Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Evidence for an association between haptoglobin and MnSOD (Val9Ala) gene polymorphisms in essential hypertension based on a Brazilian case-control study

A.L. Miranda-Vilela[1], A.K. Akimoto[1], P.C.Z. Alves[1], L.B. Ferreira[2], G.S. Lordelo[1], J.G.M. Melo[1], C.K. Grisolia[1], S.F. Oliveira[1] and M.N. Klautau-Guimarães[1]

[1]Departamento de Genética e Morfologia, Laboratório de Genética, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, DF, Brasil
[2]Centro Universitário de Brasília, Campus Universitário Asa Norte, Brasília, DF, Brasil

Corresponding author: A.L. Miranda-Vilela
E-mail: mirandavilela@unb.br

Genet. Mol. Res. 9 (4): 2166-2175 (2010)
Received July 13, 2010
Accepted August 2, 2010
Published November 3, 2010
DOI 10.4238/vol9-4gmr973

ABSTRACT. Essential hypertension is a complex and multifactorial trait; genetic and environmental factors interact to produce the final phenotype. Studies have demonstrated association of hypertension with varied gene polymorphisms. However, demonstration of common genetic causes in the general population remains elusive. We investigated a possible association between hypertension and haptoglobin, angiotensin I-converting enzyme (ACE), glutathione S-transferases GSTM1 and GSTT1, MnSOD (Val9Ala), CAT (-21A/T), and GPX1 (Pro198Leu) gene polymorphisms in an urban Brazilian population group from Brasília. Although ACE has been reported to be one of the main polymorphisms associated with hypertension, we found no association with ACE's specific genotypes. However, a possible

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association with Hp1-1 and MnSOD Val/Ala genotypes suggests that, at least for the Brazilian population, polymorphisms related to oxidative stress should be more deeply investigated.

Key words: Essential hypertension; Gene polymorphisms; Haptoglobin; Manganese superoxide dismutase

INTRODUCTION

Essential, primary, or idiopathic hypertension is defined as high blood pressure of unknown cause in which causes of secondary hypertension, including renovascular disease, renal failure, pheochromocytoma and aldosteronism, are not present. Thus, it is a heterogeneous disorder, with different patients having different causal factors that lead to high blood pressure (Carretero and Oparil, 2000; Messerli et al., 2007). Essential hypertension accounts for more than 90% of cases of hypertension and is a major risk factor for morbidity and mortality from cardiovascular causes, besides increasing the risk of cerebral and renal events (Oparil et al., 2003; Messerli et al., 2007). A number of studies have therefore focused on it worldwide, either due to its clinical and pathophysiological aspects or because it is considered to be one of the major public health problems (Caulfield et al., 1994; Carretero and Oparil, 2000; Dominiczak et al., 2000; Neder and Borges, 2006).

Human essential hypertension is a complex and multifactorial trait under polygenic control (Caulfield et al., 1994; Carretero and Oparil, 2000; Dominiczak et al., 2000; Rola and Ferreira, 2008). Hence, it is likely that several gene loci, each with a small but significant contribution, will be responsible for this genetic component (Caulfield et al., 1994; Carretero and Oparil, 2000; Dominiczak et al., 2000). Essential hypertension tends to cluster in families, and the resulting phenotypes can be modulated by various environmental factors, thereby altering the severity of blood pressure elevation and the timing of hypertension onset (Oparil et al., 2003). The genetic determinants interact with environmental factors, such as obesity, insulin resistance, high alcohol intake, cigarette-smoking, high salt intake, a sedentary lifestyle, psychosocial stress, dyslipidemia, and low potassium or calcium intake, increasing blood pressure in susceptible subjects and producing the final disease phenotype (Carretero and Oparil, 2000; Dominiczak et al., 2000).

Despite very significant recent progress in genomic and statistical tools, the genetic dissection of human essential hypertension still provides a major challenge (Dominiczak et al., 2000). In recent years, several studies have demonstrated the association of hypertension and its complications with quite varied gene polymorphisms (Rola and Ferreira, 2008). The great majority of the candidate genes studied have been chosen from the encoding enzymes and peptides of the renin-angiotensin system or other proteins related to water and sodium handling (Carretero and Oparil, 2000; Dominiczak et al., 2000; Oparil et al., 2003). However, variants of the renin-angiotensin system's genes seem to affect blood pressure only modestly, and other candidate genes have not shown consistent and reproducible associations with blood pressure or hypertension in larger populations. Thus, demonstration of common genetic causes of hypertension in the general population remains elusive (Oparil et al., 2003).

Since reactive oxygen species (ROS) play a pivotal role in the pathogenesis of endothelial dysfunction (Harrison et al., 2003), which may involve the development of hypertension and coronary artery disease (Li and Chen, 2005), polymorphisms in the antioxidant enzyme

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genes, haptoglobin (Hp) and glutathione S-transferases (GSTs), should be better analyzed in this context, although the Hp polymorphism has been suggested as a candidate genetic marker for essential hypertension (Delanghe et al., 1995). This is because while antioxidant enzymes act by directly neutralizing ROS (Ferreira and Matsubara, 1997; Hermes-Lima, 2004), Hp acts as an antioxidant to bind free hemoglobin in the plasma and block hemoglobin-induced oxidative damage (Guéye et al., 2006), and most GST substrates are xenobiotics or products of oxidative stress (Cotton et al., 2000).

In Brazil, most studies of this nature have investigated essential hypertension prevalence, and of these, the majority have been carried out by authors from the southern and southeastern regions (Neder and Borges, 2006; Passos et al., 2006). Population-based studies indicate prevalence in Brazilian cities from 22 and 44% (Boing and Boing, 2007). This indicates that essential hypertension is an important public health challenge in Brazil. Thus, the identification of variant (alleles) genes that can contribute to the development of hypertension is of extreme relevance, since the considerable variation among human populations may reflect distinctive processes of adaptation to variable environmental conditions (Barreiro et al., 2008). Therefore, this type of information would help us to better understand the complex interaction between genetic and environmental factors that contribute to the phenotypical manifestation in susceptible Brazilians, as well as improving treatment strategies.

Hence, our aim in this pilot case-control study was to investigate a possible association between essential hypertension and Hp, angiotensin I-converting enzyme (ACE), glutathione S-transferase M1 (GSTM1), glutathione S-transferase T1 (GSTT1), manganese superoxide dismutase (MnSOD Val9Ala), catalase (CAT -21A/T), and glutathione peroxidase (GPX1 Pro198Leu) gene polymorphisms in an urban Brazilian population group.

MATERIAL AND METHODS

Study design and participants

Initially, hypertensive subjects (N = 91), presenting systolic pressure equal to or greater than 140 mmHg and diastolic pressure equal to or greater than 90 mmHg in at least two readings on different days, consisted of 46 males, mean age 57.6 ± 10.5 years, and 45 females, mean age 56.5 ± 8.9 years. They were recruited from a private cardiology clinic in Brasília. Subjects presenting secondary causes of hypertension were excluded from the study, retaining only those with essential hypertension.

The initial control group (N = 110), composed of non-smoking, non-hypertensive, non-diabetic, non-dislipidemic, and apparently healthy individuals, was recruited from the same clinic and from the University of Brasília, consisting of 54 males, mean age 37.4 ± 13.4 years and 56 females, mean age 41.0 ± 12.1 years. All volunteers filled out a questionnaire about their lifestyle habits, pre-existing chronic diseases, family history of diabetes, and cardiovascular diseases.

In a second phase, hypertensive subjects and apparently healthy controls were paired according to their skin color, age and anthropometric variables [waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), and body mass index (BMI)], totaling 48 pairs for this pilot case-control study (Table 1). Among 201 registered volunteers, 96 concluded their participation (48 hypertensive subjects and 48 apparently healthy individuals chosen for pairing).

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee for Health Sciences Faculty Research of the University of Brasília and by the National Commission for Ethics in Research (CONEP). Written informed consent was obtained from all the registered volunteers, and all subjects were free to withdraw at any time during the study.

Procedures and measurements

WC, HC, WHR, and BMI were recorded, according to Wang and Hoy (2004). WCs were measured at the narrowest point below the ribs or halfway between the lowest ribs and the iliac crests. HCs were measured at the level of the anterior superior iliac spine, where this could be felt, otherwise at the broadest circumference below the waist (Table 1). About 5 mL peripheral blood was collected by venipuncture using Vacutainer tubes with EDTA as anticoagulant for the analyses of gene polymorphisms.

Genotyping of Hp, ACE, GSTM1, GSTT1, and antioxidant enzymes

DNA was isolated from the buffy-coat layer by using a Blood GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, England) purification kit and the samples were stored at -20°C until analysis. DNA samples underwent amplification in an MJ PTC-100 (MJ Research Inc.).

Hp genotypes were determined by allele-specific polymerase chain reaction (PCR) as described by Yano et al. (1998), while MnSOD, CAT and GPX1 genotypes were determined by PCR-based restriction fragment length polymorphism (RFLP) assays performed as described respectively by Mitrunen et al. (2001), Ukkola et al. (2001) and Zhao et al. (2005). DNA fragments containing I/D polymorphism in intron 16 of the ACE gene were amplified by PCR as previously described by Rigat et al. (1992), using dimethyl sulfoxide, as recommended by Odawara et al. (1997), to avoid mistyping the DD genotype. The glutathione S-transferases, GSTM1 and GSTT1 fragments, were amplified simultaneously as proposed by Chen et al. (1996), using β-globin as a positive control. The absence of an amplification product combined with the presence of a positive control band (268-bp DNA fragment of β-globin) indicated the null variants for both polymorphisms.

The PCR and PCR-based RFLP products were resolved in non-denaturing polyacrylamide gels stained with silver nitrate.

Statistical analyses

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 15.0. Except for skin color, data were reported as means ± SEM (standard error of mean). To reveal contingent differences between the groups, statistical significance was assessed by the *t*-test. Multivariate analyses of variance were used to look for possible interactions among the variables analyzed, and a *post hoc* pair-wise comparison was carried out through the Bonferroni's test. Values of $P < 0.05$ were considered to be statistically significant.

Allelic and genotypic frequencies were estimated by gene counting, and the goodness of fit of the genotype distribution for Hardy-Weinberg equilibrium was tested by the chi-square (χ^2) test. Values of $P > 0.05$ indicated Hardy-Weinberg equilibrium. Probability (P)

values for co-dominant markers (Hp, ACE, MnSOD, CAT, and GPX1) were generated using the Genepopweb Statistical Program version 4.0 (http://genepop.curtin.edu.au). For dominant markers (GSTM1 and GSTT1), the χ^2 test value was calculated using a chi-square calculator program. Since the PCR method is not suitable for distinguishing between homozygous (+/+, wild type) and heterozygous (+/–), these two groups were considered together (non-null genotypes) and compared with the variant group (null genotypes). The odds ratio (OR) with 95% confidence intervals was calculated and P values were generated by the Pearson chi-square test. Differences were considered to be significant at $P < 0.05$.

RESULTS

For the subjects chosen for pairing in the second phase, the *t*-test indicated that there was no difference between hypertensive and control groups for the variables (Table 1). There were no interactions between groups (hypertensive and control) and skin color, groups and gender, skin color and gender, or among groups, skin color and gender (Table 1). The results indicated only influence of gender (P = 0.000), which was related to height (P = 0.000), HC (P = 0.031) and WHR (P = 0.000) in the tests of between-subject effects. Concerning genetic markers, significant interactions appeared only for groups and ACE (P = 0.001). They were related to HC values (P = 0.032), but there were no significant differences between genotypes (Bonferroni's test). No interaction appeared among genetic markers.

Table 1. Skin color, age and anthropometric variables of hypertensive subjects and apparently healthy control individuals chosen for pairing.

Variables	Total (N = 48)			Males (N = 25)			Females (N = 23)		
	Hypertensive subjects	Controls	P	Hypertensive subjects	Controls	P	Hypertensive subjects	Controls	P
Skin color (white/mixed/black)	35/8/5	30/14/4	0.344	16/4/3	11/11/1	0.508	19/4/2	19/3/3	0.832
Age (years)	50.5 ± 1.18	50.6 ± 1.18	0.940	49.87 ± 1.85	49.70 ± 1.83	0.947	51.32 ± 1.51	51.24 ± 1.54	0.971
Body weight (kg)	75.6 ± 2.30	73.6 ± 2.12	0.517	78.21 ± 2.44	80.79 ± 2.74	0.486	73.26 ± 3.79	66.98 ± 2.59	0.178
Height (m)	1.66 ± 0.01	1.66 ± 0.01	0.964	1.72 ± 0.01	1.74 ± 0.01	0.367	1.61 ± 0.01	1.59 ± 0.01	0.378
BMI (kg/m^2)	27.3 ± 0.76	26.4 ± 0.58	0.349	26.31 ± 0.66	26.63 ± 0.73	0.751	28.29 ± 1.32	26.28 ± 0.89	0.211
Waist circumference (cm)	94.4 ± 1.85	91.9 ± 1.72	0.333	95.65 ± 2.03	94.96 ± 2.35	0.824	93.20 ± 3.04	89.12 ± 2.42	0.299
Hip circumference (cm)	102.2 ± 1.70	101.7 ± 1.39	0.827	99.39 ± 1.84	100.26 ± 1.83	0.739	104.80 ± 2.73	103.08 ± 2.01	0.614
Waist-to-hip ratio	0.92 ± 0.01	0.90 ± 0.01	0.155	0.96 ± 0.01	0.95 ± 0.01	0.327	0.89 ± 0.01	0.86 ± 0.01	0.159

P values were generated by the *t*-test. BMI = body mass index; N = sample size.

The P values indicated deviation from Hardy-Weinberg equilibrium, appropriate to a heterozygote excess for the MnSOD (P = 0.0001) locus in the group of hypertensive subjects, which had a higher frequency of Val/Ala genotypes than expected, a lower frequency of Val/Val genotypes than expected and an F_{IS} (inbreeding coefficient) value (F_{IS} = -0.0001) compatible with selection in favor of heterozygotes. For GSTM1 and GSTT1, results were compatible with heterozygote deficit in both groups (hypertensive and control), due to homozygous (+/+, wild type) and heterozygous (+/–) being considered together within non-null genotypes, since the PCR method is not suitable for distinguishing these genotypes. The genotypic distributions of Hp, ACE, CAT, and GPX1 loci were in accordance with Hardy-Weinberg equilibrium in both groups; MnSOD distribution was in Hardy-Weinberg equilibrium only in the control group (Table 2).

Table 2. Distribution of Hp, ACE, GSTM1, GSTT1, MnSOD, CAT, and GPX1 allele and frequencies and Hardy-Weinberg equilibrium (HWE) data for the chi-square (χ^2) test for hypertensive subjects (N = 48) and apparently healthy control individuals (N = 48) chosen for pairing.

Alleles	Allele frequencies		Genotypes	Number of observed individuals		Number of expected individuals		HWE test (P values)	
	Hypertensive subjects	Controls		Hypertensive subjects	Controls	Hypertensive subjects	Controls	Hypertensive subjects	Controls
Hp			Hp						
Hp*1	0.54	0.35	1-1	13	5	13.99	5.88	0.772	0.754
Hp*2	0.46	0.65	2-1	26	24	23.84	21.84		
			2-2	9	19	10.16	20.28		
ACE			ACE						
I	0.46	0.34	II	11	6	10.16	5.55	0.574	1.000
D	0.54	0.66	ID	22	21	23.84	21.54		
			DD	15	21	13.99	20.91		
GSTM1			GSTM1						
Null	0.69	0.75	Null	33	36	22.85	27.00	0.003	0.014
Present	0.31	0.25	Non-null	15	12	25.15	21.00		
GSTT1			GSTT1						
Null	0.42	0.33	Null	20	16	8.47	5.23	< 0.001	< 0.001
Present	0.58	0.67	Non-null	28	32	39.53	42.77		
MnSOD			MnSOD						
Val	0.52	0.59	Val/Val	6	15	12.98	16.71	< 0.001	0.366
Ala	0.48	0.41	Val/Ala	38	27	23.96	23.22		
			Ala/Ala	4	6	11.06	8.07		
CAT			CAT						
A	0.37	0.40	AA	8	10	6.57	7.68	0.541	0.144
T	0.63	0.60	AT	20	18	22.38	23.04		
			TT	20	20	19.05	17.28		
GPX1			GPX1						
Pro	0.75	0.68	Pro/Pro	27	21	27.00	22.20	1.000	0.742
Leu	0.25	0.32	Pro/Leu	18	24	18.00	20.89		
			Leu/Leu	3	3	3.00	4.92		

$P < 0.05$ indicates deviation from Hardy-Weinberg equilibrium, indicating a heterozygote excess for MnSOD ($P = 0.0001$) locus and a heterozygote deficit for GSTM1 ($P < 0.001$) and GSTT1 ($P < 0.001$) loci. For co-dominant markers (Hp, MnSOD, CAT, GPX1, and ACE), P values were generated using the statistical program Genepopweb version 4.0 (http://genepop.curtin.edu.au); for dominant markers (GSTM1 and GSTT1); they were calculated with a chi-square calculator program. GSTM1 and GSTT1 genotypes were considered: null = -/-; non-null = +/+ and +/-. Hp = haptoglobin; ACE = angiotensin I-converting enzyme; GSTM1 = glutathione S-transferase M1; GSTT1 = glutathione S-transferase T1; MnSOD = manganese superoxide dismutase; CAT = catalase; GPX1 = glutathione peroxidase.

P values obtained by the OR and the Pearson chi-square test indicated increased risk for essential hypertension related to Hp 1-1 and MnSOD Val/Ala genotypes, and reduced risk among individuals carrying Hp 2-2 and MnSOD Val/Val genotypes. No other results were significant (Table 3).

DISCUSSION

Many studies have demonstrated association of hypertension with various gene polymorphisms (Rola and Ferreira, 2008), but the demonstration of common genetic causes of hypertension in the general population remains elusive (Oparil et al., 2003). This is probably because various relevant polymorphisms have ethnic and geographic variations (Sagnella et al., 1999; Thomas et al., 2000; Pasha et al., 2002; Carter and Worwood, 2007). Since the considerable range of variation in human populations may reflect, in part, distinctive processes of

Table 3. The distributions of Hp, ACE, GSTM1, GSTT1, MnSOD, CAT, and GPX1 genotype polymorphisms plus the odds ratio (OR) with 95% confidence intervals (CI) for hypertensive subjects (N = 48) as compared to the apparently healthy control individuals (N = 48) chosen for pairing.

Genotypes	Genotypes N (%)		Hypertensive subjects *vs* controls	P
	Hypertensive subjects	Controls	OR (95%CI)	
Hp				
1-1	13 (27.1)	5 (10.4)	3.194 (1.038 to 9.827)	0.036
2-1	26 (54.2)	24 (50.0)	1.182 (0.530 to 2.634)	0.683
2-2	9 (18.8)	19 (39.6)	0.352 (0.139 to 0.890)	0.025
ACE				
II	11 (22.9)	6 (12.5)	2.081 (0.701 to 6.180)	0.181
ID	22 (45.8)	21 (43.8)	1.088 (0.487 to 2.433)	0.837
DD	15 (31.3)	21 (43.8)	0.584 (0.253 to 1.347)	0.206
GSTM1				
Null	33 (68.8)	36 (75.0)	0.733 (0.300 to 1.793)	0.496
Non-null	15 (31.3)	12 (25.0)	1.364 (0.558 to 3.334)	0.496
GSTT1				
Null	20 (41.7)	16 (33.3)	1.429 (0.623 to 3.277)	0.399
Non-null	28 (58.3)	32 (66.7)	0.700 (0.305 to 1.606)	0.399
GSTM1T1				
M1–T1–	16 (33.3)	10 (20.8)	1.900 (0.758 to 4.765)	0.168
M1–T1+	17 (35.4)	26 (54.2)	0.464 (0.204 to 1.053)	0.065
M1+T1–	4 (8.3)	5 (10.4)	0.782 (0.197 to 3.109)	0.726
M1+T1+	11 (22.9)	7 (14.6)	1.741 (0.611 to 4.960)	0.296
MnSOD				
Val/Val	6 (12.5)	15 (31.3)	0.314 (0.110 to 0.899)	0.026
Val/Ala	38 (79.2)	27 (56.3)	2.956 (1.201 to 7.271)	0.016
Ala/Ala	4 (8.3)	6 (12.5)	0.636 (0.168 to 2.416)	0.504
CAT				
AA	8 (16.7)	10 (20.8)	0.760 (0.271 to 2.129)	0.601
AT	20 (41.7)	18 (37.5)	1.190 (0.525 to 2.700)	0.676
TT	20 (41.7)	20 (41.7)	1.000 (0.444 to 2.251)	1.000
GPX1				
Pro/Pro	27 (56.3)	21 (43.8)	1.653 (0.738 to 3.703)	0.221
Pro/Leu	18 (37.5)	24 (50.0)	0.600 (0.266 to 1.353)	0.217
Leu/Leu	3 (6.3)	3 (6.3)	1.000 (0.192 to 5.222)	1.000

P values were generated by the Pearson chi-square test. For abbreviations, see legend to Table 2.

natural selection and adaptation to variable environmental conditions (Barreiro et al., 2008), common genetic causes of hypertension for particular populations are more likely to be demonstrated than those for the general population.

The Brazilian population as a whole is very mixed and heterogeneous, primarily as a result of five centuries of interethnic crosses between Europeans, Africans and Amerindians (Alves-Silva et al., 2000). Because the Federal District was formed in the late 1950s by a wide-ranging mixture of migrants from all regions of Brazil (Queiroz, 2006), its population tends to reflect the constitution of the Brazilian population better than populations of other Brazilian regions. Since genes under positive selection are thought to have an important role in human survival and to affect complex phenotypes of medical relevance (Barreiro et al., 2008) and because essential hypertension is considered to be an important public health issue in Brazil (Boing and Boing, 2007), the identification of genetic components that can contribute to the development of hypertension in the Brazilian population can help us to better understand the severity of high blood pressure in our population, as well as to improve the treatment strategies. In this context, our results indicated that Hp and MnSOD polymorphisms can be important genetic determinants that interact with environmental stimuli to produce high blood pressure in susceptible Brazilian individuals. Since some important determining environmental hypertension factors,

such as WC, HC, WHR, and BMI, as well as gender and skin color differences were found and showed no differences between hypertensive and control groups, we can conclude that, alone, these variables had no influence on the final phenotype. Although our association study investigated a relatively small number of patients, given the marked lack of confounding factors, it seems likely that the observed differences are related to genetic factors.

Hp polymorphism has been suggested as a candidate genetic marker in essential hypertension (Delanghe et al., 1995), and Hp^{*1} allele frequency is high among patients with essential hypertension (Delanghe et al., 1997). In this regard, our results corroborate this finding. On the other hand, the Hp 2-2 genotype has been associated with increased risk for essential hypertension (Prabha et al., 1987), accumulation of atherosclerotic lesions in essential hypertension, besides showing higher therapeutic needs and more refractory hypertension (Delanghe et al., 1997). Although our study did not investigate the latter two parameters, our results indicate that Brazilian individuals carrying Hp 2-2 can present a lower risk of developing hypertension than Hp 1-1 subjects. However, this could have been due to the small sample size, and more investigations of the Brazilian population should be carried out.

At the cellular and molecular levels, the endothelium is a likely central focus for the effects of hypertension and the pathogenesis of atherosclerosis. Changes in endothelial function and morphology are cardinal features of hypertension, and there is evidence that hypertension induces oxidative stress in the arterial wall (Alexander, 1995). It has even been suggested that superoxide anions might trigger the development of hypertension in some models, presumably by inactivating endothelium-derived nitric oxide and thus mitigating this important vasodilator mechanism (Nakazono et al., 1991; Alexander, 1995). Thus, the polymorphisms in the SOD genes (Mn,Cu,Zn or EC-SOD) can affect superoxide dismutase enzyme efficiency against oxidative stress due to superoxide production in the vascular endothelium, in turn favoring hypertension.

MnSOD (EC 1.15.1.1) is a mitochondrial enzyme coded by a nuclear gene located on chromosome 6q25.3 (Akyol et al., 2005; Bastaki et al., 2006). The enzyme is synthesized with a mitochondrial targeting sequence, which is cleaved in the mitochondrial matrix to produce the mature protein (Akyol et al., 2005). The valine to alanine substitution in the MnSOD mitochondrial targeting sequence induces a conformational change, which has been reported to change mitochondrial processing efficiency, to affect the transport of MnSOD to the mitochondria, and to decrease MnSOD efficiency against oxidative stress (Akyol et al., 2005). Previous studies have associated the variant -9Ala allele with diseases related to oxidative stress and abnormal free radical defense mechanisms, such as exudative age-related macular degeneration, Parkinson's disease and risk of breast, prostate and ovarian cancers (Mitrunen et al., 2001; Olson et al., 2004; Akyol et al., 2005; Choi et al., 2008). Therefore, the fact that only the hypertensive group had a higher frequency of the Ala allele and deviation from HWE for the MnSOD locus corroborates the conclusions made in the previous studies.

CONCLUSIONS

In summary, although ACE has been reported among the main polymorphisms associated with hypertension, we found no association with ACE's specific genotypes. However, a possible association with Hp 1-1 and MnSOD Val/Ala genotypes suggests that, at least for the Brazilian population, polymorphisms related to oxidative stress should be more deeply investigated.

ACKNOWLEDGMENTS

Research supported by the University of Brasília (UnB), the National Council for Technological and Scientific Development (CNPq) and the Scientific and Technological Enterprises Foundation (FINATEC). The authors gratefully acknowledge Dr. Luiz Introcaso for the hypertension anamnesis.

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