RESEARCH ARTICLE

Case–control association study of polymorphisms in the angiotensinogen and angiotensin-converting enzyme genes and coronary artery disease and systemic artery hypertension in African-Brazilians and Caucasian-Brazilians

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Abstract

The rennin–angiotensin–aldosterone system (RAAS) is a critical pathway in regulating blood pressure and salt/water homeostasis, possessing an intimate relationship with the development of systemic artery hypertension (SAH). Once hypertension is considered a risk factor for coronary artery disease (CAD), the RAAS is also related to this pathology. This investigation aimed to analyse if the frequencies of *AGT M235T* (rs699) and *ACE I/D* (rs4646994) polymorphisms are associated with CAD and SAH in African-Brazilians and Caucasian-Brazilians. In this study we analysed 714 subjects who underwent coronary angiography to detect obstructive lesions and CAD, as well as blood pressure measurement and SAH, grouped according to ethnicity: 266 African-Brazilians and 448 Caucasian-Brazilians. Among CAD and SAH cases and controls, the genotype and allele frequencies of *ACE I/D* polymorphism were similar in both ethnic groups. The *AGT 235TT* genotype and *235T* allele frequencies were higher in SAH cases (32%, 54.7%) versus controls in Caucasian-Brazilians (19.8%, 46.4%; $P = 0.038$, $P = 0.031$, respectively). The *AGT 235TT* (OR = 1.8; $P = 0.028$) demonstrated to be an independent factor risk in a multivariate logistic regression increasing SAH risk in Caucasians but not in African-Brazilians. In summary, *AGT M235T* polymorphism was associated with SAH risk in Caucasian-Brazilians, and no association was detected with CAD. No association was also observed in ACE I/D polymorphism either in CAD or SAH in African-Brazilians and Caucasian-Brazilians.

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Introduction

Systemic artery hypertension (SAH) and coronary artery disease (CAD) are some of the major problems of public health in Brazil and worldwide (Eriksson 1995). They are deeply associated; SAH leads to CAD, and less frequently, CAD gives origin to SAH (Dustan 1974). Those pathologies present multifactorial phenotypes determined by several genes, environmental factors and the interaction between them (Kato 2002).

The main pathological process of CAD is atherosclerosis, which is characterized by a chronic inflammation primarily

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due to the deposit of oxidized lipids on the inner surface of the artery wall (Fruchart *et al.* 2004).

Strong evidences correlate levels of systolic and diastolic pressure with higher probability of occurrence of ischemic heart disease, brain vascular disease and atherosclerosis (Kannel *et al.* 1971).

The studies addressing SAH pathogenesis highlight the renin–angiotensin–aldosterone system (RAAS) as a critical pathway for the control of blood pressure and renal functions (Danilczyk and Penninger 2006). This system plays an important role in the salt/water homeostasis, regulating the blood pressure and consequently presenting an intimate relationship with the development of hypertension. Once hypertension appears as a risk factor for CAD, the RAAS is also related to this pathology (Paillard *et al.* 1999).

When blood pressure decreases to values below those considered normal, the kidneys secrete the renin enzyme in the blood which converts the angiotensinogen (AGT) in the hormone angiotensin I. When this hormone passes by pulmonary vessels, it is quickly converted into another hormone, the angiotensin II, by angiotensin-converting enzyme (ACE) which stimulates aldosterone secretion (Laragh *et al.* 1972). Therefore, any dysfunction in this system causes a deregulation in blood pressure homeostasis.

The higher prevalence of SAH in African-Americans (Cooper and Rotimi 1997) and African-Brazilians (Santos *et al.* 2011) when compared to European ancestry populations, have raised speculations regarding the possibility of differences in the genetic basis of SAH among different ethnic groups (Caulfield *et al.* 1995).

Due to the aforementioned great relationship of RAAS with SAH, and consequently with CAD, polymorphisms in candidate genes of this system could confer susceptibility/protection to those diseases: the *AGT M235T* (rs699) and *ACE* insertion/deletion (I/D, rs4646994) have been analysed as possible genetic markers. Therefore, this investigation aimed to analyse if the frequencies of *AGT M235T* and *ACE I/D* polymorphisms are associated with angiographically assessed CAD and SAH, among different ethnic population groups (African-Brazilians and Caucasian-Brazilians).

Methods

Subjects

In this study, 714 subjects (266 African-Brazilians and 448 Caucasian-Brazilians) who underwent coronary angiography as described previously (Rios *et al.* 2007a, b, 2010) were investigated. They were referred to the angiography due to symptoms related to CAD, as major angina pectoris. These individuals were classified as CAD ($n = 459$) if they presented at least one obstructive lesion $\geq$50% and were considered controls ($n = 255$) if they did not show any obstructive lesion in the angiography. Patients with blood pressure $\geq$140/90 mmHg and/or those taking antihypertensive medication were classified as SAH ($n = 522$) or were considered controls ($n = 192$) if they did not present this phenotype. Among controls none presented acute myocardial infarction, cerebral vascular infarction or transient ischemic attack.

The patients provided a detailed health history and underwent physical examination. Diabetes mellitus was defined as fasting glucose level $\geq$126 mg/dL and/or patients taking antidiabetic medication. Smoking was selfreported as current or past smoking. African-Brazilians and Caucasian-Brazilians were classified by the skin pigmentation of the inner forearm and by morphological facial characteristics as previously reported (Azevédo 1980). All individuals provided written informed consent approved by the Hospital Ethics Committee.

Biochemical and DNA analyses

Blood samples were collected for DNA isolation and biochemical analysis from subjects with at least 12 h fasting. A standard salting out method was used for DNA isolation. The *AGT M235T* polymorphism was detected by polymerase chain reaction (PCR) and digested with *Tth111I* as described previously (Procopciuc *et al.* 2002). The *ECA I/D* polymorphism was detected by PCR amplification as reported (Gardemann *et al.* 1998). Total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride levels were measured by enzymatic methods using commercial kits (Wiener Lab, Rosario, Argentina) in an autoanalyser device. Low-density lipoprotein (LDL)-cholesterol levels were calculated using Friedewald formulae (Friedewald *et al*. 1972).

Statistical analysis

Allele frequencies were estimated by gene counting. The agreement of genotype frequencies with Hardy–Weinberg expectations was tested by a χ^2 goodness of fit test using Arlequin program ver. 2.000 (Schneider 2000). The Mann–Whitney U test was used to compare quantitative variables between groups. Allele and genotype frequency differences were compared among groups by Fisher's exact test using the Graphpad Prism ver. 5.0b. Other categorical variables were compared among groups by Pearson χ^2 test or Fisher's exact test when indicated using the PEPI program, ver. 4.0 (Abramson 2004). Odds ratio estimated and multivariate logistic regression were performed using the SPSS program ver. 10. A $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the patients and control samples

The clinical and biochemical parameters evaluated in the patients sample according to the presence and absence of CAD, grouped in African-Brazilians and Caucasian-Brazilians are provided in table 1. Patients with CAD were slightly older than the control individuals; however, they were in the same age range. Both African and Caucasian-Brazilian CAD cases were more frequently of male gender,

Table 1. Clinical and demographic characteristics of DAC cases and controls in African-Brazilians and Caucasian-Brazilians.

Variable	African-Brazilians			Caucasian-Brazilians		
	Case[a]	Control[a]	*P*	Case[a]	Control[a]	*P*
Number	153	113	–	306	142	–
Age (y.o.)	55.7±7.9	51.8±8.4	<0.001*	55.7±6.7	53.0±7.7	<0.001*
Male gender	64.5%	43.5%	0.001*	66.7%	45.7%	<0.001*
Diabetes mellitus	34.8%	13%	<0.001*	26.8%	7.2%	<0.001*
Hypertension	77.5%	72.2%	0.403	72.5%	63.8%	0.089
Smoking[b]	61.6%	54.8%	0.334	55.4%	40.1%	0.006*
Early CAD family history	30.4%	18.3%	0.037*	39.9%	31.2%	0.105
Total cholesterol (mg/dL)	199.2±61.8	186.8±46.7	0.065	183.2±48.2	188.6±42.2	0.153
LDL-cholesterol (mg/dL)	135.7±55.3	127.8±43.5	0.301	120.7±41.7	125.9±38.1	0.141
HDL-cholesterol (mg/dL)	29.3±8.2	31.2±9.2	0.113	27.6±8.0	30.9±7.9	<0.001*
Triglycerides (mg/dL)	170.8±108.5	138.8±82.5	0.001*	174.7±111.4	159.2±93.2	0.096
Cholesterol-lowering medication	29%	15.7%	0.018*	35.1%	18.8%	0.001*

[a]For categorical variables, the values express the % of affected individuals (the number of affected/nonaffected individuals).
[b]Current and past smoking.
*Statistically significant.

presented a higher prevalence of type 2 diabetes mellitus, and used cholesterol-lowering medication more often than their respective controls. A higher prevalence of smoking and lower HDL-cholesterol levels were observed in CAD cases when compared to controls in Caucasian but not African-Brazilians. On the other hand, a higher frequency of an early CAD family history and higher triglyceride levels were detected among CAD cases when compared to controls only in African-Brazilians. The prevalence of hypertension and the total cholesterol and LDL-cholesterol levels were similar among cases and controls in both ethnic groups.

Allele and genotype frequencies of the polymorphisms

Among CAD cases and controls, the genotype and allele frequencies of *ACE I/D* and *AGT M235T* polymorphisms were similar in both ethnic groups (table 2). The genotype and allele frequencies of *ACE I/D* polymorphism among SAH controls and cases were similar in both ethnic groups. However, the *AGT 235 TT* genotype was more frequent in cases (32%) compared to controls in Caucasian-Brazilians (19.8%; $P = 0.038$), as well as the *AGT 235 T* allele frequency was higher in cases (54.7%) when compared to controls in Caucasian-Brazilians (46.4%; $P = 0.031$) (table 3). The genotype frequencies of both polymorphisms agreed with those expected by the Hardy–Weinberg equilibrium (HWE).

Multivariate logistic regression and predictor risk

Multivariate logistic regression analyses were also performed to determine the most important CAD and SAH predictors in African-Brazilians and Caucasian-Brazilians. The *AGT 235 TT* (OR = 1.8; $P = 0.028$) increased SAH risk in Caucasian-Brazilians but not in African-Brazilians (table 4). This genotype association was independent from other SAH

Table 2. *AGT M235T* and *ACE I/D* frequencies in Caucasian-Brazilian and African-Brazilian in CAD cases and controls.

	African-Brazilians			Caucasian-Brazilians		
	Case (%)	Control (%)	*P*	Case (%)	Control (%)	*P*
AGT M235T						
MM	23 (15)	13 (11.5)		73 (23.9)	34 (23.9)	
MT	69 (45.1)	63 (55.8)		145 (47.4)	68 (47.9)	
TT	61 (39.9)	37 (32.7)	0.225	88 (28.8)	40 (28.2)	0.991
M	115 (37.6)	89 (39.4)		291 (47.6)	136 (47.9)	
T	191 (62.4)	137 (60.6)	0.718	321 (52.4)	148 (52.1)	0.943
ACE I/D						
I/I	19 (12.4)	14 (12.4)		34 (11.1)	16 (11.3)	
I/D	75 (49)	51 (45.1)		139 (45.4)	67 (47.2)	
D/D	59 (38.6)	48 (42.5)	0.796	133(43.5)	59 (41.5)	0.928
I	113 (36.9)	79 (35)		207 (33.8)	99 (34.9)	
D	193 (63.1)	147 (65)	0.649	405 (66.2)	185 (65.1)	0.763

Table 3. *AGT M235T* and *ACE I/D* frequencies in Caucasian-Brazilian and African-Brazilian in SAH cases and controls.

	African-Brazilians			Caucasian-Brazilians		
	Case (%)	Control (%)	*P*	Case (%)	Control (%)	*P*
AGT M235T						
MM	25 (12.5)	11 (16.7)		73 (22.7)	34 (27)	
MT	104 (52)	28 (42.4)		146 (45.3)	67 (53.2)	
TT	71 (35.5)	27 (40.9)	0.378	103 (32)	25 (19.8)	0.038*
M	154 (38)	50 (37.9)		292 (45.3)	135 (53.6)	
T	246 (61.5)	82 (62.1)	0.918	352 (54.7)	117 (46.4)	0.031*
ACE I/D						
I/I	22 (11)	11 (16.7)		39 (12.1)	11 (8.7)	
I/D	97 (48.5)	29 (43.9)		147 (45.7)	59 (46.8)	
D/D	81 (40.5)	26 (39.4)	0.468	136 (42.2)	56 (44.4)	0.589
I	141 (35.2)	51 (38.6)		225 (34.9)	81 (32.1)	
D	259 (64.8)	81 (61.4)	0.531	419 (65.1)	171 (67.9)	0.435

*Statistically significant.

Table 4. Multiple logistic regression for SAH risk among African-Brazilians and Caucasian-Brazilians.

	African-Brazilians			Caucasian-Brazilians		
Variable	OR	IC 95%	*P*	OR	IC 95%	*P*
Age (y.o.)	1.049	1.009–1.089	0.015*	1.034	1.002–1.067	0.038*
Male gender	0.249	0.122–0.507	<0.001*	0.581	0.360–0.939	0.027*
Diabetes mellitus	1.331	0.596–2.969	0.485	1.525	0.804–2.892	0.196
Smoking	0.889	0.468–1.689	0.720	0.499	0.319–0.782	0.002*
BMI[a]	1.082	0.996–1.175	0.061	1.121	1.058–1.189	<0.001*
CAD[b]	1.402	0.723–2.716	0.317	1.477	0.906–2.409	0.118
AGT 235TT genotype	0.729	0.391–1.360	0.321	1.800	1.065–3.041	0.028*

Variables included in the model: age, gender, diabetes mellitus, smoking (current and past smoking), BMI, CAD, *AGT 235TT* versus *MT+MM* genotypes.
*Statistically significant.
[a]BMI, body mass index: weight in kg divided by the square of the height in metres.
[b]Significant atherosclerotic lesion in coronary arteries by the coronary angiography test.

risk factors which remained significant disease predictors in the multivariate logistic analysis.

Discussion

CAD is associated with multiple risk factors, including obesity, diabetes, smoking, hypertension, poor diet and age ≥45 y.o. for men and ≥55 y.o. for women (Wilson *et al.* 1998; McPherson and Davies 2012; De Schutter *et al.* 2014). In this study, we show that diabetes, age, hyperlipidemia, smoking and CAD family history were associated with CAD sample in at least one of the two ethnic subgroups studied (Caucasian or African-Brazilians). Despite it is clearly known that those biochemical and clinical parameters are intimately associated with CAD, the molecular bases and risk factors of that pathology still lack to be understood. Therefore, in this study, we added information about the genetics background of CAD and SAH by evaluating the role of *AGT M235T* and *ACE I/D* polymorphisms in those pathologies, considering a special issue as a risk factor: the population stratification into Caucasian and African origin in Brazil.

AGT and ACE are the key components within the RAAS and studies have suggested that the activation of this system could be an important contributor to SAH and CAD (Re 2004; Gluba *et al.* 2009; Burrell *et al.* 2013). We could not observe any differences in genotype and allele frequencies for *ACE I/D* polymorphism in CAD or SAH for both populations evaluated in this study. Even though many reports agreed that the *ACE I/D* polymorphism was associated with CAD and/or SAH (Bautista *et al.* 2008; Mehri *et al.* 2012; Ellis *et al.* 2013; Guney *et al.* 2013; Moradzadegan *et al.* 2014), several other studies have failed to demonstrate an association between the *I/D* polymorphism and CAD/SAH in Indians (Pandey *et al.* 2011), Americans (Ned *et al.* 2012), Taiwaneses (Tsai *et al.* 2011) and African-Americans

(Martinez Cantarin *et al.* 2010). The *I/D* polymorphism, located within *ACE* gene (chromosome 17q23, intron 16) (Rigat *et al.* 1990), could be in linkage disequilibrium with another polymorphism or polymorphisms that directly contribute to CAD or SAH. The inconsistencies observed in different studies could be explained by differences in the genetic background among ethnicities and/or different environmental expositions.

We observed that there are significant differences in allele and genotype frequencies of *AGT M235T* polymorphism between Caucasian-Brazilians and African-Brazilians, with the *AGT 235MM* genotype and *AGT 235M* allele frequencies higher in Caucasians than in African-Brazilians (data not shown), demonstrating there is a difference in the genetic background between those ethnicities which became an important factor to be analysed in the studies. Rotimi *et al.* (1996) showed that the *TT* genotype in African-descendant populations is more frequent than in other ethnic groups (Rotimi *et al.* 1996).

When comparing cases versus controls, we suggested an association between the *AGT 235TT* genotype and *235T* allele and the SAH in Caucasian-Brazilians. Jeunemaitre *et al.* (1992) were the first to publish this association in Caucasians. Mehri *et al.* (2012) showed that Tunisian individuals carrying the TT genotype had an 1.67 ($P = 0.032$)-fold increased risk of essential hypertension (Mehri *et al.* 2012). However, there are many controversial results among the association when factors such as ethnicity and gender are considered. Associations between blood pressure deregulation and the *M235T* variant were not observed in Hispanic and Mongolian populations (Bautista *et al.* 2008; Ying *et al.* 2010), Algerian men (Meroufel *et al.* 2014), young Japanese (Miyama *et al.* 2007) and Caucasian women (Conen *et al.* 2008). This latter report, although had performed the study with an impressive large cohort, raises some limitations, such as, specificity of gender (only Caucasian female health professionals were studied), and the use of self-reported blood pressure and hypertension status.

The higher *AGT 235MM* frequency in the Caucasian-Brazilians did not work as a confounder effect on SAH risk in our study, since the *TT* genotype had increased the risk of SAH in Caucasian-Brazilians, but not in African-Brazilians in a multivariate analysis, demonstrating its relevance as a risk factor in this ethnic group. Other research publications involving Caucasian subjects reported this risk: Sethi *et al.* (2003) observed by a comprehensive meta-analysis that the *M235T* variant was associated with an increase in plasma levels of angiotensinogen in Caucasians with *MT* and *TT* genotypes, as well as a significant increase in risk of hypertension compared with *MM* homozygotes (Sethi *et al.* 2003). Further, Van den Born *et al.* (2007) described the increased risk to malignant hypertension in white subjects with the *TT* genotype when compared to hypertensive subjects, as well as slightly increased in hypertensive versus normotensive controls carrying this genotype (Van den Born *et al.* 2007).

The mechanisms underlying the association of *AGT M235T* and *ACE I/D* polymorphisms to the risk of hypertension and cardiovascular diseases are still unclear. We observed an increased risk of SAH in Caucasian-Brazilians carrying the *TT* genotype. Although *M235T* mutation is nonfunctional, Ellis *et al.* (2013) discussed that the higher concentrations of plasma AGT previously observed in *TT* homozygotes could be due to this polymorphism's strong linkage disequilibrium with a variant in the proximal promoter of AGT gene, which likely affect the interaction between at least one *trans*-acting nuclear factor and the promoter of the AGT gene (Ellis *et al.* 2013). Therefore, haplotypes analysis involving other AGT gene polymorphisms could give us more conclusive results about the relationship between that polymorphism and SAH.

In this study, we added the information that the *AGT M235T* polymorphism was associated with SAH risk in Caucasian-Brazilians, suggesting that polymorphism may have some relationship with the SAH pathology. In addition, we can speculate that differences in those frequencies could be attributed to linkage disequilibrium with other variants nearby *AGT* gene and this linkage could be dependent on ethnic background what could explain the ethnic difference in the effect on SAH detected here. However, additional studies, with a greater number of patients, haplotype analysis and comparison with angiotensinogen serum levels are required to confirm the genetic and functional associations.

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