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Identification of 27bp Variable Tandem Repeats in Endothelial Nitric Oxide Synthase (eNOS) Gene of Hypertensive Subjects in Kurdish Population from Erbil City

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Abstract: Hypertension is a multifactorial and polygenic disorder where several susceptible genes interact with the environmental factors. Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) which is involved in many physiologic regulatory functions. Variable number of tandem repeats in intron 4 of endothelial nitricoxide synthase gene are reported to be associated with blood pressure regulation. In the present study, we examined possible association between the 27 base pair (bp) repeat polymorphism in intron 4 of the eNOS3 gene and hypertension in a Kurdish population from Erbil city. Fifty eight patients with hypertension and 42 apparently healthy controls were included in the study. Genotyping was performed by polymerase chain reaction (PCR). The genotype frequencies for eNOS4b/b, eNOS4a/b and eNOS4a/a were 55%, 23.8% and 21.2% in control group, and 70.7%, 24.2% and 5.1% in hypertensive group, respectively. The eNOS bb genotype (70.7%vs 55%) was found to be significantly associated with hypertension (*P*=0.000, OR= 7.14: 95% CI; 3.025-16.85) which indicated that the odds of hypertensive is about seven times higher in bb genotype compared to control (OR= 2.38,95% CI;1.55-3.64), whereas eNOS aa were significantly more frequent in controls (21.2 % vs. 5.1 *P*=0.014) which indicated that this genotype associated with decrease in the risk of having hypertension. (*P*=0.014)

Keywords: (eNOS) gene, 27bp variable tandem repeats, polymorphism, Hypertension.

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Introduction

Hypertension is a multifactorial and polygenic disorder where several genes interact with the environmental factors. Endothelial nitric oxide synthase (eNOS), also known as nitric oxide synthase 3 (NOS3) or constitutive NOS (cNOS), is an enzyme that in humans is encoded by the *NOS3* gene located on chromosome 7 (7q35–q36), spanning 21 kb and comprising 26 exons (1,2). eNOS is primarily responsible for the generation of nitric oxide (NO) in the vascular endothelium, as (NO) is a major regulator of the cardiovascular system and control of blood pressure in humans (3), it is believable that abnormalities in the activity of the eNOS enzyme that synthesizes NO in endothelial cells may lead to NO deficiency and cause clinical hypertension (4). Elevation of arterial blood pressure in mice lacking the endothelial nitric oxide synthase (eNOS) gene, strongly suggests that alteration in NO metabolism is involved in hypertension (5). The *NOS3*gene reveals a number of polymorphic repeats that have been used for the

analysis of genetic association with various human cardiovascular disorders. The 27 bp repeat, a variable number of tandem repeats (VNTR) genetic marker in intron 4 of eNOS gene, are believed to have significance on the occurrence of essential hypertension(6). Two alleles of intron 4 VNTR have been reported: 4a/4b (4 or 5 repeats of 27 bp, respectively (7) . A new polymorphism consisting of an additional 27 bp repeat (4C) was found at very low frequency in a Caucasian population without significant association with hypertension (8). It has been suggested that eNOS 4b/a polymorphisms could increase the risk of Coronary Artery Disease(9), and also can be used as a probable marker in determining susceptibility to T2DM(10).

Variable results have been reported concerning association studies of intron 4 of eNOS gene 27 bp polymorphism and hypertension disease, significant associations were found in some studies (6,11,12,13,14) but not in others (15,16,17). Heterogeneous distributions of eNOS gene mutation among different ethnic groups or geographic areas has been found. The data on the occurrence of the gene mutations in Erbil ethnicity is not yet available, it was of interest to perform the current study to identify the association of eNOS gene polymorphism and the risk of hypertension.

Material and methods

Study participants

The study was carried out on 100 kurdish individuals from Erbil city, 42 normal and 58 hypertensive aged 30-70 years. Patients receiving antihyper-tensive medications or newly diagnosed patients were considered to be hypertensive. Pregnant females were excluded from this study.

Blood samples collection

Approximately 2ml of venous blood was collected in a screw cap tube containing ethylenediamine-tetraacetic acid (EDTA) as anticoagulant and stored at -20 until DNA extraction.

DNA extraction

DNA was extracted from whole blood using genomic DNA Mini kit (Geneaid Biotech Ltd). DNA purity and quantity were assessed by NanoDrop1000 spectrophotometer V 3.7 and checked by 0.7% Agarose gel electrophoresis.

Genotyping of the Variable Number of Tandem Repeats (VNTR) polymorphism in intron 4

Amplification of DNA samples for polymorphic analysis of variable number of tandem repeats (VNTR) in intron 4 (27 bp TR) was performed using the following primer set: 5′-AGG CCC TAT GGT AGT GCC TTT -3′ (forward) and 5′-TCT CTT AGT GCT GTG GTC AC -3′ (reverse). PCR reactions were carried out in 25 µl volumes comprising 5 µl of Taq Promega PCR reaction buffer (5x), 1 µl of each primer, 3 µl template DNA (50 ng) , 0.12 µl of Taq DNA polymerase, and 14.4 µl nuclease-free water,. The PCR reaction tubes were then placed in the thermal cycler (Perkin Elmer 9600). The PCR reaction mixtures were heated to 95 °C for 3 min for initial

denaturation, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30s. Final extension was conducted at 72 °C for 5 min.

Detection of (VNTR) polymorphism in intron 4 by electrophoresis

Five µl of the amplified PCR product was electrophorised on 3% ethidium bromide stained agarose gels, visualized and photographed. Estimation of product size was carried out with 3 µl 100 bp DNA ladder (New England Biolabs, Boston MA). For eNOS gene 27-bp repeat polymorphism in intron 4, a 393-bp band indicates four repeats of the 27 bp (a allele) and a 420-bp band indicates five repeats of the 27 bp (b allele); therefore, the wild homozygous genotype (b/b) will appear as a single band of 420 bp, while the homomutant genotype (a/a) will appear as a single band of 393 bp, and the heteromutant genotype (b/a) will appear as two bands at 420 and 393 bp as shown in figure 1.

Statistical analysis

Data analysis was done using SPSS version 11.5. Demographic details were compared using t-test for continuous data. Allele frequencies and genotype distribution of the population were compared by Chi square and/or fisher`s exact test .Odds ratios were calculated with a 95% confidence interval. In all cases statistical significance was determined at the *P* value< 0.05 was considered to be statistically significant.

Results

In the present study, a total of 100 subjects (control, 42; hypertensive, 58), aged between 30 and 70 years in kurdish population from Erbil city were included. Table (1) shows the demographic characteristics of patients and controls, sex distribution did not reveal any significant difference, and hypertensives were older (45.18±2.14) than controls (40.74±1.14, P<0.002).

Table (1): Demographic characteristics of patients and controls

Variable	Control n=42	hypertensive n=58	*P* value
Sex (M/F)	19/23	25/33	Ns
Age (years ±SD)	41.0 ±2.33	55.23±3.19	< 0.002
Age<50(years ± SD)	40.74±1.14	45.18±2.14	< 0.002
Age>50(years ± SD)	58.63±3.25	60.5±3.95	Ns

ns: not significant

The 27 bp variable number tandem repeats (VNTR) in intron 4 of eNOS gene was detected by PCR analysis. The larger allele of 420 bp has five tandem repeats (b allele) and the smaller allele of 393 bp has four repeats (a allele).

Thus, the eNOS 4*a*/*b* polymorphism results in three genotypes, *aa* homozygous (393 bp), *bb* homozygous (420 bp) and *ab* heterozygous (both 393 bp and 420 bp)., (Figure 1).

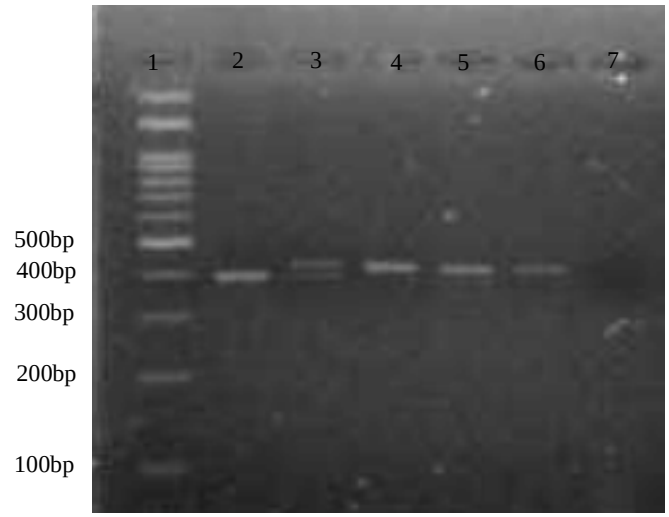

Figure (1): Ethidium bromide stained agarose gel 3% (w/v) image showing PCR products for 27 bp variable tandem repeat (VNTR) polymorphism in intron 4 of endothelial nitric oxide synthase (eNOS) gene. Lanes1:100bp DNA marker; lane 2 homozygous aa (393bp); lane 3 heterozygous ab (420bp & 393bp) lane 4, 5 and 6 homozygous bb(420bp); lane 7 (–ve) control .

The distribution of genotypes and allele frequencies were compared between patients and controls in Table 2. A comparison of genotypes and allelic frequencies of the intron 4 VNTR polymorphisms between patients and controls is shown in Table 2. eNOS 4a/b genotype frequencies in hypertensive were 3(5.1)%, 14 (24.2)%, and 41(70.7)% for aa, ab, and bb, respectively, and 9 (21.2) %, 10 (23.8) %, and 23 (55.0) % in the control group for aa, ab, and bb, respectively. The results showed that among three genotypes bb genotype was significantly more frequent in hypertensive group as compared to other genotypes group (bb *versus* aa, and ba) (OR= 7.14: 95% CI; 3.025-16.85, *P*=0.000), which indicated that the odds of hypertensive is about seven times higher in bb genotype compared to control group (RR= 2.37, 95% CI;1.55-3.64). The frequency of eNOS 4a/a genotype was rare in compare with eNOS 4a/b and 4b/b, it was more frequent in control group (n=9, 21.2%) than hypertensive (n=3, 5.1%) (*P*=0.014). The frequency of the b and a alleles were 96 (82.8%) and 20 (17.2%) respectively compared to control group (b and a alleles frequency were 56 (66.7) and 28 (33.3) respectively, there were a significant differences in alleles frequencies between hypertensive group and control group (*P*=0.007).

Table (2): Genotypes and Allele frequencies in hypertensive and control groups

Genotypes	Frequencies %		*P* Value	OR (95% CI)	RR (95% CI)
	Control =42	Hypertensive =58			
bb	23(55.0%)	41 (70.7 %)	[a]**0.000**	7.14(3.025-16.85)	2.376(1.55-3.64)
ab	10(23.8 %)	14 (24.2 %)			
aa	9 (21.2 %)	3 (5.1 %)	[b]**0.014**	3.14 (1.15-8.62)	2.16 (1.05-4.5
Alleles					
b	56 (66.7%)	96 (82.8 %)	**0.007**	2.4 (1.24-4.65)	1.24 (1.045-1.48)
a	28 (33.3 %)	20 (17.2 %)			

OR: Odds Ratio. RR: Risk Ratio. CI: Confidence Intervals. [a]:bb *vs.* ab +aa, *P*-value Fisher`s Exact test. [b]: aa *vs.* bb+ab, *P*-value Person Chi-Square test.

Discussion

Endothelial Nitric Oxide Synthase (eNOS) is an important enzyme producing endothelial-derived relaxing factor (NO) which is a major regulator of the cardiovascular system and control of blood pressure in humans, Our study was to investigate the association of the polymorphisms of eNOS gene and its activity in essential hypertension. Our results showed a significant association of bb genotype with the hypertensive group (bb *versus* aa, and ab) (odds ratio 7.14, 95% CI(3.025-16.850) P=0.000). The results also showed that aa genotype of the intron 4 VNTR variant was more frequent in the control normotensive subjects (aa *versus* bb and ab) and the difference was significant(odds ratio3.14 (1.15-8.62 95% CI(1.15-8.62) P=0.014); allele frequencies of b and a were 56 (66.7%), 96 (82.8 %)and 28 (33.3 %), 20 (17.2%) for control and hypertensive groups, respectively(odds ratio 2.4 , 95% CI (1.24-4.65) P=0.007). This study suggested a possible positive association of bb polymorphism with hypertension for study group. Presence of b allele of eNOS gene was predominant in study group with a higher prevalence of eNOS bb genotype than the others. The predominance of the 'b' allele observed in our study was in agreement with earlier studies conducted in Japanese and UK populations (18,19). However, our findings were different from the study conducted in Australian (7). The eNOS intron 4 bb genotype in the present study has been associated with an increased risk of hypertension in comparison with the eNOS intron 4 ab genotype and eNOS intron 4 aa genotype, several studies have shown this association (6,12,13,14,20,21). However, varied results have been obtained in studies of different populations, the 4 aa genotype was associated with hypertension in Indian (22) and Iranian (23) populations. However, the eNOS intron 4 polymorphism was not associated with hypertension in other studies (11, 15, 16) (17, 24, 25). The difference observed in the distribution of the eNOS intron 4a allele might be due to the genetic variability of populations, different sample sizes and different selection. Our results suggest that *eNOS4* gene polymorphisms are likely to be the major genetic susceptibility factors for essential hypertension in the kurdish population from Erbil city. It is highly recommended to employ the same study on population of Sulaimany and Duhock governorates in order to generalize the results on Kurdistan Region. For the future study it is necessary to select the other genes which involved in hypertension condition which may help better appreciate the molecular basis of hypertension.

Conclusion

The eNOS bb genotype was found to be significantly associated with hypertension and increased the risk of developing hypertension, whereas eNOS aa were significantly more frequent in controls which indicated that this genotype associated with decrease in the risk of having hypertension.

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