

The role of endothelial nitric oxide synthase gene G894T and intron 4 VNTR polymorphisms in hemodialysis patients with vascular access thrombosis

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ABSTRACT

Objective: Endothelial nitric oxide synthase (*eNOS*) gene is a candidate gene in cardiovascular and renal diseases. Several polymorphic variations have been identified in *eNOS* gene. We investigated a potential role of arteriovenous fistula (AVF) thrombosis and intron 4 and G894T polymorphisms in chronic renal failure.

Methods: We performed a case-control observational study involving 79 with/without AVF thrombosis in chronic renal failure patients. All subjects were genotyped by the polymerase chain reaction (PCR) and PCR-Restriction Fragment Length Polymorphism. Genotype distribution and allele frequencies were compared between groups using the chi-square test.

Results: Genotype frequencies in patients with thrombosis were not significantly different from those of patients without thrombosis for *eNOS* G894T polymorphism ($p=0.1$). *eNOS* gene intron 4 allele distributions seems to be associated with thrombosis in the groups.

Conclusion: This study revealed that there was an association between *eNOS* intron 4 polymorphism and thrombosis in chronic renal failure patients. This data will be helpful in planning further *eNOS* association studies in vascular access thrombosis. (*Anadolu Kardiyol Derg* 2014; 14: 239-43)

Key words: Endothelial nitric oxide synthase gene, thrombosis, chronic renal failure, polymorphism, arteriovenous fistula

Introduction

A high risk for vascular and arterial complications has been well documented and genetic factors have been generally implicated in the etiology of these complications in end-stage renal disease (ESRD) patients (1-3). Permanent vascular access is of primary importance in these groups of patients. Arteriovenous (AV) fistula seems to be convenient when compared with other vascular access alternatives such as AV grafts and central venous catheters with respect to patency, morbidity and mortality rates. Arteriovenous fistulae (AVF) which was introduced by Bessias et al. (4) in 1996, is still considered to be the optimal vascular access for hemodialysis (HD) therapy. Vascular access thrombosis is the main problem in HD population (5, 6). Complications of vascular access contribute to increased morbidity in HD patients. Vascular access for HD might be influenced in part by genetic polymorphisms (7).

The endothelial nitric oxide synthase (*eNOS*) gene (previously named *NOS3*) is located on chromosome 7 (7q35-q36)

and *eNOS* plays a key role in the regulation of normal function of the vessel wall. The vascular endothelium is recognized as an important factor in a healthy cardiovascular system, this system synthesizes nitric oxide (NO) which provides local regulation of vasomotor tone and anti-thrombotic actions (8-10). NO is also a potent regulator of intrarenal hemodynamics (11, 12). Several polymorphic variations of the *eNOS* gene are known and their association with human diseases has been studied (9). Specific allelic variations in the *eNOS* gene might either directly affect the properties of the eNOS enzyme activity and ethnic differences can be seen (8, 10). G894T polymorphism within exon 7 is mostly associated with coronary artery disease, Alzheimer's disease, essential hypertension and ischemic stroke (13, 14). A functional 27-bp variable number of tandem repeats (VNTR) in intron 4 (intron 4b/a) of *eNOS* gene has been reported (8, 15). 4a allele is associated with coronary heart disease and renal disease (11, 12). Carriers of the 4a allele had lower NO levels than 4b/4b homozygous sub-



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Accepted Date: 20.05.2013 **Available Online Date:** 20.02.2014

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DOI:10.5152/akd.2013.4779

jects (16). The allele frequencies of these polymorphisms were 45.3% and 62.7% for GG of exon 7 and bb of intron 4 in 300 healthy Turkish individuals respectively (17).

Chronic renal failure (CRF) is a vascular disorder and investigating *eNOS* gene polymorphisms might shed some light on the pathophysiology of renal diseases. Various renal diseases (including ESRD, glomerulonephritis, diabetic nephropathy and IgA nephropathy) have been studied for associations with *eNOS* polymorphisms (18). Different gene polymorphisms especially thrombotic factors (*ACE*, *PAI-1*, *MTHFR*, *Prothrombin*, *Factor V*, and *HPA*) and cytokine genes (*TGF β 1*, *TNF α*) were mostly studied in AVF thrombosis affecting HD patients (7, 19-22). G894T variant alters the primary structure of the protein and has the potential to alter one or more functional properties of the enzyme directly and it has not been studied in vascular access thrombosis. Intron 4b/a polymorphism could affect the rates of *eNOS* transcription and/or *eNOS* enzyme levels (10). In the present study we attempted to examine a relevance of the *eNOS* gene polymorphisms in CRF patients with/without AVF thrombosis.

Methods

Study design and population

This study designed as an observational case-controlled study and *eNOS* gene polymorphisms were analyzed in 79 Turkish patients with a diagnosis of chronic renal failure. AVF were implemented in the Department of Cardiovascular Surgery, Erciyes University. The fistula from radial artery to cephalic vein (Brescia-Cimino Fistula) is made at the wrist. The cephalic vein is mobilized first and its patency and quality assessed. The anastomosis was constructed in a side-to-side fashion, by using 6-0 or 7-0 monofilament suture. After flow is established and a thrill is palpated in the proximal vein, the distal vein is ligated to pathway for venous hypertension. The fistula is allowed to mature for 6 to 8 weeks prior to puncture. AVF were implemented on the wrist of all participants. Patients who implemented AVF for the first time were enrolled. After the observation of 8 weeks 30 participants with thrombosis (21 male and 9 female) were defined as patients' group and 49 participants without thrombosis (21 male and 28 female) were defined as control group. Clinical, demographic and biochemical features of the groups (age, gender, total cholesterol, HDL, LDL, Triglycerides, BUN, creatinine, WBC) were evaluated. The study protocol was approved by the local ethics committee. This study has been performed in accordance with Helsinki Declaration. Written informed consents were obtained from all of the patients before the study.

Genotyping studies

Blood samples were obtained in EDTA tubes to study polymorphisms. Genomic DNA was extracted from peripheral blood samples using standard procedures of Magna Pure LC (Roche, Germany). Amplifications of *eNOS* intron 4 polymorphism were

performed using primers forward 5'-AGGCCCTATGGTAGTGCCTT-3' and reverse 5'-TCTCTTAGTGTGGTGCAC-3'. The 50 μ L reaction mixture contained 5 μ L of 10X Taq buffer; 3 μ L of 25 mM MgCl₂ solution; 3 μ L each of the 10 μ M primers; 3 μ L of 10 mM deoxy-nucleotide triphosphates (dNTP), specifically deoxyadenosine triphosphate, guanosine triphosphate, cytosine triphosphate, and thymidine triphosphate; and 1.5U of Taq DNA polymerase. Denaturation was performed at 94°C for 5 min. Thirty five cycles of thermocycling with 30 sec denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C, followed by 7 min of final extension at 72°C were performed and detected on 2% agarose gel. The final products were as follows: 420 bp for 4b/4b, 420 and 393 bp for 4b/4a and 393 bp for 4a/4a genotypes. For genotyping of *eNOS* G894T polymorphism of each participant, 0.1 μ g of genomic DNA was amplified by PCR. The 50 μ L reaction mixture contained 5 μ L of 10X Taq buffer; 3 μ L of 25mM MgCl₂; 3 μ L each of the 10 μ M sense and antisense primers; 4 μ L of 2.5 mM dNTP; and 1U of Taq DNA polymerase. Amplification was proceeded at 95°C for 5 min, followed by 30 cycles (60 sec at 95°C, 60 sec at 60°C and 60 sec at 70°C) and a final extension at 70°C for 7 min. The primer sequences were as follows: 5'-CATGAG-GCTCAGCCCCAGAAC-3' and 5'-AGTCAATCCCTTTGGTGCTCAC-3'. This PCR amplification yielded a 206-bp product which were reconstituted in 15 μ L of nuclease-free water and 10X Buffer Tango and then subjected to overnight incubation at 37°C with 1U of the restriction endonuclease *Mbo*I (Fermentas). In the presence of a T nucleotide at position 894 corresponding to Asp 298, the 206 bp PCR product was cleaved into two fragments of 119 and 87 bp; whereas the presence of a G nucleotide removed the restriction site recovering a single fragment of 206 bp after incubation with *Mbo*I. The digestion products were separated using 2.5% agarose gel electrophoresis and stained in ethidium bromide.

Statistical analysis

The differences of mutation types between thrombosis/no thrombosis were analyzed using chi-square analysis. Shapiro Wilk's test was used and histogram q-q plots were examined to assess the data normality. A two-way independent samples t test and Mann-Whitney U tests were applied to compare the differences between continuous variables and chi-square analyses were used for categorical variables. Values are expressed as frequencies and percentages, mean and standard deviation or median and 25th-75th percentiles. Moreover, odds ratios were calculated with 95% confidence intervals to estimate the renal failure risk for each variable. Analysis was conducted using SPSS (Statistical Package for Social Sciences) version 15.0 software (SPSS, Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

We have genotyped 79 patients with the diagnosis of CRF for the *eNOS* gene polymorphisms in this study. The genotypes of the groups were determined by PCR and RFLP. Table 1 shows the

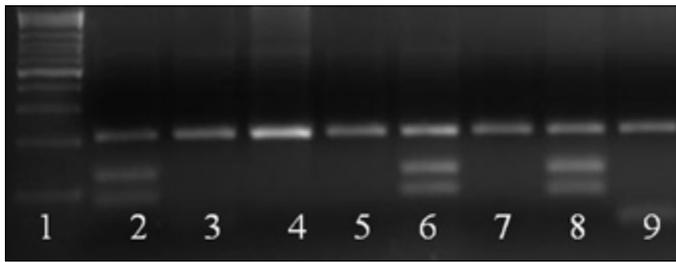


Figure 1. Analysis of the G894T polymorphism of the eNOS gene. Lane 1: 100 base pair (bp) DNA Ladder Lane 2,6,8: GT genotype Lane 3,4,5,7,9: GG genotype

genotypes of the polymorphisms in the groups. Agarose gel electrophoresis results of these polymorphisms were presented in Figure 1, 2.

eNOS intron 4 b/b genotype were 98% and 56.7% in the groups (Table 1). We could not define 4b/4a genotype in the control group. The association between *eNOS* intron 4 genotypes and the groups were found significant ($p < 0.05$). In addition intron 4 polymorphism was significantly different between the groups according to gender and this data was not shown in Table 1. 4b/4a genotype is more prevalent in males in thrombosis group. Allele frequencies of intron 4 polymorphism were shown as Figure 3. *eNOS* G894T genotype was found with a frequency of 26.7% in thrombosis group. There was no significant difference ($p > 0.05$) between patient and control groups (Table 1). It seems that G/G genotype was more prevalent in patients group than controls. No significant differences were detected according to gender but G894T genotype was more prevalent in males in thrombosis group. Table 2 indicates the clinical, demographic and biochemical features of the groups and risk factors were summarized as OR (95%CI) in Table 3 and were not different in the groups. Creatinine and BUN levels were higher in patients with AVF.

Discussion

In this study we investigated the relevance of the *eNOS* gene intron 4 and G894T polymorphisms in CRF with/without AVF thrombosis and showed a relationship between the groups for intron 4 polymorphism.

An impairment of nitric oxide production causes abnormalities in vascular function in many diseases including thrombosis, hypertension, atherosclerosis and renal disease (23, 24). In the kidney, nitric oxide dilates renal blood vessels and modulates renin secretion. The polymorphisms of *eNOS*, particularly G894T (Glu298Asp), have already been associated with hypertension, coronary artery disease and atherosclerosis (13, 24). The frequencies of Glu298Asp mutation in both non-diabetic and diabetic end-stage renal disease (ESRD) groups are significantly higher than that in healthy controls according to Nagase et al. (25)'s study. In another study no association of the G894T polymorphism with renal disease was observed in Caucasian-Brazilians with type 2 diabetes (26). Suzuki et al. (27) examined the G894T polymorphism in 159 patients with ESRD undergoing maintenance HD and 270 unrelated healthy controls. There was

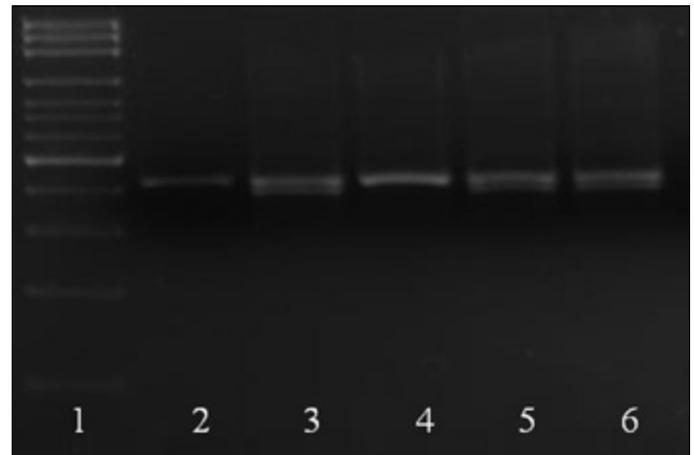


Figure 2. Analysis of the variable number of tandem repeats (VNTR) polymorphism in intron 4 of the eNOS gene. The PCR product was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The 420-bp band indicates five 27-bp repeats, and the 393-bp band indicates four 27-bp repeats. Lane 1: 100 bp DNA Ladder Lane 2: PCR product Lane 3, 5, 6: 4b/4a Lane 4: 4b/4b

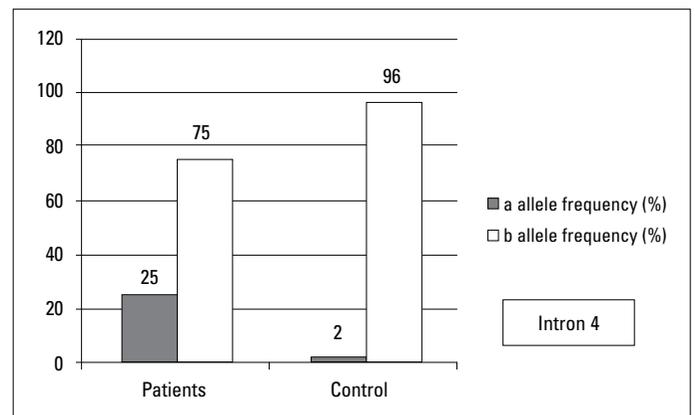


Figure 3. Distribution of the allele frequencies of eNOS gene intron 4 polymorphism in the CRF patients and control groups. The dark gray bars represent the frequency of an allele and white bars represent the frequency of b allele in both groups

significantly increased risk for nondiabetic ESRD and ESRD associated with diabetic nephropathy groups. The odds ratio results [3.2 (CI, 1.8-5.8)] indicated a similarly strong association between ESRD and the Glu298Asp variant. Tang et al. (1) revealed that *eNOS* T alleles in ESRD patients with vascular disease were significantly higher than controls. They suggested *eNOS* G894T polymorphism was increasing the risk of vascular complications in ESRD patients (1). This polymorphism was not investigated in AVF thrombosis to our knowledge. In our study, there was no significant difference between the groups according to *eNOS* G894T genotypes. If we had greater sample size these results could be changed.

The frequency of the *eNOS* genotype *aa* was significantly different in ESRD patients and in controls. This study showed that the *eNOS* *a* allele frequency is significantly higher in ESRD patients than in controls (12). Akçay et al. (24) investigated intron 4 polymorphism in 125 patients who underwent renal transplantation and they revealed that the graft function was not affected

Table 1. Genotype frequencies of eNOS G894T and intron 4 polymorphisms in the groups

Variable	Patients without thrombosis (n=49)		Patients with thrombosis (n=30)	
	n	(%)	n	(%)
eNOS intron 4				
Genotypes				
b/b	48	98	17	56.7
b/a	-	-	11	36.7
a/a	1	2	2	6.6
p<0.05 [£]				
eNOS G894T				
Genotypes				
G/G	26	53.1	22	73.3
G/T	19	38.7	8	26.7
T/T	4	8.2	-	-
P>0.05 [£]				
£p value expressed according to chi-square analysis				

Table 2. Clinical and demographic characteristics of patients

Features	Patients with AVF (n=30)	Patients without AVF (n=49)	P	P [£]
Age, years	57.07±16.32	53.75±15.78	0.374	0.298
Gender, M/F	21 (70)/9 (30)	21 (42.8)/28 (57.2)		
Total cholesterol, mg/dL	183.6±46.45	199.3±36.35	0.098	0.108
HDL cholesterol, mg/dL	33.88±11.82	38.87±11.68	0.071	0.226
LDL cholesterol, mg/dL	97.84±30.72	102.84±35.67	0.526	0.652
Triglycerides, mg/dL	183 (115-221)	190 (130-255)	0.264	0.937
WBC	6.15 (5.14-8.24)	6.0 (4.98-7.04)	0.249	0.310
BUN, mg/dL	43.5 (26.3-59.25)	37 (19-57)	0.342	0.205
Creatinine, mg/dL	5.54 (3.53-7.63)	2.63 (1.18-7.05)	0.196	0.224
Values are expressed as n (%), mean±SD or median (25 th -75 th percentiles) BUN - blood urea nitrogen; F - female, HDL - high density lipoprotein; LDL - low density lipoprotein; M - male; WBC - white blood cell £adjusted by gender.				

by this variation (24). In Santos et al. (26) study, the VNTR intron 4 a/b polymorphism was not associated with renal disease in Caucasian-Brazilians. Elshamaa et al. (23) studied 78 children with chronic kidney disease (CKD) and 30 healthy controls. They showed that an allele was a high-risk allele for ESRD and this data were significantly different (p<0.05). Also they measured serum nitric oxide level and it was found to be lower in carriers of the eNOS 4a allele than in noncarriers. So they asserted that the eNOS intron 4 polymorphism may be associated with an increased risk of CRF. Genetic polymorphism in the eNOS intron 4 short allele may play a role development of vascular access thrombosis according to Brophy et al. (5). In our study we revealed that an allele of the eNOS intron 4 gene polymorphism showed a significantly higher frequency in our

Table 3. Risk factors of patients according to OR (95% CI)

Features	OR (95% CI)	P
Age, years	1.013 (0.984-1.043)	0.720
Gender	0.321 (0.123-0.843)	0.021
Total cholesterol, mg/dL	0.999 (0.977-1.002)	0.104
HDL cholesterol, mg/dL	0.961 (0.920-1.004)	0.078
LDL cholesterol, mg/dL	0.995 (0.982-1.009)	0.521
Triglycerides, mg/dL	1.0 (0.997-1.003)	0.843
WBC	1.079 (0.942-1.235)	0.271
BUN, mg/dL	1.01 (0.992-1.03)	0.270
Creatinine, mg/dL	1.072 (0.942-1.220)	0.291
CI - confidence interval		

patient group. This was significantly different between the groups.

Study limitations

Our study has some limitations. First, there were relatively small numbers of patients in the groups. This could lead to either estimate the significance of the association of genotypes with the disease. There were a relatively small number of controls with the eNOS TT allele and we could not determine TT allele in other group. We can indicate that the small sample size limited the statistical power of our study. Secondly, our patient group was not matched for gender, although the age range for both groups is very similar. The male:female ratio is 2.33 for patients with thrombosis and 0.75 for patients without thrombosis. Risk factors of thrombosis were not significantly different in groups. As an alternative approach, serum levels may be determined in these groups.

Conclusion

In this study, our results failed to show a significant correlation between eNOS G894T gene polymorphism in Turkish renal patients. According to our data, eNOS intron 4 polymorphism can serve as a useful genetic marker for evaluation of susceptibility to CRF. However, the interactions between this genetic predisposition, clinical features of the patients and other environmental factors require further studies. As a conclusion, this study needs to be examined with a larger sample groups to confirm and see the exact association between these polymorphisms and thrombosis in CRF patients.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept - Ö.N.E., E.F.Ş.; Design - Y.Ö., E.F.Ş.; Supervision - E.F.Ş., Y.Ö., Ö.N.E.; Resource - Y.Ö., E.F.Ş.; Materials - Ö.N.E., F.S.; Data collection&/or processing - E.F.Ş., F.S.; Analysis &/or interpretation - E.F.Ş., Y.Ö., Ö.N.E.; Literature search - E.F.Ş., F.S., Y.Ö.; Writing - E.F.Ş., Ö.N.E., Y.Ö., F.S.; Critical review - E.F.Ş., Ö.N.E.; Other - Ö.N.E.

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