Distribution of Endothelial Nitric Oxide Synthase Gene Polymorphisms in Turkish Population

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Abstract: Nitric oxide (NO) is the major mediator in the regulation of vascular homeostasis. It is synthesized by endothelium from arginine with the catalytic help of endothelial NO synthase (*eNOS*). Polymorphisms in the *eNOS* gene have been associated inconsistently with many vascular diseases. Conflicting results may arise from ethnic variations, notably in control-case studies. Therefore, to obtain reliable information and improve the design of such genetic association studies, we examined the distribution of genetic alleles of 3 clinically relevant *eNOS* polymorphisms (the variable number of tandem 27–base pair [bp] repeats in intron 4, T-786C in promoter, and G894T in exon 7) in 300 healthy Turkish individuals with a mean age of 50.07 (SD 12.15) years (range, 22–78 years; 50.7% men and 49.3% women). The haplotype frequency was estimated and associations between 3 polymorphisms were evaluated. We discussed the similarity and differences in the distribution of alleles with other populations. The allele frequencies were 45.3%, 48%, and 62.7% for GG of exon 7, TT of promoter, and bb of intron 4 (wild types), respectively. The most common haplotype combines the wild-type alleles (G-T-b) for all 3 polymorphisms (estimated frequency, 37.6%). The linkage analysis between each pairwise combination showed specific associations between T-786C polymorphism in promotor and G894T polymorphism in exon 7 (positive D' value, +0.3387). No significant correlation was found in the genotype distribution of the 3 polymorphisms with age, sex, and obesity. Our *eNOS* gene allele distributions resemble those of Caucasians and white populations. Our data showed ethnic differences with other populations in the allele distribution of *eNOS* gene besides conserved distribution of these polymorphisms in Turkish population. This study produced a reference control data for the 3 *eNOS* polymorphisms in Turkish population and will be helpful in planning *eNOS* association studies in vascular disease.

Key Words: nitric oxide, *eNOS* gene, exon 7, promoter, VNTR intron

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In the endothelium, the synthesis of nitric oxide (NO) from amino acid L-arginine is catalyzed by the endothelial NO synthase (*eNOS*) and the continuously generated NO serves to maintain basal vascular tone. Besides maintaining the basal vasodilator tone, NO inhibits platelet aggregation, attenuates leukocyte adhesion to the endothelium, and modulates smooth muscle proliferation.[1] This readily diffusible gas has limited stability in biological settings (t1/2 of several seconds) and is known to participate in a variety of chemical reactions with metals, thiols, and other reactive oxygen species.[2] Given the numerous physiological roles for NO and its rapid reaction and inactivation in cellular systems, strict control of NO production is crucial for its selective actions. Mammalian endothelial NO production is governed by the expression and activity of the *eNOS* (the oxidoreduction of arginine to NO and citrulline). *eNOS* is a functional homodimer (monomer molecular weight, 135 kd), and its activity is regulated by changes in intracellular Ca^{+2}.[3] The human *eNOS* gene is located at 7q35 to 7q36 containing 26 exons that span 21 kilobases.[4]

The pivotal role of NO in the regulation of the vasculature, mainly cardiovascular system, motivated a number of researchers to investigate if polymorphisms in the *eNOS* gene are associated with many cardiovascular diseases.[5] Three clinically relevant polymorphisms in the *eNOS* gene have been widely studied: a variable number of tandem 27–base pair (bp) repeats (VNTRs) in intron 4, T-786C polymorphism in promoter, and G894T polymorphism in exon 7. Yet, inconsistent links among these 3 *eNOS* polymorphisms and vascular diseases have been found.[6–8] The existing discrepancies possibly reflect ethnic differences. The power of case-control studies that are usually designed to identify genetic risk factors for a disease is becoming vital, especially when ethnic variations in allele frequencies in cases and controls are not matched.[9] Reliable information regarding the distribution of *eNOS* variants in different ethnic populations is required to improve the design of association studies. Although interethnic differences in the distribution of *eNOS* genetic variants exist in the American population,[10] it is not known whether such ethnic dissimilarities exist among other populations.

In this study, the distributions of genetic variants of 3 clinically relevant *eNOS* polymorphisms (VNTR in intron 4, T-786C in promoter, and the G894T in exon 7) in the Turkish population were determined. We also estimated the haplotype frequency and evaluated the association between these variants in the Turkish population.

MATERIALS AND METHODS

Subjects

Three hundred healthy controls with a mean age of 50.07 (SD 12.15) years (range, 22–78 years) and free of any symptomatic vascular disease were recruited for this study. Complete personal, family, and medical histories were taken, and detailed physical examinations were performed. Cerebrovascular, cardiovascular, peripheral vascular, large and small vessel, pulmonary, chronic liver or renal, collagen tissue, and Behçet diseases; hypertension; metabolic syndrome; diabetes mellitus; pulmonary hypertension; malignancy; and hyperthyroidism or hypothyroidism were excluded. A sample of peripheral blood was obtained from all individuals. The study was approved by the ethical committee of Hacettepe University Faculty of Medicine, and an informed consent was obtained from all participants.

Genotyping

Genomic DNA was isolated from the whole blood using NucleoSpin Blood QuickPure Kit (Machenery-Nagel, Düren,

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Germany). Polymerase chain reactions (PCRs) were carried out for the 3 polymorphisms of *eNOS* gene using specific primers and conditions.[11]

Variable Number of Tandem Repeat (27-bp Repeat) Polymorphism in Intron 4

Sense 5′-AGG CCC TAT GGT AGT GCC TTT-3′ and antisense 5′-TCT CTT AGT GCT GTG GTC AC-3′ primers were used for PCR. The PCR was performed in a 40-μL reaction volume that included approximately 400-ng template genomic DNA, 10 pmol/μL of each primer, 0.2 mmol/L of each deoxyribonucleotide triphosphate (dNTP), 4-μL 10× PCR buffer, 2.5-mmol/L $MgCl_2$, and 3-U Taq DNA Polymerase (Fermentas Life Sciences, Leon-Rot, Germany). The PCR mixtures were heated to 94°C for 30 seconds for denaturation and underwent 35 cycles at 94°C for 30 seconds for

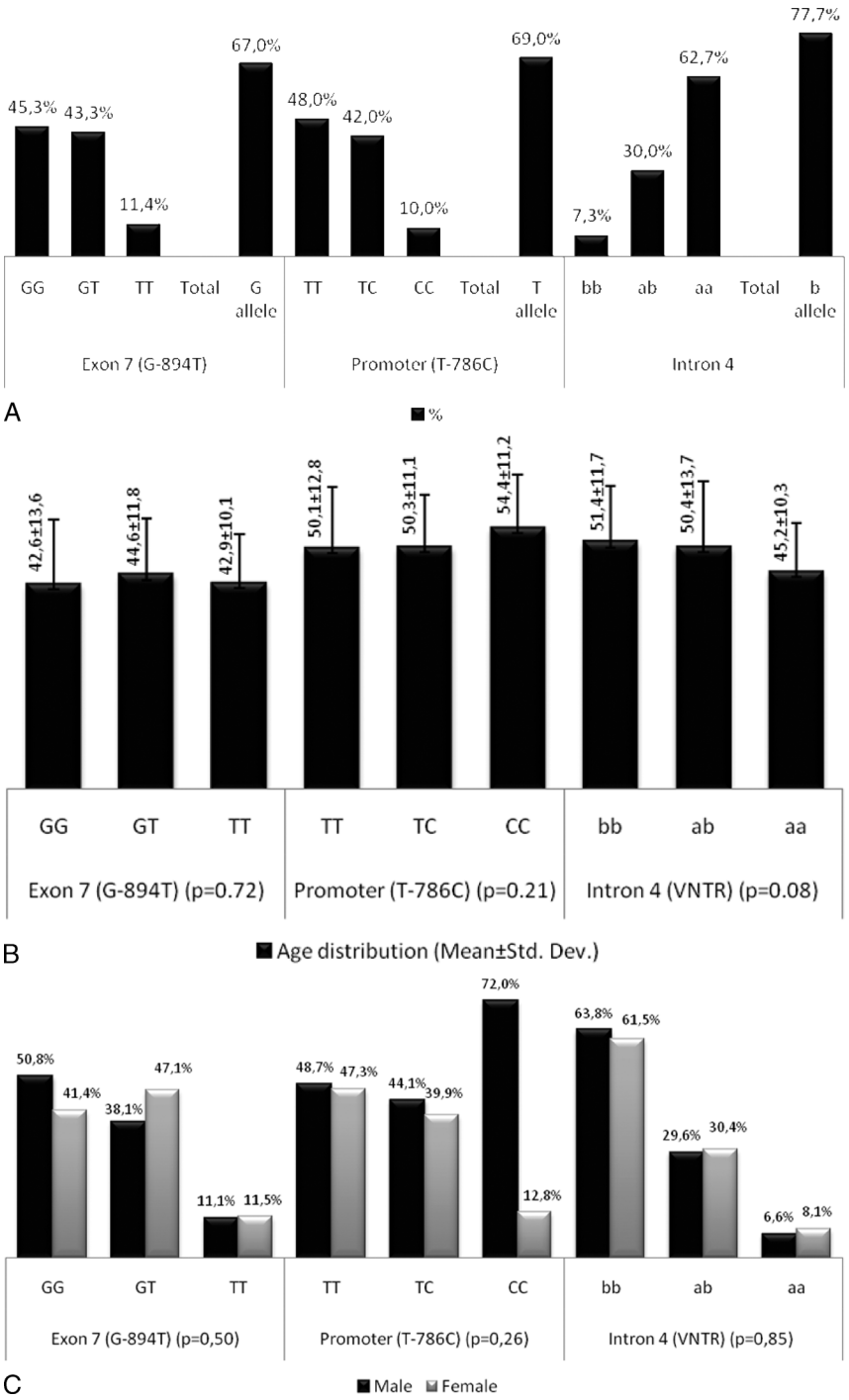

FIGURE 1. A, Percentile distribution of genotypes of 3 *eNOS* gene polymorphisms in the Turkish population. B, Genotype distribution of 3 *eNOS* gene polymorphisms with age. C, Genotype distribution of 3 *eNOS* gene polymorphisms with sex.

TABLE 1. Genotypes and Allele Frequency of 3 *eNOS* Polymorphisms in Turkish Population

	n	%
Exon 7 (G-894T)		
GG	68	45.3
GT	65	43.3
TT	17	11.4
Total	150	
G allele		67
Promoter (T-786C)		
TT	144	48
TC	126	42
CC	30	10
Total	300	
T allele		69
Intron 4 (VNTR)		
bb	188	62.7
ab	90	30
aa	22	7.3
Total	300	
b allele		77.7

denaturation, 63°C for 30 seconds for annealing, and 72°C for 1 minute for extension (ICycler; BioRad, München, Germany). Finally, extension was conducted at 72°C for 5 minutes. Fragments of 393 and 420 bp correspond to the *eNOS* alleles 4a (4 repeats of 27 bp) and 4b (5 repeats of 27 bp), respectively. DNA fragments were separated on a 3% NuSieve agarose gel and visualized by ethidium bromide (Fig. 1). DNA marker was PhiX174 DNA/*Bsu*R1 (*Hae*III) (Fermentas Life Sciences, Leon-Rot, Germany).

T-786C Polymorphism in the 5′-Flanking Region (Promoter)

The sense 5′-TGG AGA GTG CTG GTG TAC CCC A-3′ and antisense 5′-GCC TCC ACC CCC ACC CTG TC-3′ primers were used in a total volume of 50 μL, containing 400-ng template DNA, 6.25 pmol/μL of each primer, 0.25 mmol/L of each dNTP, 5-μL 10× PCR buffer, 1.5-mmol/L $MgCl_2$, and 3-U Taq DNA Polymerase (Fermentas Life Sciences). The reaction conditions used were 1 step of denaturation at 94°C for 5 minutes followed by 40 cycles comprising 1 minute at 94°C for denaturation, 1 minute at 61°C for annealing, 1 minute at 72°C for extension, and 1 step of extension at 72°C for 5 minutes using ICycler (BioRad). Polymerase chain reaction products were checked on a 2% agarose gel. Amplified products were digested with *Msp*I (Fermentas Life Sciences) for 3 hours at 37°C. Resulting fragments of 140 and 40 bp for the wild-type allele (T) or 90, 50, and 40 bp in case of polymorphic allele (C) were determined by separating them on a 2% agarose gel.

G894T Polymorphism in Exon 7

For the detection of G894T polymorphism in exon 7, the sequence of the sense and antisense primers were 5′-AAG GCA GGA GAC AGT GGA TGG A-3′ and 5′-CCC AGT CAA TCC CTT TGG TGC TCA-3′, respectively. Polymerase chain reactions were performed in a total volume of 50 μL, containing 400-ng template DNA, 6.25 pmol/μL of each primer, 0.25 mmol/L of each dNTP, 10-μL 10× PCR buffer, 1.5-mmol/L $MgCl_2$ and 3-U Taq DNA Polymerase (Fermentas Life Sciences) with the same cycles as described previously for the promoter region. The resulting 268-bp fragment was digested with *Mbo*I (Fermentas Life Sciences) for 3 hours at 37°C, producing 178 and 90 bp fragments (polymorphic allele) (T) or no digestion (wild type) (G). Restriction sites were confirmed on 2% agarose gel.

Statistical Analysis

Statistical analysis was carried out with SPSS for Windows version 15.0 statistical software (SPSS Inc, Chicago, IL). Age distribution was presented as mean ± (SD) and sex and obesity genotype distribution as percentages. Association between sex, obesity, genotype distribution is examined by χ^2 test. One-way analysis of variance is used for determining the association between age and genotype distribution. Hardy-Weinberg equilibrium for genotype frequencies was assessed by χ^2 test. $P < 0.05$ was considered to be statistically significant.

Estimation of Linkage Disequilibrium

Linkage disequilibrium analyzer program was used to perform a linkage analysis between each pairwise combination of variants. This program calculates D' (the maximum likelihood estimate of disequilibrium), which is a standard measure of linkage disequilibrium. The estimated disequilibrium D' values for each pairwise combination of variants were calculated as $D' = D/D_{max}$, where $D = h - pq$. In this study, p and q are the frequencies for the rarer variants of the 2 polymorphisms being tested for linkage, such that $P < q < 0.5$, and h is the frequency of the haplotype, including 2 specific variants. When $D < 0$, $D_{max} = -pq$. When $D > 0$, $D_{max} = p(1 - q)$. Thus, D' values can vary from +1 to −1, with a positive D' indicating that the rarer variants are associated and a negative D' indicating that the rarer variant of one polymorphism is associated with the common variant at the other locus.

RESULTS

Sex and obesity distribution in our study population comprises 152 men (50.7%) and 148 women (49.3%) and 101 obese (33.7%) and 199 nonobese (66.3%) individuals. The distribution of genotypes for each polymorphism showed no deviation from Hardy-Weinberg equilibrium except VNTR in intron 4 (G894T in exon 7, $P = 0.80$; T-786C in promotor, $P = 0.75$; and VNTR in intron 4, $P = 0.019$). Table 1 shows the frequency of 3 *eNOS* genotypes and alleles in the Turkish population (Fig. 1A). No significant correlation was found in the genotype distribution of the 3 polymorphisms with age (Fig. 1B), sex (Fig. 1C), and obesity. The estimated haplotype

TABLE 2. Estimated Haplotype Frequency

Haplotypes			
Exon 7 (G-894T)	Promoter (T-786C)	Intron 4 (VNTR)	n = 300
T	T	a	0.066
T	C	a	0.02
T	T	b	0.204
T	C	b	0.04
G	T	a	0.254
G	C	a	0.024
G	T	b	0.376
G	C	b	0.016

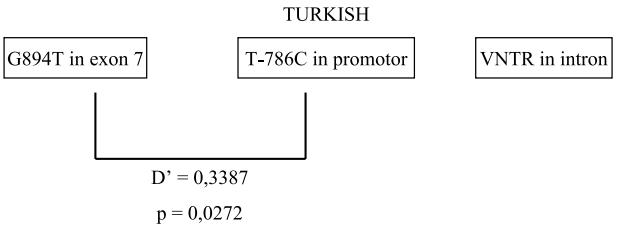

FIGURE 2. Maximum likelihood estimate of disequilibrium (*D*′) for pairwise combinations of polymorphic *eNOS* gene alleles in Turkish population. A positive *D*′ value indicates that the rarer alleles of the 2 polymorphisms are associated.

frequency is shown in Table 2. As anticipated, the most common haplotype combines the wild-type alleles (G-T-b) for all 3 polymorphisms (estimated frequency, 37.6%). The second most common haplotype includes intron 4a allele with wild-type alleles for the other 2 polymorphisms (G-T-a; estimated frequency, 25.4%). The linkage analysis between each pairwise combination showed specific associations between T-786C polymorphism in promotor and G894T polymorphism in exon 7. A positive *D*′ value (+0.3387) for association between the rarer alleles in the promoter region and in exon 7 (-786C and 894T, respectively) was found (Fig. 2), thereby indicating that these rare variants are associated (P = 0,0272).

DISCUSSION

In this study, we determined the genotype and haplotype distribution of 3 *eNOS* gene polymorphisms (VNTR in intron 4, T-786C in promoter, and the G894T in exon 7) in healthy Turkish population, which is important in establishing the relationship between these polymorphisms and genetic susceptibility to developing many vascular diseases.

To date, 22 Turkish studies with *eNOS* gene polymorphisms in different diseases (Behçet and coronary artery disease, hypertension, myocardial infarction, ischemic stroke, sepsis, deep vein thrombosis, vascular access graft thrombosis, thrombosis in the perioperative period of congenital cardiac surgery,

TABLE 3. *eNOS* Gene Polymorphism Studies in Turkish Population

		n	%	Total	Age, mean (SD), yr
Intron 4 (VNTR)					
Akar et al.[12]	bb	72	75.8	95	—
	ab	20	21		
	aa	3	3.16		
Cine et al.[13]	bb	249	81.4	306	37.27 (11.86)
	ab	55	18		
	aa	2	0.6		
Karasneh et al.[14]	bb	63	61	104	—
	ab	39	38		
	aa	2	2		
Matyar et al.[15]	bb	97	72.9	133	—
	ab	35	26.3		
	aa	1	0.8		
Olcay et al.[16]	bb	70	80.5	87	40.53 (15.83)
	ab	16	18.4		
	aa	1	1.1		
Agirbasli et al.[17]	bb	—	79	102	57.6 (13.6)
	ab	—	21		
	aa	—	0		
Dursun et al.[18]	bb	53	80	66	18–55
	ab	13	20		
	aa	0	0		
Celik et al.[19]	bb	97	72	134	—
	ab	35	26		
	aa	2	2		
Promoter (T-786C)					
Karasneh et al.[14]	TT	43	42	102	—
	TC	44	43		
	CC	15	15		
Tangurek et al.[20]	TT	31	59.6	52	56.8 (9.5)
	TC	15	28.8		
	CC	6	11.5		
Ciftçi et al.[21]	TT	31	59.6	31	59 (1.68)
	TC	21	67.7		
	CC	8	25.8		
Exon 7 (G-894T)					
Akar et al.[22]	GG	46	56.1	82	—
	GT	33	40.2		
	TT	3	3.6		
Aras et al.[23]	GG	60	51.3	117	—
	GT	48	41		
	TT	9	7.7		
Afrasyap et al.[24]	GG	74	49.3	150	60.71 (9.14)
	GT	62	41.3		
	TT	14	9.3		
Karasneh et al.[14]	GG	54	51	105	—
	GT	45	43		
	TT	6	6		
Cam et al.[25]	GG	57	68.7	83	44.6 (1.4)
	GT	24	28.9		
	TT	2	2.4		
Kara et al.[26]	GG	59	59.2	100	48.4 (11.7)
	GT	34	33.7		
	TT	7	7.1		
Berdeli et al.[27]	GG	100	57.8	173	—
	GT	50	28.9		
	TT	23	13.3		
Alaşehirli et al.[28]	GG	50	63.3	79	42.47 (12.1)
	GT	12	15.2		
	TT	17	21.5		
Oksel et al.[29]	GG	59	65	91	—
	GT	27	29		
	TT	5	6		
Balat et al.[30]	GG	—	61	114	—
	GT	—	26		
	TT	—	13		
Guldiken et al.[31]	GG	—	49.3	133	66.2 (10.7)
	GT	—	45.8		
	TT	—	4.9		

fibromyalgia syndrome, psoriasis, renal graft survival and renal artery atherosclerosis, acute poststreptococcal glomerulonephritis, and primary nocturnal enuresis) have been presented.[11–33] Nonetheless, this is the first study evaluating the distribution of *eNOS* gene polymorphisms in healthy Turkish population. The frequencies that were found in the occurrence of the alleles of the 3 *eNOS* gene polymorphisms were quite similar to those reported controls in the previous association studies (Table 3 and Fig. 3). We found no statistical difference in the distribution of promoter alleles in controls of all Turkish studies with our controls (Fig. 3B). Only 4 Turkish studies for exons 7 and 5 for intron 4 polymorphisms conflicted with our findings (Fig. 3A,C). Our data were almost identical to those previously reported for the Turkish studies, showing the conserved distribution of these polymorphisms in Turkish population (Fig. 3).

However, there was a statistically notable disparity in the distribution of *eNOS* genotypes between other populations[10–34] (Fig. 4). Interestingly, both G894T polymorphism in exon 7 and VNTR polymorphism in intron 4 distribution resemble the distribution of Caucasians and white population. This relevance is valuable because the Turkish population is white and geographically closer to Caucasians.

Haplotype analysis is a more powerful approach in genetic studies than the analysis of single polymorphisms.[35] The distribution of estimated haplotype frequency may help us to understand how the combination of single-nucleotide polymorphisms and distinct haplotypes influence a number of diseases in different populations.[36] Thus, it is important to determine the haplotype frequency in healthy population. As expected, the highest frequency of the haplotype for the 3 *eNOS*

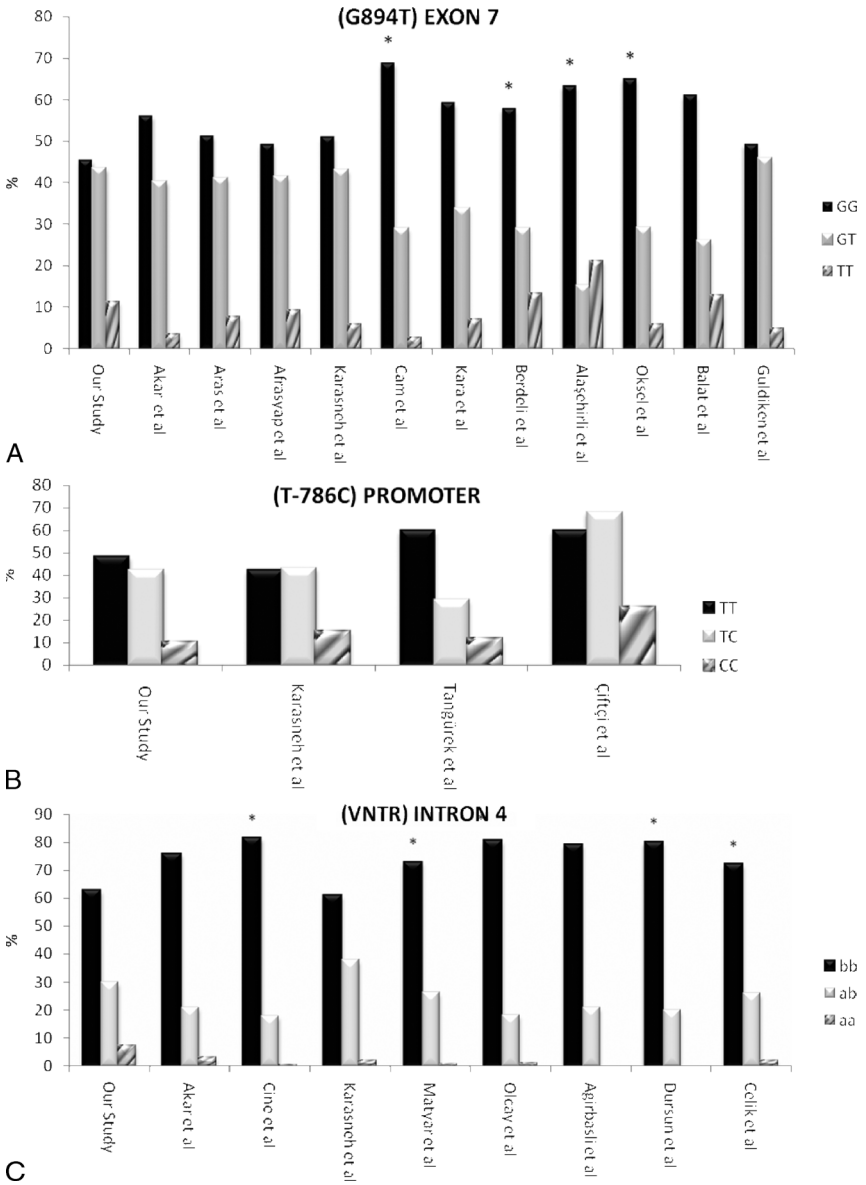

FIGURE 3. Percentile distribution of *eNOS* gene polymorphisms in Turkish studies. *Statistical differences exist as compared with our study (χ^2 tests; $P < 0.01$).

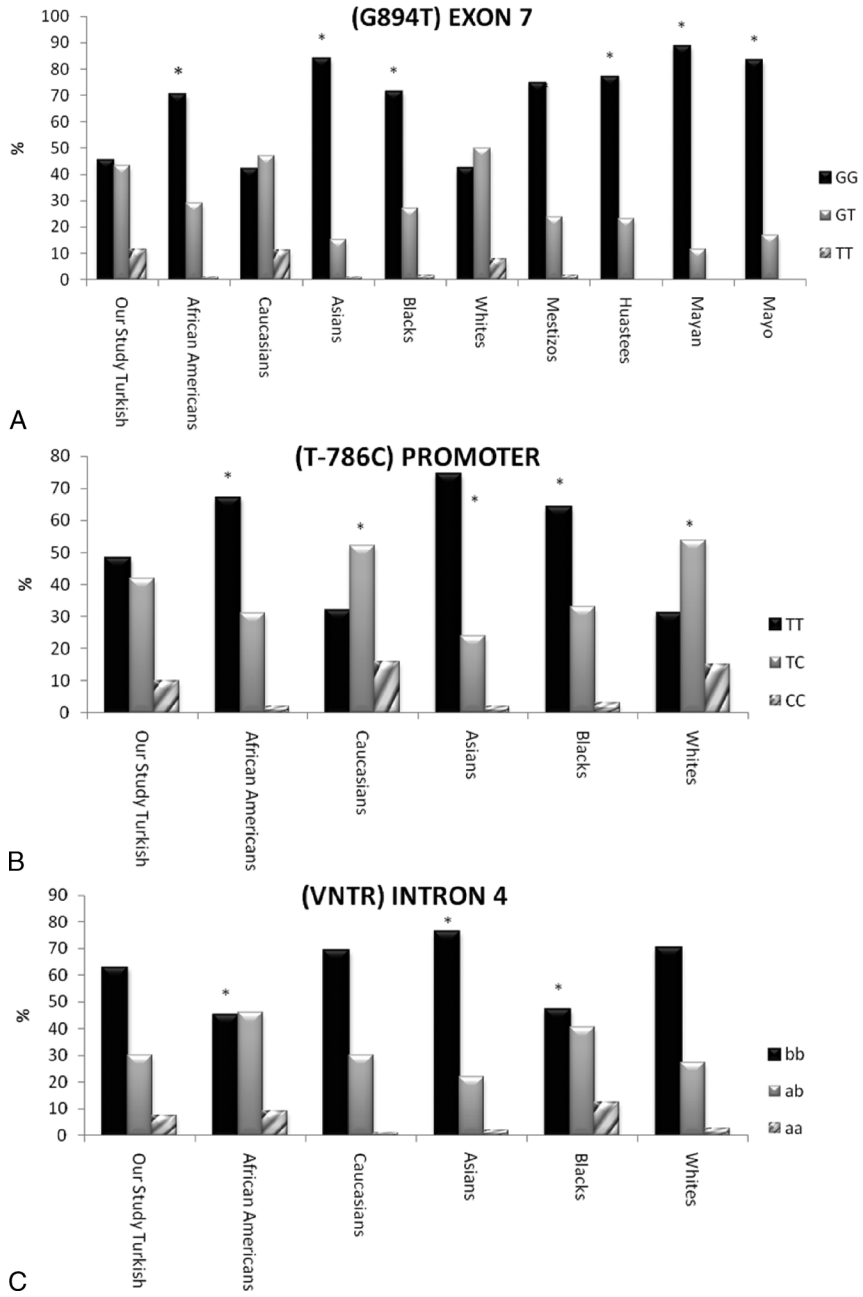

FIGURE 4. Comparison of percentile distribution of *eNOS* gene polymorphisms in different populations with our study (Turkish population). *Statistical differences exist as compared with other populations (χ^2 tests; $P < 0.01$).

gene polymorphisms in Turkish population includes only wild-type alleles.

In functional studies, 894T polymorphism in exon 7 was found more susceptible to proteolytic cleavage than its wild-type allele (G894), and this might have contributed to abnormally low NO generation in carriers of polymorphic allele.[37] However, the glutamate (G894) and aspartate (894T) are conservative substitutions; therefore, another possibility would be that this polymorphism serves as a marker for a functional effect elsewhere in the *eNOS* gene or in its vicinity.[14,38] Therefore, we do concede that the conservative substitution in exon 7 may not have a direct functional effect, but it may be in linkage disequilibrium with a regulatory polymorphism on the same haplotype. Based on our linkage analysis, as we detected an association of the rarer allele in the promoter region with the rarer allele in exon 7 in the Turkish population, the regulatory polymorphism may be located in this pattern.

It is valuable to evaluate the genetic association and population studies because various environmental factors that affect the level of oxidative stress may also modify the phenotypic expression potentials of DNA genotypes in the *eNOS* gene, making functional studies of the *eNOS* gene more complicated to conduct.[14] Moreover, population differences may in part explain the population disparities in bioavailability of NO and response to drugs.[39–41] The identification of the distribution of *eNOS* gene polymorphism underlying population

differences can help to predict the drug effects and improve the therapy.[42] Although it is possible that this issue cannot be completely clarified by only addressing the interethnic differences in the distribution of *eNOS* polymorphisms, it is valuable to determine the genetic predispositions to diseases.

Sex-specific differences in morbidity and mortality may be mediated in part by genetic factors. However, no significant correlation was found in the genotype distribution of the 3 *eNOS* gene polymorphisms with age and sex, suggesting that these polymorphisms are not expressed in a sexually dimorphic manner.

Methodological limitation of this study lays in the existence of obesity in the 33.7% of the study group, although other confounders such as hypertension, metabolic syndrome, diabetes mellitus, cerebrovascular and cardiovascular diseases, and so on have been excluded. However, no significant correlation was found in the genotype distribution of the 3 polymorphisms with obesity, supporting that our study group is confidential. In a study of adolescents with obesity-associated hypertension, no association was found with T-786C promoter and VNTR polymorphisms, assisting the characteristics of our control group.[43]

For such polymorphism studies, the fundamental issue includes adequate statistical power, appropriateness of controls, and multigene interactions. Thus, we analyzed 300 healthy controls looking for the gene interactions in the Turkish population.

The results of the present work contribute to the accumulated knowledge about the allele distribution of the 3 *eNOS* gene polymorphisms in the Turkish population and would be helpful to elucidate the true relationship of these polymorphisms with different vascular diseases in the Turkish population.

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