Endothelial NOS G894 T and MMP-3 5A/6A Gene Polymorphisms and Hypertension in Serbian Population

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The incidence of hypertension is increasing and it is more common in man than in women. Up to date, MMP-3 5A/6A polymorphism has been associated with artery stiffening and elevated blood pressure, whereas results considering association of endothelial NOS (eNOS) G894 T polymorphism with hypertension are controversial. The aim of our study was to analyze the possible association of eNOS G894 T and MMP-3 5A/6A gene polymorphisms with hypertension in Serbian population. Study sample consisted of 172 hypertensive and 200 normotensive subjects divided by gender. Both female and male group was truncated according to age. All subjects were genotyped for MMP-3 5A/6A and eNOS G894 T polymorphism. There was a

significantly higher ($P < 0.05$) prevalence of 5A/5A genotype in hypertensive females compared to normotensive ones (19.30 % vs. 10.84%) even more pronounced in group ≥ 50 years, according to its recessive effect. In young males (< 40 years), we found 3.7-fold increased risk for hypertension associated with allele 6A ($P < 0.01$), and 8.1-fold with genotype 6A/6A ($P = 0.01$) according to recessive model. We found no association of eNOS G894 T polymorphism with hypertension. These results indicate that there were gender- and age-specific differences in association of MMP-3 5A/6A polymorphism with hypertension in Serbian population. *J. Clin. Lab. Anal.* 19:241–246, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Hypertension is one of the most common polygenic disorders and is a hallmark risk factor for coronary heart disease, stroke and congestive heart failure. The incidence of hypertension is increasing and it is more common in man than in women (1). Vascular remodeling of arteries contributes to increased peripheral resistance and the development of hypertension (2). Variations in mechanical forces (e.g., shear stress and tensile stress) cause the endothelium-mediated release of active substances, such as nitric oxide (NO), which in short term modulate the vascular tone by restoring basal stress levels and in long term, induce vascular remodeling (3). There is a fine balance between matrix degradation and deposition to maintain optimum vessel wall structure in the face of prevailing mechanical stresses (4). Vascular fibrosis caused by the accumulation of collagen and other extracellular matrix (ECM)

compounds in the vessel wall is an important part of the ECM remodeling in hypertension (2). Stromelysin-1 (MMP-3) as a key member of metalloproteinase family is involved in the turnover of the ECM components contributing to alterations in collagen, elastin, laminin and fibronectin turnover (5) and activates other members of the matrix metalloproteinase (MMP) family (6).

NO as a potent vasodilator is important regulator of vascular tone, thus its reduced bioavailability should play role in the development or progression of

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hypertension (7). It is produced in endothelial cells by the endothelial nitric oxide synthase (eNOS) encoded by polymorphic eNOS gene. Targeted deletion of the eNOS gene in mice results in both hypertension and impaired vascular remodeling (8). Inhibition of eNOS elevates blood pressure in nonhypertensive humans (9). The G to T polymorphism at position 894 of the human eNOS gene is the only polymorphism identified to date that changes the eNOS protein sequence (10). There are controversial data regarding association of this polymorphism with hypertension in different populations (11–14).

A common polymorphism in the promoter region of MMP-3 gene (5A/6A) has been identified (15). In transfection experiments, the 5A allele had two-fold higher protein expression compared to 6A allele in both cultured fibroblast and vascular smooth muscle cells (16). The 5A/5A genotype has been associated in vivo with higher and 6A/6A genotype with lower MMP-3 levels in arterial walls, compared with the heterozygotes (4). Recently, the 5A/5A genotype was linked with stiffer large arteries (4,17) and with elevated blood pressure level (18).

Results regarding association of eNOS gene polymorphism with hypertension are controversial, and at present little is known about association of MMP-3 5A/6A gene polymorphism and hypertension. Understanding that ethnic origin should be carefully considered in studying the association of gene polymorphism and disease etiology, the aim of our study was to analyze the possible association of eNOS G894T and MMP-3 5A/6A gene polymorphisms with hypertension in Serbian population.

MATERIALS AND METHODS

Study Population and Study Design

A total of 372 individuals, 172 hypertensive and 200 normotensive, were included in the study. All of them were Caucasians, Serbian origin, from the Belgrade area. The hypertensive participants were recruited from the outpatient clinics in a period of two years, with entry criteria: systolic blood pressure (SBP) >140 mm Hg and diastolic blood pressure (DBP) >90 mm Hg without antihypertensive treatment; diagnosis of hypertension before the age of 60. Control group consisted of normotensive volunteers who were undergoing the annual medical check-up at The Occupational Medicine Center, INN Vinča and had satisfied the entry criteria: SBP <140 mm Hg or diastolic blood pressure DBP <90 mm Hg. All subjects fulfilled a questionnaire designed to obtain relevant social (smoking, alcohol consumption, physical activity), medical (general health,

cardiovascular state, physician visits), and family (general health and cardiovascular status of parents and siblings) history of participants. For all participants additional inclusion criteria were serum glucose <6 mmol/L, normal sedimentation rate and absence of acute or chronic disease and drug intake. None of them did have medical record of cardiovascular diseases, hypertrophic cardiomyopathy, diabetes mellitus and renal insufficiency. An ethical review committee approved the study and each participant gave their written informed consent to participate in the study. Because of the gender- and age-specific development of hypertension, we divided our sample on females and males and truncated the both groups according to age (in females the cutoff point was the age of 50 years; in males the cutoff point was the age of 40 years) in order to assess the more profound relationship between genotypes and hypertension.

Resting Blood Pressure Measurement

Resting blood pressure (BP) was measured at two occasions, three times, at three minutes intervals in the sitting position, after a 30 min rest. BP was read by mercury column sphygmomanometer according to the WHO/American Society of Hypertension recommendations and the mean value of these measurements were used for the analysis.

Lipids Measurement

Lipid concentrations were determined in sera collected after overnight fasting. The total plasma cholesterol (TC) and triglyceride (TG) levels were determined on Monarch Plus apparatus (Instrumentation Laboratory, Lexington, KY) using enzymatic colorimetric methods. High-density lipoprotein cholesterol (HDL-C) was determined after dextran sulfate-Mg²⁺ precipitation of VLDL and low-density lipoprotein cholesterol (LDL-C), using the CHOD-PAP method. LDL cholesterol was calculated according to the Friedewald formula for participants with TG levels <4.5 mmol/L (19). All reagent kits were provided by Instrumentation Laboratory.

Determination of Genotypes

Genomic DNA was isolated from whole blood samples collected with EDTA and purified by the proteinase K/phenol extraction method (20). MMP-3 5A/6A gene polymorphism was detected according to previously described method (21) using oligonucleotide primers: forward, 5′GATTACAGACATGGGTCAC3′ and reverse 5′TTTCAATCAGGACAAGACGAAGT TT3′. The 110 bp DNA fragment was amplified by polymerase chain reaction (PCR) in Touch Down™

(Hybaid, Teddington, UK) and digested with 5U of *PdmI* restriction endonuclease (MBI Fermentas, Vilnius, Lithuania) at 37°C overnight. eNOS G894T gene polymorphism was identified by PCR and *MboI* (MBI Fermentas) restriction digestion, as previously described (22).

The digestion products were loaded on an 8% polyacrylamide gel for genotyping and run for 2 hr in electric field of 12V/cm. Gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products, Inc., Upland).

Statistical Analysis

Statistical analysis was performed using Statistica Version 5 (1997) software package (StatSoft, Inc.). In all tests, differences with two-tailed alpha-probability ($P \leq 0.05$) were considered significant. The allelic frequencies and genotype distribution were estimated using the gene counting method. Differences in both allele and genotype frequency distribution between the studied groups were estimated by chi-square (χ^2) test. Deviation from Hardy-Weinberg equilibrium was also assessed using χ^2 test.

Means of normally distributed continuous variables were compared in an unpaired *t*-test. Due to skewed TG distribution, TG analysis was performed by logarithm-transformed values. As a measure of strength of association between MMP-3 and eNOS genotypes and hypertension multiple logistic regression analysis was used, and expressed in terms of adjusted OR and 95% confidence interval (CI). Relative risk for hypertension was adjusted for BMI, LDLC, TG, and smoking status. The genotype effects were examined according to a dominant, additive, and recessive model for both polymorphisms.

RESULTS

Description of the Population

The main characteristics of the study sample for hypertensive and normotensive group divided by gender are shown in Table 1. Difference between hypertensive and normotensive females was significant for age, BMI, and TG values, which were all higher in hypertensive women. Compared with the normotensive, hypertensive males had a significantly higher BMI values.

MMP-3 gene 5A/6A Polymorphism and Hypertensive Status

Genotype frequencies for MMP-3 5A/6A gene polymorphism did not deviate from Hardy-Weinberg equilibrium in any of the studied groups. Distribution of MMP-3 genotype and allele frequencies were not significantly different between hypertensive and normotensive neither males nor females (Table 2). There was a significantly higher ($P < 0.05$) frequency of 5A/5A genotype (10.84%) in hypertensive females compared to normotensive ones (19.30%), according to recessive model (5A/5A vs. 5A/6A + 6A/6A). After dividing the female group by age (see materials and methods), difference in 5A/5A genotype frequency remained significant ($P < 0.001$) only in the group of females older than 50 years of age (NT = 5.9% vs. HT = 24.2%). In the males, we found significantly different genotype distribution ($P < 0.001$) only in the group aged up to 40 years (NT: $n = 26$, HT: $n = 34$), with significantly higher presence of 6A/6A genotype in the hypertensives (NT = 15.4% vs. HT = 41.2%).

The females with 5A/5A genotype had higher adjusted risk for hypertension (OR = 1.86), and more so in the group ≥ 50 years (OR = 4.62), but it did not reach

TABLE 1. Biochemical characteristics of participants according to hypertensive status divided by gender

	Female			Male		
	Normotensive	Hypertensive	P^*	Normotensive	Hypertensive	P^*
n	83	57		117	115	
Age, y	43.63 $\pm$ 11.74	52.60 $\pm$ 12.41	0.0001	46.91 $\pm$ 10.95	45.73 $\pm$ 11.86	NS
BMI (kg/m ²)	24.06 $\pm$ 3.64	26.26 $\pm$ 3.46	0.003	26.50 $\pm$ 2.47	27.46 $\pm$ 3.34	0.03
TC (mmol/L)	5.83 $\pm$ 1.41	6.20 $\pm$ 1.36	NS	5.74 $\pm$ 1.24	5.87 $\pm$ 1.34	NS
LDLC (mmol/L)	3.65 $\pm$ 1.25	3.91 $\pm$ 1.24	NS	3.56 $\pm$ 1.10	3.60 $\pm$ 1.14	NS
HDLC (mmol/L)	1.49 $\pm$ 0.36	1.43 $\pm$ 0.42	NS	1.31 $\pm$ 0.39	1.31 $\pm$ 0.38	NS
TG (mmol/L)	1.34 $\pm$ 0.88	1.75 $\pm$ 1.37	0.009 ^a	1.60 $\pm$ 0.91	2.03 $\pm$ 1.81	NS
Smokers, n (%)	36 (43.37)	24 (42.11)	NS	62 (52.30)	56 (48.70)	NS

*Students' *t*-test was used to compare values for hypertensive and normotensive subjects.

^aAnalysis were performed on logarithm transformed values.

Values are mean $\pm$ SD, for age, TC, LDLC, HDLC and TG.

NS, Non significant.

TABLE 2. Genotype distribution and allele frequencies for MMP-3 5A/6A and eNOS G894T polymorphisms in normotensive/hypertensive male/female

	Male			Female		
	Normotensive n (%)	Hypertensive n (%)	<i>P</i> ^a	Normotensive n (%)	Hypertensive n (%)	<i>P</i> ^a
MMP-3 genotypes						
5A/5A	24 (20.51)	16 (13.91)	0.12	9 (10.84)	11 (19.30)	0.12
5A/6A	62 (52.99)	61 (53.04)		51 (61.45)	31 (54.38)	
6A/6A	31 (26.50)	38 (33.04)		23 (27.71)	15 (26.32)	
MMP-3 allele						
5A/6A	0.47/0.53	0.40/0.60	0.89	0.42/0.58	0.46/0.54	0.94
eNOS genotypes						
GG	61 (52.13)	56 (48.69)	0.75	32 (38.56)	28 (49.12)	0.18
GT	46 (39.32)	49 (42.61)		42 (50.60)	22 (38.60)	
TT	10 (8.55)	10 (8.70)		9 (10.84)	7 (12.28)	
eNOS allele						
G/T	0.72/0.28	0.70/0.30	0.96	0.64/0.36	0.68/0.32	0.93

^a*P*- χ^2 test.**TABLE 3. MMP-3 gene polymorphism odds ratios (ORs) for hypertension in female subgroups adjusted for differences in significant clinical risk factors^a**

Genotype classes	Females < 50 years	<i>P</i>	Females ≥ 50 years	<i>P</i>	Females overall	<i>P</i>
	OR (95% CI)		OR (95% CI)		OR (95% CI)	
5A/5A vs. 5A/6A + 6A/6A	1.0 (0.2–4.9)	0.99	4.62 (0.47–45.2)	0.17	1.86 (0.7–5.1)	0.22
5A/5A vs. 5A/6A vs. 6A/6A	0.9 (0.4–1.9) ^b	0.73	2.1 (0.7–6.0) ^b	0.18	1.3 (0.7–2.3) ^b	0.33

^aAdjusted for BMI, LDL-C, TG and smoking status.^bRisk change adding 1 mutant allele at a time.**TABLE 4. MMP-3 gene polymorphism odds ratios (ORs) for hypertension in male subgroups adjusted for differences in significant clinical risk factors^a**

Genotype classes	Males < 40 years	<i>P</i>	Males ≥ 40 years	<i>P</i>	Males overall	<i>P</i>
	OR (95% CI)		OR (95% CI)		OR (95% CI)	
6A/6A vs. 5A/5A + 5A/6A	8.1 (1.5–44.3)	0.01	1.3 (0.6–2.9)	0.6	1.12 (0.6–2.2)	0.7
6A/6A vs. 5A/6A vs. 5A/5A	3.7 (1.4–9.7) ^b	0.006	1.1 (0.7–1.8) ^b	0.7	1.16 (0.7–1.9) ^b	0.5

^aAdjusted for BMI, LDL-C, TG and smoking status.^bRisk change adding 1 mutant allele at a time.

statistical significance (Table 3). In young males (<40 years), we found 3.7-fold increased risk for hypertension associated with allele 6A ($P < 0.01$), and 8.1-fold with genotype 6A/6A ($P = 0.01$) considering its recessive effect (Table 4).

eNOS Gene G894T Polymorphism and Hypertensive Status

The observed genotype frequencies for G894T polymorphism were in Hardy-Weinberg equilibrium. There were no significant differences in distribution of both

genotype and allele frequencies either between hypertensive and normotensive males or females (Table 2). Further stratification by age did not show any significance considering association of this polymorphism with hypertensive status in any of the subgroups (data not shown).

DISCUSSION

Up to this time, none of the studies investigated association of MMP-3 5A/6A polymorphism with hypertension. Recently, it has been reported that

5A/5A genotype was associated with elevated blood pressure level in large community-based population study (18). In addition, the study of 213 healthy individuals showed the association of 5A/5A genotype with stiffer large arteries and higher systolic blood pressure level in older subjects (17). Gender-specific as well as age-specific differences in the pathophysiology, risk, and treatment of essential hypertension have been noted. Before the age of menopause, female sex hormones may offer risk protection for hypertension unavailable to men (23). We had two approaches in this study, one focusing on gender and other on age related risk for hypertension. In females with 5A/5A genotype we found a non-significant trend toward higher risk of hypertension. Even the risk was elevating with age it did not reach statistical significance. It is known that postmenopausal hypertensive women have stiffer large arteries compared to men (24). It was reported that with loss of estrogen at the menopause there is no abrupt increase in BP (25), but there is elastin fragmentation and collagen accumulation in the arterial tree with a substantial increase in the intrinsic rigidity of the arterial wall (26). Both higher and lower proteinase activity of MMP-3 homozygotes (5A/5A and 6A/6A, respectively) is contributing to alterations in ECM turnover (4). Variety of studies has demonstrated altered collagen composition in vascular tree in hypertension (27). Existing significantly higher prevalence of 5A/5A genotype in hypertensive women, but only a trend toward higher risk for hypertension with age in our study, suggests that there could be another risk factor in association with genotype leading to hypertension. One of the possible explanations may be the association of MMP-3 5A/5A genotype with arterial stiffening in older women. In addition, our results suggest that 5A/5A genotype may not be useful predictive factor for hypertension in females younger than 50 years of age.

In males up to 40 years of age, we found significant 6A allele dose dependent association with hypertension. The gender-specific association of gene polymorphism with hypertension in males has been already reported for the ACE I/D gene polymorphism (28,29), with more pronounced association in younger males (30). In vivo it has been proved that MMP-3 6A/6A genotype is associated with 2-fold lower gene expression than 5A/6A heterozygotes in humans with corresponding changes in MMP-3 protein levels (4) and enzyme activity (31). So far, 6A/6A genotype has been associated with larger intima media thickness in healthy males (32). Also, 6A allele has been associated with accelerated progression of atherosclerosis, which is connected with a reduced enzyme activity and followed by greater accumulation of ECM (33,16). MMP activity may modulate hypertension-related accumulation of

extracellular matrix proteins in resistance arteries (2). It was suggested that the extracellular degradation of collagen type I is depressed in essential hypertension (34). In young spontaneous hypertensive rat (SHR) vessels, a significant decrease in MMP-3 activity could result in decreased ECM turnover and increased collagen, fibronectin and proteoglycans accumulation (2). Thus, it is reasonable that the genotype related to greater ECM accumulation (e.g. 6A/6A) might be in association with hypertension. Our results clearly present MMP-3 6A/6A genotype as an independent risk factor for hypertension in males up to 40 years of age in Serbian population.

We found no association of G894T eNOS gene polymorphism with hypertension similar to some of the studies (13,35). Previous results regarding association between eNOS genotype and hypertension have been controversial (11–14,35). It might be due to different ethnical origin; T894 allele frequency was increased in Japanese hypertensive subjects (11,12), whereas that of G894 was increased in hypertensive Caucasians (14); as well as due to selection bias. In our population allele frequency of G894 were similar in both hypertensive and normotensive group, but generally higher than previously reported (14) for Caucasians with the same inclusion criteria for participants. Thus, we concluded that in Serbian population G894T eNOS gene polymorphism is not a useful marker for susceptibility to hypertension.

In conclusion, we analyzed the possible association of MMP-3 5A/6A and eNOS G894T polymorphisms with hypertension in Serbian population and revealed that there were gender- and age-specific differences in association of MMP-3 5A/6A polymorphism with hypertension. These results are the first indicating that 6A allele is likely to be important factor in the development of hypertension in young males. The limiting factor in our study should be the number of participants, so we consider our results preliminary. Nevertheless, given the fact that association studies are often irreproducible and because the present study was conducted in relatively small population, replication studies in large and different populations will be indispensable for establishing the association of MMP-3 5A/6A polymorphism with hypertension. Finally, in the future it would be worth to assess the candidate gene–gene interactions, which may also modulate the evaluation of an association.

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