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ASSOCIATION OF ACE I/D AND MMP-3 5A/6A GENE POLYMORPHISMS WITH HYPERTENSION IN MEN FROM SERBIA

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Abstract – The ACE and MMP-3 loci are involved in the vascular remodeling, increased intima media thickness and arterial stiffness associated with hypertension. We determined ACE I/D and MMP-3 5A/6A gene polymorphisms in 231 Caucasian males (126/105, hypertensive/normotensive). Owing to age-related differences hypertension, the sample was truncated with respect to age (the cut-off point was the age of 40). Our results indicate that ACE I/D and MMP-3 5A/6A polymorphisms are likely to be risk factors for hypertension in men from Serbia = 40 years of age. In the same group, the combined effect of DD/6A+ genotypes on hypertension was more pronounced than their separate effect.

Key words: ACE, age-dependence, hypertension, men, MMP-3, gene polymorphism.

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INTRODUCTION

Arterial hypertension is associated with cardiovascular remodeling, which is characterized by qualitative and quantitative changes in extracellular matrix (ECM) content (B a s h e y *et al*., 1989). In hypertension, an important aspect of ECM remodelling is accumulation of collagen, fibronectin, and other ECM components in the vessel wall (I n t e n g a n *et al*., 2001). After injury crucial factors involved in ECM turnover in tissue remodeling during development of atherosclerosis or in pathological hypertensive remodeling are angiotensin II (Ang II), as a powerful effector peptide of the renin-angiotensin system (RAS), and matrix metalloproteinases (MMPs) (C h e n *et al*., 2004; G h i l a r d i *et al*., 2002; P a r m l e y, 1998).

The angiotensin I-converting enzyme (ACE) that is target for ACE inhibition (ACEI) is the key enzyme of RAS. An insertion/deletion (I/D) polymorphism in the ACE gene correlates with the levels of circulating (C a m b i e n *et al*., 1988; R i g a t *et al*., 1990) and tissue localized ACE (C o s t e r o u s s e *et al*., 1993), whereas the DD genotype shows the highest levels (R i g a t *et al*., 1990). So far, there has been evidence for both positive (T u r n e r *et al*., 1999; H i g a k i *et al*., 2000; O' D o n n e l *et al*., 1998; S t a n k o v i ć *et al*., 2002) and negative (K i e m a *et al*., 1996; B e r g and B e r g, 1994) links of ACE I/D polymorphism with hypertension. Recently, association of the DD genotype with hypertension or blood pressure levels was found in men, but not in women (H i g a k i *et al*., 2000; O' D o n n e l *et al*., 1998; S t a n k o v i ć *et al*., 2002), suggesting gender specific influence of this polymorphism on hypertension.

Matrix metalloproteinases are catabolic enzymes involved in the degradation of ECM proteins, and their activity may modulate hypertension-related accumulation of extracellular matrix proteins in resistance arteries (I n t e n g a n *et al*., 2001). The enzyme MMP-3 (stromelysin-1) is a key member of the metalloproteinase family because of its broad substrate specificity (W o e s s n e r, 1991) and ability to activate other MMPs (W o e s s n e r, 1991; M u r p h y *et al*., 1992). It has been shown that the 5A/6A polymorphism in the promoter of gene MMP-3 affects the level of its transcription. The 5A allele had two-times higher protein expression compared to the 6A allele in both cultured fibroblast and vascular smooth muscle cells (Y e *et al*., 1996). The 6A/6A genotype has been linked with both carotid intima-media thickening in healthy males (G n a s s o *et al*., 2000) and accelerated growth of coronary atheromas in men, which is associat-

ed with reduced enzyme activity and followed by greater accumulation of ECM (R a u r a m a a *et al*., 2000).

In view of the proposed role of RAS and MMPs in hypertensive remodeling, the aim of our study was to analyze the possible separate and combined effect of ACE I/D and MMP-3 5A/6A gene polymorphisms on hypertension in Serbian males.

MATERIALS AND METHODS

Subjects and study design

The study population consisted of 231 male Caucasian subjects from Belgrade, Serbia. One hundred and twenty six hypertensive participants were randomly recruited from outpatient clinics over a period of two years. The following served as entry criteria: systolic blood pressure (SBP) > 140 mm Hg or diastolic blood pressure (DBP) > 90 mm Hg without antihypertensive treatment; and diagnosis of hypertension before the age of 60. The control group (n=105) consisted of normotensive volunteers who were undergoing the annual medical check-up at the Occupational Medicine Center, INN Vinča, and who satisfied the following entry criteria: SBP < 140 mm Hg; and DBP < 90 mm Hg. Additional inclusion criteria were a serum glucose level of < 6 mmol/L, a normal sedimentation rate, absence of acute or chronic disease, and absence of drug intake. None of them had coronary heart disease, hypertrophic cardiomiopathy, diabetes mellitus, on renal insufficiency, nor did they suffer from high blood pressure complications. Because of the age-specific development of hypertension, we divided our sample according to age (the cut off point was the age of 40, n=61) in order to assess the more profound relationship between genotypes and hypertension.

Lipids and blood pressure measurement

Biochemical and clinical data were obtained from the same physician, who was provided with a queried. All the participants were inquired in detail about their smoking habits, alcohol consumption, physical activity, use of medications, and family medical history. Lipid concentrations were determined in fresh sera after overnight fasting. Total plasma cholesterol (TC) and triglyceride (TG) levels were determined on a Monarch Plus apparatus (Instrumentation Laboratory, Lexington, USA) using enzymatic colorimetric methods. High-density lipoprotein cholesterol (HDLC) was determined after dextran sulfate-Mg^{2+} precipitation of VLDL, while low-density lipoprotein cholesterol (LDLC) was determined using the CHOD-PAP method. Low density lipoprotein cholesterol was calculated according to the Friedewald formula (F r i e d e w a l d *et al*., 1972) for participants with TG levels < 4.5 mmol/L. All reagent kits were from Instrumentation Laboratory (Lexington, USA).

Blood pressure measurements

Blood pressure (BP) was measured in the morning (readings were taken three times, at least 2 min apart). This was done on the right arm in the line of the heart in a sitting position after 30-min rest at three separate clinic visits. Blood pressure was read using a mercury column sphygmomanometer according to the WHO/American Society of Hypertension recommendations. All efforts were made to minimize factors which might affect BP. The mean value of these measurements was used for analysis.

Determination of genotypes

Genomic DNA was purified by the proteinase K/phenol extraction method as described elsewhere (K u n k e l *et al*., 1977) from whole blood samples, which were collected with EDTA as an anticoagulant.

The ACE I/D gene polymorphism was detected as previously described (S t a n k o v i ć *et al*., 2002) using three primers, separated by agarose (1.8 % w/V) gel electrophoresis in an electric field of 7.5 V/cm, and stained with ethidium bromide, while MMP-3 5A/6A gene polymorphism was detected according to a previously described method (D u n l e a v e y *et al*., 2000). The 110 bp DNA amplified by the polymerase chain reaction (PCR) in Touch Down™ (Hybaid, Teddington, UK) was digested by 5U of *Pdm*I restriction endonuclease (Fermentas, Vilnius, Lithuania). The digestion products were loaded on an 8 % polyacrylamide gel for genotyping and run in an electric field of 12V/cm. Gels were stained with silver nitrate. All gels were visualized by the GDS8000 gel documentation system (Ultra Violet Products Inc, Upland, CA, USA).

Statistical methods

Statistical analysis was performed using the Statistica software package, version 5 (StatSoft Inc, 1997). In all tests, differences with two-tailed alpha–probability (p) ≤ 0.05 were considered significant. The allelic frequencies and genotype distribution were estimated by the gene-counting method. Analysis of differences in proportions

between genotypes and alleles was conducted by the chi-square (χ^2) test. Deviation from the Hardy-Weinberg equilibrium was also assessed using the χ^2 test.

Means of normally distributed continuous variables were compared using the unpaired *t*-test. If the departure from normal distribution was significant (TG, systolic BP, diastolic BP), comparisons were done with logarithm-transformed values.

In multiple logistic regression analysis, after full adjustment for confounding factors (smoking status, BMI, LDLC, TG), the maximum likelihood estimation procedure was used to obtain associations between hypertension and ACE I/D and MMP-3 5A/6A genotypes and expressed in terms of the adjusted odds ratio (OR) and a 95% confidence interval (CI). Hypertensive status was entered as the dichotomous dependent variable and genotype classes were coded on a ratio scale (0 and 1) according to three models of inheritance: model 1 – assumes a dominant effect of the *D* or *6A* allele, model 2 – a recessive effect of the *D* or *6A* allele, and model 3 – an additive effect of the *D* or *6A* allele. In model 1 the codes were: 1 for ID+DD and 5A/6A + 6A/6A, 0 for II and 5A/5A; in model 2 they were: 1 for DD and 6A/6A, 0 for II+ID and 5A/5A + 5A/6A; and in model 3 they were: 0 for II and 5A/5A, 1 for ID and 5A/6A, 2 for DD and 6A/6A.

RESULTS

Description of the population

The main characteristics of the study sample for hypertensive and normotensive groups in the study sample are shown in Table 1. Differences between hypertensive and normotensive men were significant for age, BMI, SBP, and DBP level. In general, the control group was older than the hypertensive one, thereby minimizing the possibility of later development of hypertension.

ACE I/D and MMP-3 5A/6A gene polymorphism and hypertensive status

Distribution of the ACE I/D and MMP-3 5A/6A genotypes and allele frequencies in normotensive and hypertensive males are shown in Table 2. For both polymorphisms observed, the genotype distributions were in Hardy-Weinberg equilibrium. There was a significant difference of ACE I/D genotype distribution between hypertensive and normotensive males ($p<0.05$), but the difference in D allele frequency did not reach statistical significance ($p=0.07$). Distribution of the MMP-3 genotype and allele frequencies did not differ significantly between the two studied groups. After dividing the study sample by age, hypertensive men aged = 40 years had significantly higher presence of the DD genotype (NT=15.79% vs. HT=45.24%; $p<0.001$) and D allele (NT=0.39 vs. HT=0.65; $p<0.001$) compared to normotensive ones. In the same age group, significantly greater ($p<0.01$) prevalence of the MMP-3 6A/6A genotype (NT=21.05% vs. HT=35.71%) and higher 6A allele frequency (NT=0.45 vs. HT=0.62; $p<0.001$) were discovered in hypertensive men.

Table 1. Clinical parameters of normotensive and hypertensive men
Values are mean ± SD for age, BMI, SBP, DBP, TC, LDLC, HDLC, and TG; [a] analyses were performed on logarithm transformed values; [*]χ^2 –test; NS non-significant.

Parameter	Normotensive (n =105)	Hypertensive (n =126)	t-test (p)
Age (years)	48.1 ± 10.5	44.9 ± 11.8	<0.05
Body-mass index (kg m^{-2})	26.3 ± 2.3	27.5 ± 3.3	<0.05
Smokers – n (%)[*]	58 (54.7)	62 (48.4)	NS
SBP (mmHg) [a]	119.4 ± 8.7	145.6 ± 13.6	<0.05
DBP (mmHg) [a]	77.9 ± 6.5	95.4 ± 7.8	<0.05
TC (mmol L^{-1})	5.7 ± 1.3	5.8 ± 1.3	NS
LDLC (mmol L^{-1})	3.5 ± 1.1	3.6 ± 1.1	NS
HDLC (mmol L^{-1})	1.3 ± 0.4	1.3 ± 0.4	NS
TG (mmol L^{-1})[a]	1.6 ± 0.9	1.9 ± 1.5	NS

Table 2. Distribution of ACE and MMP-3 genotypes and allele frequencies in normotensive and hypertensive men; p value from χ^2 test; NS non-significant.

	Normotensive		Hypertensive		p
ACE	n	%	n	%	
	105		126		
II	26	24.76	22	17.46	
ID	53	50.48	60	47.62	0.02
DD	26	24.76	44	34.92	
Allele I/D	0.5/0.5		0.41/0.59		NS
MMP-3	n	%	n	%	p
	105		126		
5A5A	22	20.95	19	14.84	
5A6A	54	51.43	68	54.69	NS
6A6A	29	27.62	39	30.47	
Allele 5A/6A	0.47/0.53		0.42/0.58		NS

Logistic regression analysis revealed a non-significant trend toward higher risk for occurrence of hypertension in men carrying the ACE DD genotype based on its recessive effect (adjusted OR=1.66; 95 % CI 0.92-2.99; p=0.09), as well as in men carrying one or more MMP-3 6A allele (model of inheritance 1) (OR=1.47; 95 % CI 0.73-2.96; p=0.28). Estimating the combined effect of DD/6A+ genotypes on hypertension, we found an increase of relative risk, but it did not reach statistical significance (adjusted OR=1.83; CI 0.97-3.48; p=0.06). When truncated by age, in the group of men = 40 years old, the DD genotype (adjusted OR=5.88, CI 1.29-26.73, p=0.02) as well as one or more 6A allele (adjusted OR=3.96; CI 0.95-16-46; p=0.05) became significant risk factors for hypertension. Moreover, the combined effect of DD/6A+ genotypes was more pronounced than their separate effects (adjusted OR=7.97; CI 1.29-49.26; p=0.02) (Fig. 1).

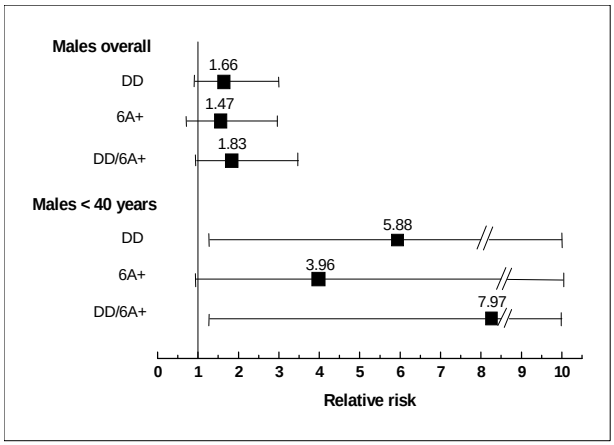

Fig. 1. Adjusted relative risk of hypertension associated with ACE and MMP-3 genotypes. Separate effects of DD and 6A+ and the combined effect of DD/6A+ genotypes are presented in men overall and in men = 40 years old.

DISCUSSION

Regulation of blood pressure is achieved through a delicate balance of several physiological and biochemical systems, each of which is under complex genetic control. Hence the multifactorial nature of hypertension. Gender-specific as well as age-specific differences in the pathophysiology, risk, and treatment of hypertension have been noted. Association of the ACE DD genotype with hypertension in men (F o r n a g e *et al*., 1998; S t a n k o v i ć *et al*., 2002; H i g a k i *et al*., 2000; Y o o, 2005) was confirmed in this study. By focusing on a younger population, we have minimized environmental and blunting influences pertaining to lifestyle. In men = 40 years of age, we found a significant relative risk of hypertension (OR=5.88) in subjects with the ACE DD genotype. This is consistent with some previous studies (H i g a k i *et al*., 2000; S t a n k o v i ć *et al*., 2002), but with a difference in the age cut-off point, which we set more rigorously (at the age of 40) compared to others, who considered "young" men to be those less than 50 (S t a n k o v i ć *et al*., 2002) or 60 (H i g a k i *et al*., 2000) years old.

To date, besides atherosclerosis (Y e *et al*., 1996), MMP-3 5A/6A polymorphism has been linked to an elevated blood pressure level (B e i l b y *et al*., 2005) and stiffer large arteries (M e d l e y *et al*., 2003); little is known about association of MMP-3 5A/6A polymorphism with hypertension. In men = 40 years old, we found significant association of the 6A allele with hypertension based on its dominant effect. The 6A/6A genotype has been linked with lower MMP-3 levels in arterial walls compared with the 5A/5A genotype in humans (M e d l e y *et al*., 2003), the given linkage corresponding with changes in enzyme activity (G a l i s *et al*., 1994). Lower proteinase activity of MMP-3 6A/6A homozygotes contributes to alterations of ECM turnover (M e d l e y *et al*., 2003). It is followed by greater accumulation of ECM (R a u r a m a a *et al*., 2000; Y e *et al*., 1996) and altered collagen composition of the vascular tree in hypertension (I w a t s u k i *et al*., 1977). Thus, the 6A/6A genotype could be a reasonable putative risk factor for hypertension, as our results suggest.

We showed that in younger men the additive effect of the ACE DD and MMP-3 6A+ genotypes contributes to increased relative risk of hypertension compared to their separate effects. It is known that tissue ACE exert influence on arterial structure and remodeling (H i l g e r s *et al*., 2004). The ACE DD genotype may have a more pronounced effect on extracellular matrix synthesis due to higher local levels of angiotensin II (R i g a t *et al*., 1990). It has been reported that angiotensin II suppresses MMP-1 activity, thus leading to collagen accumulation (T u n o n *et al*., 2000). In addition, decreased circulating levels of MMP-1, together with depressed extracellular collagen type I degradation have been observed in hypertensive patients compared with normotensive subjects (L a v i a d e s *et al*., 1998). In young spontaneously hypertensive rat vessels, significant decrease of MMP-3 activity can result in decreased ECM turnover and increased collagen, fibronectin, and proteoglycans

accumulation (C a s t r o *et al*., 1999). Thus, it is reasonable that younger men carrying both the DD and 6A+ genotypes, which are predisposed to greater ECM accumulation, could be under higher risk of developing hypertension, as well as hypertension related secondary organ damage.

Angiotensin II at the cellular level stimulates fibroblast-mediated collagen synthesis in a dose-dependent manner and suppresses collagenase activity, synergistically leading to progressive collagen accumulation (S u n and W e b e r, 1998; T u n o n *et al*., 2000). The ACEI or Ang II type 1 receptor antagonists (AT1RA) reduce intima-media thickness and improve arterial function in hypertension with or without a reduction in blood pressure level (H u a n g *et al*., 1998). Compared to antihypertensive treatment with vasodilator hydralazine, only ACEI corrected the altered distribution of myocardial collagen phenotypes I and III (M u k h e r j e e and S e n, 1993), supporting the concept that pathological collagen content can be regressed in hypertensive patients (S c h w a r t z - k o p f f *et al*., 2000) and suggesting a possible connection between ACE and MMPs on the tissue level.

In conclusion, age-specific differences in association of ACE I/D and MMP-3 5A/6A polymorphisms with hypertension in Serbian men point to a possible additive effect of some genes involved in increased risk of occurrence of hypertension, one of the group of enigmatic polygenic diseases. Further replication studies in large and different populations will be necessary to confirm the observed relationship in younger men, as well as the risk of developing hypertension-related end organ damage.

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АСОЦИЈАЦИЈА ПОЛИМОРФИЗАМА I/D I 5A/6A У ГЕНИМА ЗА ACE И MMP-3 СА ХИПЕРТЕНЗИЈОМ КОД МУШКАРАЦА У СРБИЈИ

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ACE и MMP-3 су укључени у процесе ремоделовања зида крвних судова, задебљања интима-медије и губитка еластичности артерија који су повезани са хипертензијом. Утврђивање генотипова полиморфизама I/D и 5A/6A у генима за ACE и MMP-3 је урађено на узорку од 231 мушкарца, Кавказоида (126/105, хипертензивни/нормотензивни). Обзиром на повезаност настанка хипертензије са годинама старости испитивана популација је подељена у две старосне групе (до и преко 40-е године старости). Наши резултати указују да су полиморфизми у генима за ACE (I/D) и MMP-3 (5A/6A) могући фактори ризика за настанак хипертензије код мушкараца = 40 година старости у популацији Србије. У истој старосној групи заједнички утицај генотипова DD/6A+ на настанак хипертензије је био већи од њиховог појединачног утицаја.