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# Endothelial Constitutive Nitric Oxide Synthase Gene Polymorphism in the Turkish Population

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***TÜRK POPULASYONUNDA ENDOTELYAL KONSTİTÜTİF NİTRİK OKSİD SENTAZ GEN POLİMORFİZMİ***

***ÖZET***

*Endotel disfonksiyonu koroner arter hastalığı (KAH) risk faktörleri ve kronik kalp yetersizliği (KKY) patogenezinde önemli rol oynamaktadır. Çalışmanın amacı, endotelyal konstitütif nitrik oksit sentaz geninin (ecNOS) 4. intronundaki "variable number of tandem repeats (VNTRs)" polimorfizmi ile esansiyel hipertansiyon (EH), KKY ve KAH risk faktör dağılımındaki ilişkiyi incelemekti. Çalışmaya 65 sağlıklı kontrol, 57 EH ve 50 KKY hastası alındı. ecNOS geninin VNTRs'i polimeraz zincir reaksiyonu (PCR) ile tekrar sayısını belirlemek için çoğaltıldı ve kontrol ile hasta grupları arasındaki alel frekansı değerlendirildi. Dört ve 5 tekrarlı 2 alel (a, b) tespit edildi. Kontrol grubu ile karşılaştırıldığında, ab genotipi EH ile anlamlı bir şekilde ilişkili bulundu (p=0.048, odds ratio= 2.54 (%95 güvenirlik intervali (CI) 0.99-6.55), dilate KKY'de istatistiki anlamlılık kazanmamakla beraber daha sık görüldü, iskemik KKY'de ise aynı oranda saptandı. ab genotipli iskemik KKY hastaları bb genotipi ile karşılaştırıldığında, anlamlı olarak daha genç (55±10 vs 65±80 yaş; p=0.047) ve daha düşük HDL-kolesterollü (0.7±0.16 vs 1.09±0.21 mmol/L (27±6 vs 42±8 mg/dl); p=0.005) bulundu. Sonuçta, araştırılan bu Türk popülasyonunda ecNOS geni iskemik KKY ile ilişkisiz, EH ile ilişkili bulunurken, dilate KKY ile olası bir ilişki olabileceğine karar verildi. ab genotipli genç ve düşük HDL-kolesterollü hastalar KAH ve KKY açısından yüksek derecede risk taşıyabilirler.*

***Anahtar kelimeler:*** *ecNos gen polimorfizmi, Türk popülasyonu, esansiel hipertansion, kronik kalp yetersizliği*

The endothelial constitutive nitric oxide synthase (ecNOS) gene is important for the maintenance of a continuous vasodilator tone in vessels due to basal nitric oxide (NO) production [1]. Inhibition of NO production increases blood pressure [2], and hypertensive subjects might have abnormal endothelium dependent responses to vasoactive substances [3,4]. A study with ecNOS gene knockout mice revealed that the disruption of the ecNOS gene leads to hypertension [5]. Studies of some common variants of the ecNOS gene were not involved in essential hypertension (EH) [6,7,8]. Other studies found positive association of the ecNOS gene with EH [9,10,11]. Endothelial dysfunction is now recognized as an early, perhaps initiating event in the pathogenesis of coronary artery disease (CAD), and has been shown to be present in patients with hypertension, other risk factors for CAD, and chronic heart failure (CHF) [3,4,12-19].

In this study, the possible association between a variable number of tandem repeats (VNTRs) polymorphism in intron 4 of the ecNOS gene, and EH, CHF, and the distribution of risk factors for CAD in Turkish population were investigated.

## MATERIAL and METHODS

### Study population

A total of 57 hypertensive patients were selected according to the following criteria: 1) patient age >20 years, 2) onset of EH occured at <50 years of age, 3) established EH according to the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VI) [20], 4) absence of secondary forms of hypertension, 5) family history of EH occurring before 60 years of age, with at least one parent or one sibling affected. All patients with EH were under treatment for a duration of 64±70 months; 44% were on monotherapy, 56% were on combination therapy. Among drugs used were 54% angiotensin converting enzyme inhibitors (ACEi), 13% angiotensin II type 1 receptor blockers (ATIIRb), 27% beta blockers (BB), 29% statins, 28% aspirin, 40% calcium channel blockers (CCB), and 29% diuretics.

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A total of 50 CHF patients with predominantly sistolic dysfunction (EF <40%) were grouped as ischemic or non ischemic. Patients with pathologic coronary angiography, a history of myocardial infarction, or coronary artery bypass surgery were grouped as ischemic, whereas patients with normal coronary angiography and no history of HT and other possible causes were grouped as non ischemic. All patients with CHF were under treatment for the duration of 57±42 months. Among drugs used were 82% ACEi, 18% ATIIRb, 18% BB, 24% statins, 86% aspirin, 18% CCB, 56% digitalis, 70% diuretics, 74% nitrates, 22% anticoagulants, and 26% spironolactone.

Sixty-five healthy control subjects were selected according to the following criteria: 1) subject aged between 20 to 50 years, 2) absence of antihypertensive treatment, 3) blood pressure <140/90 mm Hg, 4) absence of CHF, 5) absence of other concomitant diseases.

Informed consent was obtained from each individual according to a protocol approved by the local committee of Istanbul University.

### Laboratory analysis

Risk factors for CAD according to the National Cholesterol Education Program [21,22] criteria and the following possible risk factors were evaluated: triglycerid >2.26mmol/L (200mg/dl), uric acid >446μmol/L (7.5 mg/dl), obesity (BMI >27kg/m$^2$), alcohol intake, fibrinogen >4g/L (400 mg/dl), left ventricular hypertrophy (mass index cuttoffs of 120g/m$^2$ for men, 100g/m$^2$ for women) [23,24]. Hemograms, electrolytes, renal and liver functions, serum lipids, glucose, uric acid, and fibrinogen were recorded. Biochemical analysis were measured by standard methods in the clinical laboratory department of the university hospital.

### Analysis of the VNTRs polymorphism of the ecNOS gene

ecNOS genotypes were determined by polymerase chain reaction (PCR) using oligonucleotide primers (sense: 5'-AGGCCCTATGGTAGTGCCTTT-3'; antisense: 5'-TCTCTTAGTGCTGTGGTCAT-3') that flank the region of the 27 base pair (bp) direct repeat in intron 4 as described previously with minor modifications [25]. Reactions were performed in a total volume of 50 μl containing 500ng genomic DNA, 10 pmol of each primer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (MBI Fermentas Inc., New York, NY), 5μl PCR buffer (500mmol/l KCl, 100 mmol trihidroxymethylaminomethan chlorid and 0.8% Nonidet P40; MBI Fermentas Inc.). The thermocycling procedure consisted of initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute. The PCR products were analyzed using 3% agorose gel electrophoresis and visualized by ethidium bromide staining.

### Statistical analysis

Numerical variables were compared with Student's t test. Non-numerical variables were compared with $\chi 2$ test and Fischer's exact test when appropiate. A p value less than 0.05 was considered significant. The statistical analysis was done by using Statistical Package for Social Sciences (SPSS) for Windows 10.0.

## RESULTS

The large allele, ecNOS4b contained 5 tandem 27bp repeats, and the smaller allele, ecNOS4a contained 4 repeats. The size of the PCR products were 393 bps and 420 bps for the ecNOS4a and ecNOS4b alleles, respectively (Figure 1). Frequencies of aa, ab, and bb genotypes in the control group, in the EH group, and in the CHF group are shown in Table 1. The distribution of the genotypes were within the Hardy-Weinberg equilibrium.

Cardiovascular risk factors of patients and controls are shown in Table 2. Hypertensive patients and CHF patients had numerically (data not shown) and percentwise more risk factors compared to controls. In the subgroup analyses of CHF, the group with ischemic CHF had significantly more males and smokers (24/29 vs 9/21, p=0.003; 20/29 vs 6/21, p=0.004, respectively), and had significantly more positive family history (18/29 vs 7/21, p=0.04). No patient or control had increased alcohol consumption. Significantly more females with left ventricular hypertrophy in EH were detected (28/32 vs 13/25; p=0.003).

The ecNOS4ab genotype was associated with EH (p=0.048, odds ratio=2.54 (95% confidence interval (CI) 0.99-6.55)), was more frequent in dilated CHF

**Table 1. Genotype and allele frequencies in hypertensive, ischemic and non ischemic dilated chronic heart failure (CHF) patients and controls in Turkish population**

| | bb genotype | ab genotype | aa genotype | a allele | b allele |
|---|---|---|---|---|---|
| Controls | 57 (88%) | 8 (12%) | 0 (0%) | 0.06 | 0.94 |
| Non ischemic CHF | 16 (76%) | 5 (24%) | 0 (0%) | 0.12 | 0.88 |
| Ischemic CHF | 26 (90%) | 3 (10%) | 0 (0%) | 0.05 | 0.95 |
| Hypertension | 42 (74%) | 14 (24%) | 1 (2%) | 0.14 | 0.86 |

**Table 2. Cardiovascular risk factors of the patients and controls**

| | Controls n=65 | Hypertension n=57 | Heart failure n=50 | P value |
|---|---|---|---|---|
| Age (Years) | 37.03 ± 8.1 | 44.7 ± 6.9 | 65.9 ± 8.9 | a*,b*: p<0.001 |
| Sex (M/F) | 26/39 | 25/32 | 33/17 | a:p: NS, b: p=0.005 |
| Smoking (n, %) | 19 (29%) | 18 (32%) | 26 (52%) | a:p: NS, b: p=0.02 |
| Diabetes mellitus* (n,%) | 0 (0%) | 5 (9%) | 15 (30%) | a:p<0.01, b: p<0.001 |
| High TC* (n,%) | 0 (0%) | 24 (42%) | 22 (44%) | a,b: p<0.001 |
| High LDL* (n,%) | 0 (0%) | 15 (26%) | 14 (28%) | a,b: p<0.001 |
| Low HDL* (n,%) | 13 (20%) | 14 (25%) | 12(24%) | a, b: p=NS |
| High TG* (n,%) | 3 (5%) | 19 (33%) | 15 (30%) | a,b: p<0.001 |
| High UA* (n,%) | 0 (0%) | 0 (0%) | 9 (18%) | a:p=NS, b:p<0.001 |
| High fibrinogen* (n,%) | 5 (8%) | 13(23%) | 27 (54%) | a:p=0.02, b: p<0.001 |
| Positive family history* | 10 (15%) | 17 (30%) | 24 (48%) | a:p=NS, b: p<0.001 |
| BMI>27kg/m$^2$ (n,%) | 17 (26%) | 27 (47%) | 18 (36%) | a:p=0.02, b:p=NS |

** Glucose>7mmol/L (126mg/dl), TC (Total cholesterol>5.17 mmol/L (200mg/dl), LDL (Low density lipoprotein cholesterol>3.36 mmol/L (130mg/dl), HDL (High density lipoprotein cholesterol<0.9 mmol/L (35mg/dl), TG (Triglycerid>2.26 mmol/L (200mg/dl), UA (Uric acid>446μmol/L (7.5mg/dl), Fibrinogen>4g/L (400mg/dl), Myocardial infarction or sudden death in first degree consanguity in men before 55, in women before 65 years of age.*
*a: controls compared with hypertensives, b: controls compared with heart failure patients*

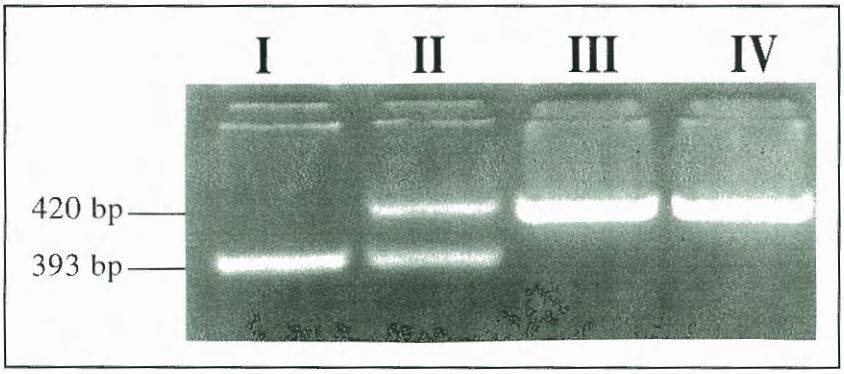


**Figure 1.** Genotyping of the polymorphism in intron 4 of the endothelial constitutive nitric oxide synthase gene. **Lane I:** a/a homozygous, **lane II:** a/b heterozygous, **lane III and Lane IV:** b/b homozygous. The 420 base pairs (bp)-band indicates five repeats and the 393 bp-band indicates four repeats of the 27 bp consensus sequence

without reaching statistical significance (p=0.2) compared to controls. Ischemic CHF had similar prevalence of ab genotype compared to controls (p=0.8). Ab genotype patients with ischemic CHF were significantly younger (55±10 vs 65±80 years of age; p=0.047), had significantly lower high density lipoprotein-cholesterol (HDL) levels (0.7±0.16 vs 1.09±0.21 mmol/L (27±6 vs 42±8 mg/dl); p=0.005) compared to bb genotype. According to the genotype (ab or bb) there was no significant difference in age, sex, smoking, diabetes mellitus, lipoprotein-cholesterol, triglycerid, uric acid, fibrinogen, obesity, positive family history, left ventricular hypertrophy, creatinine, electrolytes, and hemogram parameters.

## DISCUSSION

We found, that the ecNOS gene was associated with EH in this Turkish population studied. Admittedly, this observation was of borderline significance. While the same gene may play an important role in the pathogenesis of dilated CHF, it showed no association with ischemic CHF.

Among the many candidate genes for a genetic basis of EH, the genes of the renin-angiotensin system, including angiotensinogen [26], angiotensin-converting enzyme [27], angiotensin II type 1 receptor [28], and the atrial natriuretic peptide genes [29] are reported to be positively associated with EH. Despite the promise of earlier research, studies of some common

**Table 3. Endothelial constitutive nitric oxide synthase gene allele frequencies in studies reported in the literature**

| | CONTROLS | | | PATIENTS | | | |
|---|---|---|---|---|---|---|---|
| | n | a allele | b allele | n | a allele | b allele | |
| Uwabo[10] | 120 | 0.04 | 0.96 | 123 | 0.11 | 0.89 | EH* |
| Ichihara[30] | 550 | 0.10 | 0.90 | 455 | 0.14 | 0.86 | MI* |
| Wang[25] | 153 | 0.17 | 0.83 | 549 | 0.14 | 0.86 | CAD* |
| Park[31] | 206 | 0.12 | 0.88 | 121 | 0.14 | 0.86 | MI |

**EH: Essential hypertension, MI: Myocardial infarction, CAD: Coronary artery disease*

variants of the ecNOS gene had showed conflicting results. While some studies reported negative results [6,7,8], other studies found a positive association of the ecNOS gene with EH [9,10,11]. There is only one study which demostrated reduced gene expression of vascular ecNOS in CHF patients [19]. To the best of our knowledge the present study is the first study that investigated ecNOS gene polymorphism in non ischemic CHF.

We used VNTRs polymorphism in intron 4 of the ecNOS gene. The same polymorphism was used by many investigators in CAD, in myocardial infarction (MI), and in EH before.

Wang et al showed a positive association between 4a polymorphism and smoking-dependent risk of CAD in whites in Australia, but not with EH [25]. Uwabo et al found that the frequency of the four-repeat allele was lower in Japanese than in Australians, so he suggested that the discrepancy between Wang's and his study may be attributable to various factors such as diagnostic criteria or race [10]. Ichihara et al demonstrated that smoking did not affect the association of the ecNOS4a allele with MI in the Japanese population in contrast to Wang. He showed that the ecNOS4a allele was an independent risk factor for MI in the Japanese population, especially in those lacking other conventional risk factors [30]. Park et al found that young persons who smoke or have ecNOSaa genotype may have an increased risk of developing MI [31]. The a,b allele frequencies in our study were found similar to frequencies reported by Uwabo et al, either in controls, or in EH patients. The allele frequencies of ischemic CHF patients were similar to our controls, but in dilated CHF patients the frequencies were similar only to those reported in the literature (Table 3). The molecular mechanism by which the ecNOS gene polymorphism interact with the development of EH or CHF is not clear. However, we found, that ab genotype patients with ischemic CHF were significantly younger and had lower HDL levels compared to bb genotype patients. The former finding was similar to reported by Park et al [31]. The latter finding can be explained by inherently low HDL levels of Turkish population [32].

As with any genetic study, these findings apply only to the population studied. Based on these results, we conclude that in this Turkish population the ecNOS gene is not associated with the ischemic CHF, but with EH, and may be associated with dilated CHF. Young ab genotype patients with low HDL levels are at increased risk for CAD and CHF. Further studies with more patients, especially in dilated CHF are needed to clarify the power of this association.

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