n = 75)	EHT (n = 91)	P
Male/Female (n/n)	35/40	34/57	NS
Age (years)	55.43 ± 8.7	56.23 ± 9.0	NS
Systolic BP (mmHg)	<140	≥140	<0.01
Diastolic BP (mmHg)	<90	≥90	<0.01
Smoking (n)	27 (36%)	58 (64%)	<0.001
Total-C (mmol/L)	5.07 ± 0.242	5.14 ± 1.017	NS
LDL-C (mmol/L)	3.07 ± 0.66	3.13 ± 0.65	NS
HDL-C (mmol/L)	1.09 ± 0.33	1.13 ± 0.19	NS
TG (mmol/L)	2.21 ± 0.87	2.24 ± 1.19	NS

(The values of lipids and ages are expressed as mean ± standard deviation.)
EHT: essential hypertension; BP: blood pressure; Total-C: total cholesterol; LDL-C: low-density lipoprotein choesterol; HDL-C: high-density lipoprotein cholesterol; TG: trigycerides.

added to a mixture containing 0.2 µmol/L each of dATP, dCTP, dGTP, and dTTP; 5 µL of 10× Cetus buffer (pH 8.3); 5 µL of DMSO (100%); and 0.5 units of Taq DNA Polymerase (Perkin Elmer Cetus), in a final volume of 20 µL.

The PCR was initiated with a denaturation by first heating the samples for 5 min at 94 °C. Thirty-five cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 56 °C, primer extension for 2 min at 72 °C, and a last extension for 5 min at 72 °C were applied for amplification. The PCR products of the NO gene locus were examined by gel electrophoresis (2% NuSieve agarose-agarose) at 150 V for 30 min and visualized at room temperature under UV light after ethidium bromide staining (Figure 1).

Determination of G10-T polymorphism in intron 23

For the G10-T polymorphism in intron 23 of the eNOS gene, the primer pair for PCR was as follows: forward, 5'-CCC CTG AGT CAT CTA AGT ATT C-3'; reverse, 5'-AGC TCT GGC ACA GTC AAG-3'. The resulting 676 bp amplification products were incubated at 37 °C for 2 h with 10 U of the restriction enzyme HindII (Bioron). The amplified DNAs were then digested by HindII into fragments (577 and 99 bp). In the case of a G-to-T substitution at position 10 of intron 23 of the eNOS gene, an additional HindII restriction site was produced and the amplified fragments were digested into smaller fragments (374, 203, and 99 bp) (Figure 2). This polymorphism could be determined in 90 patients and 61 controls.

Statistical analysis

The data was analyzed using the Statistical Package for the Social Sciences (SPSS, Version 10.0). Genotypic and allelic frequencies were determined using the gene counting method. The chi-squared test and Fisher's exact test were performed for independent relationships between variables. Statistical significance was accepted at $P < 0.05$.

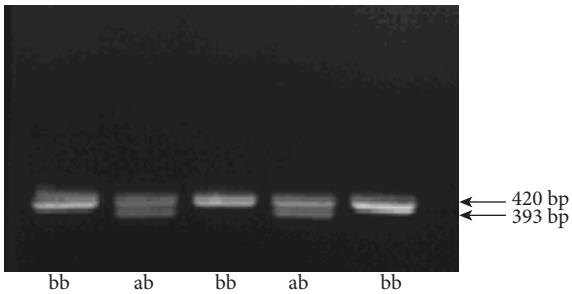

Figure 1. Genotyping of the VNTR in intron 4 of eNOS gene.

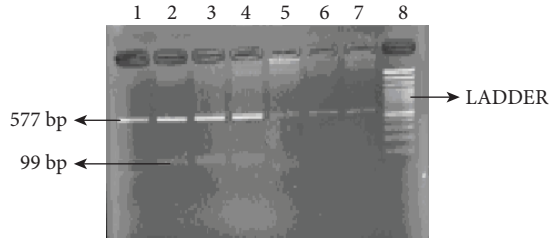

Figure 2. Genotyping of the intron 23 of eNOS gene.

Results

The distribution of genotypes and allele frequencies are compared between patients and controls in Table 2. The genotype frequencies were in agreement with Hardy-Weinberg equilibrium.

A comparison of genotypes and allelic frequencies of the G10-T and intron 4 VNTR polymorphisms between EHT patients and controls is shown in Table 2. There was no significant difference between the G-allele frequency of the G10-T polymorphism in intron 23 and intron 4 VNTR polymorphisms of the eNOS gene in EHT patients and in controls.

eNOS 4a/b genotype frequencies in EHT patients were 3.3%, 20.88%, and 75.82% for aa, ab, and bb, respectively, and 1.3%, 21.33%, and 77.33% in the control group for aa, ab, and bb, respectively. Genotype frequencies were not significantly different between groups ($\chi^2 = 4.59$, P = 0.10).

Moreover, we found no significant difference between the allelic frequencies of the intron 4 VNTR polymorphism in the EHT patients and in the controls.

Discussion

The incidence of HT may differ in each race or ethnic group and the frequencies of eNOS gene polymorphisms have been reported to vary among ethnic groups (10-12,21). Therefore, findings about the association between genetic polymorphisms of eNOS genes and EHT are conflicting.

In our study, we found no significant difference between the EHT patients and controls in terms of the allelic frequencies of the intron 4 VNTR or G10-T in intron 23. Zintzaras et al. (22) have reported an association between HT and eNOS 4b/a polymorphism: under a recessive model, the allele b provided evidence of protection, mainly when analysis was confined to whites. However, they reported no detectable influence of the G894T, T786C, and G23T polymorphisms.

Miyamoto et al. (23) found that the Glu298Asp missense variant was significantly associated with EHT, but found no such association between VNTR in intron 4 (eNOS 4b/4a) and the disease. Benjafield and Morris (24) have found no association of NOS3 markers (a biallelic VNTR in intron 4 and an exon 7 variant) in patients with EHT. Zhao et al. (13) found no association between the T-786C, intron 4b/a, and G894T polymorphisms of the eNOS gene in the development of HT in a northern Han Chinese population. Our findings about the eNOS gene intron 4 a/b VNTR were similar to the findings reported by Miyamoto et al. (23), Zhao et al. (13), and Benjafield and Morris (24).

The difference between findings may be explained in view of the genetic variability of populations. Furthermore, it has been suggested that the difference observed in the distribution of the eNOS intron 4a allele might be due to the different sample sizes and different selection criteria adopted for patients and controls, in particular the clinical presentation, age, race, geographic area, and environmental risk factors (10).

Table 2. Genotypic and allelic frequencies of eNOS gene intron 4a/b VNTR and intron 23 polymorphisms in controls and hypertensive patients.

Polymorphisms	Groups	Genotypic frequency*			Allelic frequency*		
		4a/4a	4a/4b	4b/4b	4a	4b	P
Intron 4 VNTR	Control	1 (1.3%)	16 (21.33%)	58 (77.33%)	0.12	0.88	NS
	EHT	3 (3.3%)	19 (20.88%)	69 (75.82%)	0.14	0.86	NS
G10-T		GG	GT	TT	G	T	P
Polymorphism in	Control	61 (100%)	-	-	1	0	NS
intron 23	EHT	90 (100%)	-	-	1	0	NS

*Genotypic and allelic frequencies were determined using the gene counting method.
EHT: essential hypertension; eNOS: endothelial nitric oxide synthase; NS: not significant; VNTR: variable number of tandem repeats.

A previous study showed that eNOS 4a/b gene polymorphism was not associated with HT in Turkish patients (25). However, to our knowledge, no data are available yet about the association between intron 23 polymorphism and EHT in the Turkish population.

Our results showed that there was no significant association between the G10-T polymorphism in intron 23 (G10-T) and EHT. However, Yoon et al. (15), in their study on a Japanese population, found that the genotypic frequency of G10-T polymorphism in intron 23 of the controls was 87.5% GG and 12.5% GT. This difference may be explained in view of the genetic variability of the populations. However, the influence of this difference on EHT needs to be investigated further.

Acknowledgments

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