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# INVESTIGATION OF ENOS GENE INTRON 4 A/B VNTR POLYMORPHISMS IN PATIENTS WITH ESSENTIAL HYPERTENSION

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## ABSTRACT

Hypertension is a multi-factor disease involving interaction of both environment and genetic components. It is a major risk factor for Coronary Artery Disease (CAD), which is associated with high mortality rate. The aim of this case control study was to investigate the possible role of genetic variants of the endothelial Nitric Oxide Synthase (eNOS) gene (VNTR) in the pathogenesis of essential hypertension. The study included 103subjects (52 hypertensive patients and 51 normal control)**.** Randomly selected was conducted to assess the association of SNP eNOS intron 4a/b VNTR polymorphism gene with essential hypertension in Iraqi population. Blood samples from subjects and controls were analyzed to investigate the eNOS genotypes No significant differences were found in the frequency of eNOS genotypes between hypertensive patients and controls ($p > 0.05$).

**KEYWORDS:** Polymorphisms, Investigation

## INTRODUCTION

Essential hypertension, or hypertension of unknown cause, accounts for more than 90% of cases of hypertension. It tends to cluster in families and represents a collection of genetically based diseases or syndromes with several resultant inherited biochemical abnormalitie1-4. The resulting phenotypes can be modulated by various environmental factors, thereby altering the severity of blood pressure elevation and the timing of hypertension onset.

Many pathophysiologic factors have been implicated in the genesis of essential hypertension: increased sympathetic nervous system activity, perhaps related to heightened exposure or response to psychosocial stress; overproduction of sodium- retaining hormones and vasoconstrictors; long-term high sodium intake; inadequate dietary intake of potassium and calcium; increased or inappropriate renin secretion with resultant increased production of angiotensin II and aldosterone; deficiencies of vasodilators, such as prostacyclin, nitric oxide (NO), and the natriuretic peptides; alter- ations in expression of the kallikrein– kinin system that affect vascular tone and renal salt handling; abnormalities of resistance vessels, including selective lesions in the renal microvasculature; diabetes mellitus; insulin resistance; obesity; increased activity of vascular growth factors; alterations in adrenergic receptors that influence heart rate, inotropic properties of the heart, and vascular tone; and altered cellular ion transport (Figure 1)2.

The novel concept that structural and functional abnormalities in the vasculature, including endothelial dysfunction, increased oxidative stress, vascular remodeling, in addition, decreased compliance, may antedate hypertension and contribute to its pathogenesis has gained support in recent years.

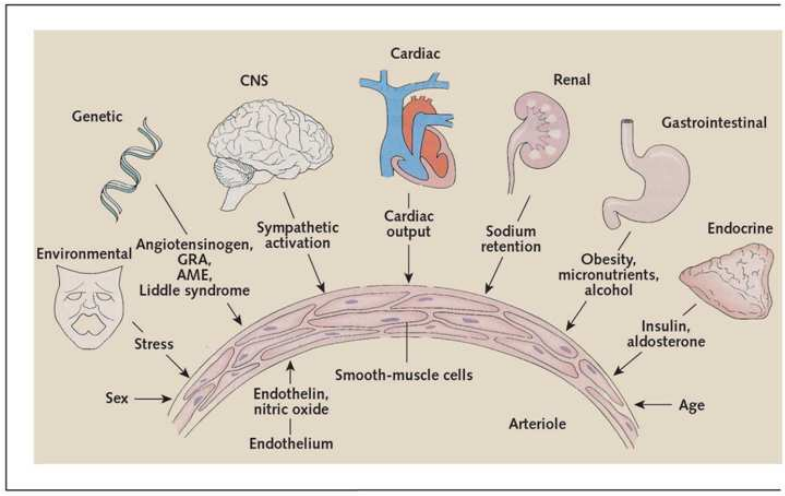


**Figure 1: Pathophysiologic Mechanisms of Hypertension**

NO is synthesized from the amino acid l-arginine by a family of enzymes, the nitric oxide synthases (NOS), through a metabolic route, namely, the l-arginine-nitric oxide pathway5. The synthesis of NO by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure6. NO also contributes to the control of platelet aggregation and the regulation of cardiac contractility7. These actions are all mediated by the activation of soluble guanylate cyclase, and the consequent increase in the concentration of cyclic GMP in target cells8.

The endothelial constitutive NO synthase (ecNOS), which produces NO from L- arginine, is encoded by a gene located on chromosome 7q35-36, expressed in endothelium9. There are two alleles identified in intron 4 of the ecNOS gene. The larger allele, 4b, consists of five tandem 27-bp repeats and the smaller one, 4a, has four repeats10.


**Figure 2: Location of Enos Intron 4a/B VNTR Gene in Chromosome 7q3536 (11)**

## RESEARCH DESIGN AND METHODS

The study population included 52 individuals with essential hypertension diagnosed according to World Health Organization (WHO) criteria. Subjects included in the study comprised of those who were diagnosed as primary hypertensives by physicians (based on clinical and other investigations) and those who were already on antihypertensive drugs at the time of study. Subjects diagnosed with secondary hypertension arising due to CAD, renal failure and other associated conditions were excluded from the study. The data generated was analyzed in comparison to that found in 51 normotensive healthy subjects that formed a control group.

Detailed information relating to the age, sex, BMI, Family history, consanguinity, diet and habits like alcohol consumption and smoking were collected from both patients and controls. Of the cases and controls registered for the study, 5 ml of venous blood was collected in EDTA vaccutainers. Informed consent was obtained from individuals for their participation in the study, which was approved by the Ethical Committee of our institution

**DNA Extraction**

Peripheral blood samples of patient and control groups were collected in EDTA tubes, and then DNA was extracted from whole-blood samples using Genomic DNA Mini Kit (Blood / cultured cell) (Geneaid) 12.

The eNOS gene intron 4, 27 bp VNTR polymorphism was detected by polymerase chain reaction (PCR) according to the method described by Shah *et al* 13. 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 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 75 V for 120 min and visualized at room temperature under UV light after ethidium bromide staining.

**Statistical Analysis**

Continuous variables expressed as mean ±SD and Student's t-test was used to determine differences in means between control and T2DM groups. ANOVA test and Student's t-test was used to compare mean levels of continuous characteristics across genotypes using SPSS v. 20.0 software (SPSS Inc., Chicago, IL).Categorical data (genotypes and alleles) were expressed as frequency. In all statistical analysis the level of significance was <0.05.

**Clinical and Biochemical Characteristics of Study Subjects**

The current study included 103 subjects (52 hypertensive patients and 51 control individuals). The clinical and biochemical characteristics of the recruited individuals were presented in table 1. It shows significant differences in BMI, FBS, lipid profile, in the hypertensive patients when compared with those of the control group. However, no significant difference was seen in age and sex.

**Table 1: Clinical and Biochemical Characteristics of Study Subjects**

| | Control Subjects | Hypertensive Patients | P Value |
|---|---|---|---|
| No (M/F) | 51(29/22) | 52(24/28) | 0.7 |
| Age (y) | 53.47± 4.97 | 53.09±7.14 | 0.52 |
| BMI (kg/m2) | 25.39± 2.51 | 30.02± 5.45 | 0.000 |
| FBS (mg/dl) | 105.98±6.25 | 100.83±4.36 | 0.3 |
| Cholesterol(mg/dl) | 130.73± 30.8 | 165.83±34.77 | 0.000 |
| Triglycerides(mg/dl) | 110.15±44.81 | 145.9± 95.23 | 0.000 |
| VLDL (mg/dl) | 21.03±9.96 | 30.05± 16.11 | 0.000 |
| LDL (mg/dl) | 64.31± 36.93 | 80.45± 40.43 | 0.000 |
| HDL (mg/dl) | 66.58± 8.9 | 55.32±19.72 | 0.000 |

## GENOTYPING

### Estimation of DNA Concentration and Purity

DNA concentration and purity was estimated by measuring A260/A280 ratio. Results were clarified in table 3. and figures 3.1 and 3.3 respectively. DNA samples were found to be pure and the concentrations ranged from 30-100 µg/ml.

**Table 2 DNA Concentration and Purity**

| | Mean ± SD |
|---|---|
| DNA Concentration(µg/ml) | 66.87 ± 20.2 |
| DNA Purity | 1.76 ± 0.22 |

### Results of amplification reactions

DNA was extracted from blood,The PCR product was electrophoresed on 0.8 % agarose (75V and 120 min.) and directly visualized with ethidium bromide under UV light, (figure 3).

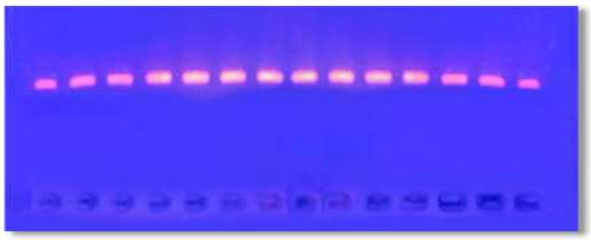

**Figure 3.1: PCR Product of DNA Analyzed by Agarose Gel Electrophoresis.**

The amplification product of eNOS(VNTR) gene polymorphism i.e, the 4a/4b as described by Shah *et al.*(13) . Results were confirmed by agarose gel electrophoresis (figure 4).

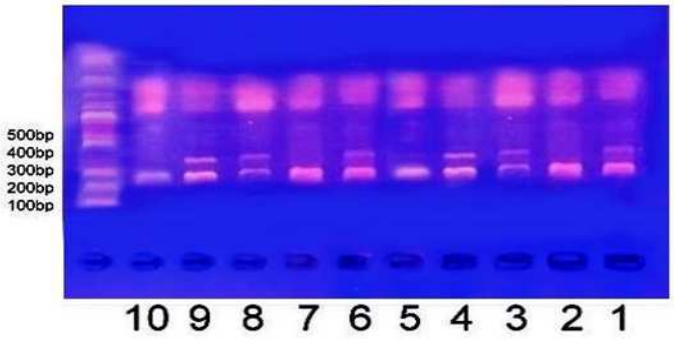


**Figure 3.3: Results of Enos Gene Polymorphism Product on Agarose Gel Electrophoresis**

PCR product of eNOS gene were electrophoresed on 3% agarose (75V and120 min) and directly visualized with ethidium bromide under UV light.

Lines: DNA Marker

Lines 1, 4, 6, 9 : ab genotype 420,390 bp

Lines 2, 5,7,10 :aa genotype 390 bp

Lines 3, 8: bb genotype 420 bp

### Genotype and Allele Frequencies

To determine the genotyping error rate, a random duplication in 10% of the samples was reanalyzed. The concordance was obtained to be 100%. The genotype and allele frequencies of eNOS gene polymorphism in T2DM with nephropathy and normal control subjects in codominant, dominant and recessive models were examined by multinominal logistic regression analysis.

The genotype and allele frequencies of eNOS (VNTR) gene polymorphism in hypertension stated no significance association of hypertension with normal control control (table 3).

**Table 3: Results of Genotype and Allele Frequency of Enos Gene Polymorphism in Patient and Normal Control Groups**

| Enos (A/B) | Control -Ve N=51 | HT N=52 | Unadjusted or (95% CI) | P Value | Adjusted or (95% CI) | P Val Ue |
|---|---|---|---|---|---|---|
| Codominant | | | | | | |
| bb (Reference) | 25 | 30 | | | | |
| ab | 16 | 9 | 0.4 (0.1 -1.2) | 0.1 | 1.1 (1.0 - 1.2) | 0.0 3 |
| aa | 10 | 13 | 1.0 (0.4 – 2.8) | 0.8 | 0.9 (0.9 – 1.0) | 0.7 |
| Dominant | | | | | | |
| aa+ab (Reference) | 26 | 22 | 0.7 (0.3- 1.5) | 0.3 | 1.0 (0.9 -1.1) | 0.2 |
| Recessive | | | | | | |
| ab+bb (Reference) | 41 | 39 | | | | |
| aa | 10 | 13 | 1.3 (0.5- 3.4) | 0.5 | 1.0 (0.9- 1.1) | 0. 2 |

**Table 4: Clinical Characteristics of T2DM Subjects According to Enos Gene Polymorphism Genotype (Codominant Model)**

| Clinical Characteristics | Bb (N=30) | Ab(N=9) | Aa (N=13) | P Value |
|---|---|---|---|---|
| BMI (kg/m²) | 29.22 ± 3.38 | 28.09 ± 5.09 | 28.21 ± 5.06 | 0.95 |
| FBS (mg/dl) | 95.41± 20.03 | 98.36± 18.13 | 100.99± 15.83 | .83 |
| Cholesterol(mg/dl) | 230.16± 45.85 | 190.4 ± 40.62 | 232.81 ± 55.13 | .013 |
| Triglycerides(mg/dl) | 245. 6± 67.61 | 205.65 ±60.48 | 225.86 ± 60.64 | .035 |
| VLDL (mg/dl) | 52.61± 16.68 | 48.05± 20.46 | 52.53± 19.6 | .34 |
| LDL(mg/dl) | 134.9± 45.5 | 105.4 ± 38.53 | 132.76± 55.93 | .058 |
| HDL(mg/dl) | 53.54± 10.87 | 53.00± 12.10 | 51.52± 11.65 | .85 |

## DISCUSSIONS

### SNPS of ENOS (VNTR) Genes in Patients and Control Group

It was demonstrated that 27bp repeat in the *eNOS* gene could bind nuclear proteins as an enhancer/repressor to promote/suppress the transcription efficiency14. Functional significance of this polymorphism was also identified in cases with endothelial dysfunction15.

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 groups16-19. 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 . Zintzaras et al.20 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.

Miyamoto et al.21 found that VNTR in intron 4 (eNOS 4b/4a) was not significantly associated with EHT . Benjafield and Morris 22 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 intron 4b/a, and 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.21, Zhao et al.23, Benjafield and Morris22 and Patkar et al24.

Our results were Inconsistent with the results of Hou et al.25, Li R et al26.

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 factors22.

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