Association of Angiotensinogen M235T and A(-6)G Gene Polymorphisms With Coronary Heart Disease With Independence of Essential Hypertension: The PROCAGENE Study

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OBJECTIVES

We examined the relationship between the angiotensinogen (AGT) gene M235T polymorphism, the variant promoter of the AGT gene A(-6)G and the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism and coronary heart disease (CHD) in native Gran Canaria Island habitants, who have the highest rates of CHD in Spain.

BACKGROUND

Some studies subject that the ACE (I/D) polymorphism could be associated with CHD, while AGT (M235T) has been related to essential hypertension.

METHODS

We studied 304 subjects with angiographic evidence of coronary artery disease and a clinical diagnosis of myocardial infarction or unstable angina and 315 age- and gender-matched controls. Blood was drawn and DNA extracted. Angiotensin-converting enzyme (I/D) gene polymorphism was analyzed by polymerase chain reaction (PCR) and AGT gene polymorphisms by restriction fragment length polymorphism-PCR and mutagenically-separated PCR.

RESULTS

The ACE (I/D) polymorphism showed no association with CHD, whereas the frequency distribution of AGT (M235T) genotypes among patients and controls (235T: 29.1% and 19.0%; M235T: 48.5% and 50.2%; M235: 22.4% and 30.8%, respectively) was statistically different (p = 0.005) and not related to the presence of essential hypertension. Similar results were observed with the AGT A(-6)G polymorphism. In multiple logistic regression analysis, CHD odds ratio associated with 235T and M235 homozygotes were 1.7 (1.1 to 2.6) and 0.54 (0.36 to 0.82), respectively.

CONCLUSIONS

This study shows that genetic variation of the AGT (M235T), but not the ACE (I/D), genotypes contributes to the presence of CHD independently of blood pressure profile in a subset of the Spanish population with a high prevalence of cardiovascular disease. (J Am Coll Cardiol 2001;37:1536–42) © 2001 by the American College of Cardiology

The World Health Organization estimates that six million people will die from cardiovascular diseases (CVD) in industrialized countries annually (1). While the role of risk factors (smoking, high blood pressure [BP] and cholesterol) for coronary heart disease (CHD) is acknowledged (2), emerging evidence suggests a contribution of genetic factors. Among various candidates, the genes coding for the proteins of the renin-angiotensin system may contribute to the evolution of CHD (3).

Identification of a polymorphism in the gene coding for the angiotensin-converting enzyme (ACE) gene involving an insertion (I) or deletion (D) on intron 16 (4) has been followed with considerable interest in genetic association studies. The D allele was associated with higher plasma levels of ACE (4). A retrospective multicenter case-control study showed that the frequency of the DD genotype was higher in subjects with myocardial infarction (MI) recruited between three and nine months after the event (5). Although subsequent studies did not confirm this finding, a meta-analysis (6) of the literature suggested an association between the D allele and ischemic heart disease.

The angiotensinogen (AGT) gene has been associated with heart disease progression. A polymorphism in exon 2 of the AGT gene, a threonine to methionine substitution at position 235 (M235T), has been associated with higher BP and higher AGT levels in populations of Caucasian (7,8) and African–Caribbean ancestry (9), as well as the syndrome of preeclampsia (10). Further investigation of the association of 235T homozygosity showed a link with increased risk of CHD (11). A molecular variant in the proximal promoter of the AGT gene, an adenine instead of a guanine, six nucleotides upstream from the site of transcription initiation, A(-6)G, has been reported. Studies of
binding between AGT promoter region oligonucleotides and nuclear proteins strongly suggest that substitution at nucleotide -6 affects specific interactions between at least one transacting nuclear factor and the promoter of AGT (12). Since A(-6)G is in very tight linkage disequilibrium with AGT (M235T) polymorphism could simply reflect the modifications in the promoter activity induced by nucleotide substitution at the -6 position. These observations provide a biological insight on possible mechanisms for a genetic predisposition to essential hypertension.

The Prospective Cardiac Gene (PROCAGENE) study is a large case-control study designed to examine the relationship between CVD risk factors, genetic markers in the renin-angiotensin system and the presence of CHD in individuals residing in Gran Canaria Island, Spain.

**METHODS**

**Study population.** The PROCAGENE study included 619 randomly selected persons (469 men and 150 women) aged 25 to 79 years, identified prospectively in participating centers in Gran Canaria Island (Canary Islands, Spain) between January 1996 and December 1998. All subjects completed the established protocol study. A case was defined as an individual of Canarian/Spanish ancestry admitted to any hospital in Gran Canaria with a diagnosis of MI or unstable angina and documented evidence of coronary artery disease by angiography. All consecutive incident cases admitted to the coronary unit were studied within the study period to minimize selection bias. Age and gender-matched controls were selected from the Gran Canaria population (total population 713,768). Controls were randomly ascertained by a two-stage stratified selection process. Cities in Gran Canaria were randomly selected according to population (total population 713,768). Controls were randomly selected according to population size (5,000; 5,000 to 19,999; 20,000 to 49,999; 50,000 to 99,999; >99,999 habitants), and potential controls were selected from each of the cities. These underwent diagnostic tests to exclude CVD.

Canarian/Spanish ancestry was defined as being born in the Canaries to Canarian parents or subjects of Spanish ancestry residing in the island for more than 10 years. The Canarian population is considered to be relatively homogeneous since it was in isolation until the seventies. Urban dwelling was defined as living in cities with more than 100,000 habitants. All participants were interviewed by a trained nurse who followed a standardized questionnaire to collect demographic, lifestyle, anthropometry and medication use. Smoking status was classified as current smoker or nonsmoker. Obesity was defined as a body mass index (BMI) \( \geq 26 \text{ kg/m}^2 \), a value that reflects the Canarian population BMI distribution.

Subjects underwent three supine BP measurements according to the criteria established by the American Heart Association using an Omron Hem 705CP semiautomatic device calibrated in accordance with the protocol described by the British Society of Hypertension (13). Blood pressure was measured in the right arm after subjects had voided, abstained from smoking for at least 15 min and rested for 5 min in the decubitus position. Triplicate measurements spaced 30 s apart were taken on the same arm by a trained nurse. Fasting blood and urine samples were obtained for genotyping, biochemistry, urinary creatinine and albumin excretion determinations.

The study was approved by our institutional research ethics committee, and informed consent was obtained from all participants.

**Laboratory methods.** **BIOCHEMICAL MEASUREMENTS.** Plasma glucose, serum creatinine, total cholesterol, high-density lipoprotein cholesterol and triglycerides were measured by enzymatic-colorimetric methods. Low-density lipoprotein cholesterol was calculated according to the Friedewald formula when triglyceride levels were \( \leq 400 \text{ mg/dl} \). Lipoprotein (a) (Lp [a]) was analyzed with an immunoturbidimetric method (Boehringer-Mannheim, Germany), and plasma homocysteine was measured by the Fluorescence Polarization Immunoassay (Abbott, Diagnostic Division, Chicago, Illinois), with an intraassay coefficient of variation of 1.9%. Microalbuminuria was measured by an immunoturbidimetric assay (Boehringer-Mannheim, Germany).

**GENOTYPING OF THE ACE (I/D) AND AGT (M235T) AND AC(-6)G POLYMORPHISMS.** DNA was extracted from leukocytes by standard procedures (14). The ACE (I/D) genotypes were determined according to previously described conditions (15) with minor modifications. The reaction was performed with 10 pmol of primers 5' CAG ACC ACT CCC ATC CTT TCT 3' and 5' CAT GTG GCC ATC ACA TTC GTC AGA T 3'. Because previous reports suggested that the D allele is preferentially amplified in heterozygotes (16), each sample expressing the ACE-DD genotype was subjected to a second independent polymerase chain reaction (PCR) amplification with insertion-specific primers (17): hace5a, 5' TGG GAC CAC AGC GCC CGC CAC TAC 3' and hace5c, 5' TCG CCA GCC CTC CCA TGC CCA TAA 3'. Data were corrected for this misclassification. We used an amplification protocol described...
previously (18), I/D and I/I samples as amplification controls.

The AGT (M235T) polymorphism was typed by the mismatch method previously described (8). Primers used were: AGT1, 5’ GAT GCG CAC AAG GTC CTG TC 3’ and AGT2, 5’ CAG GGT GCT GTC CAC ACT GGC TCG C 3’.

The AGT A(-6)G polymorphism was determined by mutagenically separated PCR as described by Morgan et al. (19) with minor modifications. In brief, three primers were used: 1) AGT-6MS5': 5’ GTG TCG CTT CTG AGA TCT GTC CTT CTG G 3’ a common forward primer; 2) AGT-6MS3’S: 5’ TAC CCA GAA CAA CGG CAG TCT CTT CCA CT 3’ for the detection of allele A(-6); and 3) AGT-6MS3’L: 5’ CCG GTT ACC TTC TGC TGT AGA GCC CAG AAC AAC GGC AGC TTC TCT GTC CTT CTG GCA 3’ for the detection of allele (-6)G.

Two pmol of AGT-6MS5’, 4 pmol of AGT-6MS3’S and 8 pmol of AGT-6MS3’L were used. Since a limited amount of DNA was obtained, we failed to analyze four subjects for ACE (I/D), five for AGT (M235T) and five for AGT A(-6)G polymorphisms.

Statistical analyses. The SPSS statistical software package (SPSS 8.0, Chicago, Illinois) was used for analysis of the data. A p value < 0.05 was considered statistically significant. All continuous variables are reported as mean ± SD. The unpaired t test was used for comparison of group's means. Frequencies of gene variants with coronary risk factors were evaluated by chi-square analysis. The Kolmogorov-Smirnov test was employed for estimation of the normal distribution of the variables. Mantel-Haenszel chi-square and odds ratios (OR) with 95% confidence intervals (CI) analysis were carried out to estimate the risk of CHD associated with continuous variables (age < 50 or ≥ 50 years old; BMI < 26 or ≥ 26; alcohol consumption < 30 or ≥ 30 g/day; systolic BP < 140 or ≥ 140 mm Hg; diastolic BP < 90 or ≥ 90 mm Hg; cholesterol ≤ 200 or > 200 mg/dl; high-density lipoprotein cholesterol ≤ 65 or > 65 mg/dl; low-density lipoprotein cholesterol ≥ 160 or > 160 mg/dl; triglycerides ≤ 150 or > 150 mg/dl; total cholesterol/high-density lipoprotein cholesterol ratio ≤ 5 or > 5; Lp (a) ≤ 30 or > 30 mg/dl; glucose ≤ 126 or > 126 mg/dl; creatinine < 1 or ≥ 1 mg/dl; homocysteine ≤ 15 or > 15 μmol/L; microalbuminuria < 30 or ≥ 30 mg/g of creatinine) and the categorical variables: gender, urban/rural life, smoking, sedentary habit, diabetes and hypertension.

The Hardy-Weinberg equilibrium for the frequencies of the ACE and AGT genotypes was tested by chi-square analysis. Odds ratios and 95% CI were calculated to estimate the relative risks of CHD associated with ACE (I/D), AGT (M235T) and AGT A(-6)G polymorphisms.

To assess the independent variable predictor ability for CHD, we performed multiple logistic regression analysis with the backward stepwise method. The contribution of analyzed genotypes and reported CHD risk factors in predicting the dependent variable (coronary event) when all other independent variables were allowed for was expressed as an OR (e^b) with 95% CIs (e^b ± 1.96 × SE). The model included the classical risk factors that attained statistical significance in the univariate analysis (urban/rural dwelling, smoking, alcohol intake, diabetes, hypertension, glucemia, Lp (a), high-density lipoprotein cholesterol, total cholesterol/high-density lipoprotein cholesterol ratio) and ACE and AGT genotypes. All the variables were transformed in dummy variables, taking the value 1 for the presence of the characteristic and 0 for its absence. Taking a cut-off point of 50%, the established model classified correctly 71% of individuals.

RESULTS

Study patients, lifestyle and cardiovascular risk factors. Three hundred and four cases (mean age 56 ± 10 years, 22% women) and 315 randomly selected age- and gender-matched community controls (mean age 54 ± 10 years, 26% women) were included in the study. Table 1 shows the main characteristics of our case and control groups. Hypertension (chi-square = 13.84; p < 0.001), diabetes (chi-square = 41.86; p < 0.001), smoking (chi-square = 33.67; p < 0.001) and urban habitat (chi-square = 7.13; p = 0.008) were significantly higher in the group of patients compared with the group of controls. The control group presented higher levels of high-density lipoprotein cholesterol (p < 0.001).

Cases and controls did not differ significantly with regard to BMI, systolic BP and microalbuminuria. However, diastolic BP values, total cholesterol, triglycerides and low-density lipoprotein cholesterol were lower in cases than they were in controls, most likely due to a higher frequency of treatment with antihypertensive and lipid-lowering agents among cases. Patients with a diagnosis of coronary artery disease showed a significant increase in total cholesterol/high-density lipoprotein cholesterol ratio and plasma Lp (a) (t = 7.68 and t = 3.63, respectively, both p < 0.001). Paradoxically, plasma homocysteine levels were higher in controls compared with cases, albeit within normal range for both groups.

Table 1. Main Clinical Characteristics of Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 304)</th>
<th>Controls (n = 315)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76 ± 13</td>
<td>84 ± 12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>16 ± 27</td>
<td>11 ± 19</td>
<td>0.012</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>35 ± 9</td>
<td>50 ± 12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total chol./HDL-ratio</td>
<td>5.9 ± 1.7</td>
<td>4.9 ± 1.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>14.5 ± 5.2</td>
<td>15.7 ± 7.3</td>
<td>0.029</td>
</tr>
<tr>
<td>Lp (a) (mg/dl)</td>
<td>37 ± 43</td>
<td>37 ± 43</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>112 ± 50</td>
<td>105 ± 28</td>
<td>0.041</td>
</tr>
</tbody>
</table>

BP = blood pressure; chol. = cholesterol; HDL = high-density lipoprotein; Lp(a) = lipoprotein (a).
Distribution of ACE and AGT genotypes among cases and controls. For each polymorphism ACE (I/D), AGT (M235T) and AGT A(-6)G, we confirmed that the genotype proportions fit the Hardy-Weinberg equilibrium estimated by chi-square test. The ACE-DD genotype frequency was higher in our population when compared with those described for other Caucasian populations (17,20–23), probably because of ethnicity, although this genotype was at nonsignificant increased frequency among CHD patients. Examination of this distribution showed a significant difference in the AGT (M235T) polymorphism (chi-square = 10.59; p = 0.005) and A(-6)G (chi-square = 8.09; p = 0.01) among patients and controls (Table 2). The frequency of 235T and A(-6) homozygotes was higher (chi-square = 8.50; p = 0.004 and chi-square = 6.78; p = 0.009) in cases than it was in controls. The allele frequencies were p (T) = 0.53 and 0.44, p (A) = 0.54 and 0.46 for patients and controls, respectively.

The molecular variant in the proximal promoter of the AGT gene, A(-6)G, was in tight linkage disequilibrium with M235T, consistent with previously reported results (12). G and A alleles were closely associated with M and T alleles, respectively, as revealed by the haplotype frequencies in case (G-M: 0.449, G-T: 0.010, A-T: 0.522 and A-M: 0.019) and control (G-M: 0.533, G-T: 0.005, A-T: 0.438 and A-M: 0.024) populations.

The OR for CHD among individuals with the analyzed genotypes is depicted in Figure 1. The analyses did not show a statistically significant difference for the ACE (I/D) polymorphism between cases and controls. The CHD OR associated with 235T homozygosity was 1.74 (CI: 1.20 to 2.54; p = 0.004) and 1.63 (CI: 1.22 to 2.35; p = 0.009) for A(-6) homozygotes. The ORs for M235 and (-6)G homozygosity were 0.64 (CI: 0.45 to 0.93; p = 0.01) and 0.69 (CI: 0.48 to 1.00; p = 0.05), respectively. No differences between patients and controls were observed in heterozygote subjects (Fig. 1A). Finally, we analyzed the association of ACE (I/D) and AGT (M235T) as well as ACE (I/D) and AGT A(-6)G genotype combinations with CHD risk (Fig. 1B). The CHD OR associated with AGT-235T and with AGT A(-6) was 2.06 (CI: 1.20 to 3.51; p = 0.001) and 1.8 (CI: 1.07 to 3.02; p = 0.02), respectively, among subjects with ACE-I/D.

Since AGT (M235T) has been associated with arterial hypertension in previous analyses, we studied the distribution of this gene polymorphism in the hypertensive and normotensive groups of our population. As shown in Table 3, no statistically significant differences in the genotype distribution between hypertensive subjects and normotensive subjects were observed, both in case and control groups (β = 0.004). Thus, genotype distribution differences between cases and controls for hypertensive and normotensive groups were the same as that previously determined for the total population.

On multiple logistic regression analysis and in agreement with the previous data, we found an association between CHD and the homozygotes for AGT (M235T) polymorphism (Table 4). On the other hand, we determined an OR = 1.5 (CI: 0.98 to 2.3; p = 0.06) for the AGT A(-6) homozygote and OR = 0.6 (CI: 0.39 to 0.91; p = 0.02) for the AGT (-6)G homozygote. Of interest is that analysis of genotype combinations revealed an enhanced protection for CHD in those subjects with AGT-M235 + ACE-II (OR = 0.24; CI: 0.07 to 0.77; p = 0.02) or AGT (-6)G + ACE-II (OR = 0.24; CI: 0.07 to 0.81; p = 0.02).

**DISCUSSION**

The principles of genetic approaches to complex diseases have been reviewed (24). The field is complicated by issues related to sample size, definition of ethnicity, publication bias toward positive results and inaccurate definition of disease phenotypes (6,25). The PROCAGENE study is based on clear characterization of the phenotype of ischemic heart disease in an isolated Spanish subpopulation with the highest rate of CHD (26) in Spain. The study design minimized potential confounding by differences in ethnicity by limiting, by design, the inclusion of individuals from other geographical regions.

**ACE (I/D) polymorphism in CHD.** In contrast with other studies (5,6,27,28), our results show no evidence of an association between ACE polymorphisms and CHD in the population of Gran Canaria Island. Consistent with previously reported data, we found a trend towards a decrease in CHD of those subjects with ACE-II (OR = 0.70), although this did not reach statistical difference, probably reflecting a lower frequency of this genotype (17). Cambien et al. (5) first described an association between the DD genotype of the ACE gene polymorphism and MI. Other studies found an association with CHD (29), MI (30), hypertrophic cardiomyopathy (31), coronary artery restenosis (32), dilated cardiomyopathy (33) and parental history of MI (27). However, conclusions derived from these studies remain controversial due to the lack of association between
DD genotype and CHD in other reports (6,17,34). These findings stress the necessity of considering ethnic factors in the assessment of genetic risk identifiers.

**AGT polymorphisms in CHD.** Angiotensinogen-235T was present in 19% of our control population compared with 15% for individuals in Western populations (7). Our results provide strong evidence of an association between the AGT gene and the risk for CHD. In keeping with other studies (11), homozygosity for 235T was associated with increased risk for CHD (unadjusted OR = 1.74), while the M235 homozygote seems to be associated with a decreased risk for CHD (OR = 0.64). The OR of 235T homozygosity for CHD was similar in the multivariate analyses without BP or other variables overlapping its predictor information. Similar results were found with the molecular variant localized in the proximal promoter of the AGT. It has been suggested in previous studies that the T allele might be associated with the cardiovascular complications of hypertension. Angiotensinogen-235T homozygosity was associated with essential hypertension in Japanese populations (35) and with controversy in white Europeans (7,8). Although the association studies suggest a role of the AGT gene, the linkage of the AGT gene and essential hypertension first reported by Jeunemaitre et al. (7) has not been replicated in others.

**Table 3.** AGT (M235T) Genotype Distribution in Hypertensives and Normotensives

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Cases n (%)</th>
<th>Controls n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypertensives</td>
<td>Normotensives</td>
</tr>
<tr>
<td></td>
<td>[Chi-Square = 0.33; p = 0.85]</td>
<td>[Chi-Square = 0.47; p = 0.79]</td>
</tr>
<tr>
<td>AGT-235T</td>
<td>42 (29.4)</td>
<td>45 (28.8)</td>
</tr>
<tr>
<td>AGT-M235T</td>
<td>71 (49.7)</td>
<td>74 (47.4)</td>
</tr>
<tr>
<td>AGT-M235</td>
<td>31 (21.0)</td>
<td>37 (23.7)</td>
</tr>
<tr>
<td>Totals:</td>
<td>143</td>
<td>156</td>
</tr>
</tbody>
</table>
Table 4. Estimates of Risk of CHD Associated With Clinical, Biochemical and Angiotensinogen Genotypes (Adjusted for Multiple Risk Factors)

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>4.3 (2.7 to 7.0)</td>
</tr>
<tr>
<td>Smoking</td>
<td>2.9 (1.9 to 4.3)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.9 (1.3 to 2.8)</td>
</tr>
<tr>
<td>AGT-235T homozygotes</td>
<td>1.7 (1.1 to 2.6)</td>
</tr>
<tr>
<td>Lp (a) &gt;30 mg/dl</td>
<td>1.7 (1.07 to 2.2)</td>
</tr>
<tr>
<td>Urban habitat</td>
<td>1.7 (1.1 to 2.5)</td>
</tr>
<tr>
<td>Total chol/HDL-chol. ratio &gt;5</td>
<td>1.5 (1.1 to 2.0)</td>
</tr>
<tr>
<td>AGT-M235T homozygotes</td>
<td>0.54 (0.36 to 0.82)</td>
</tr>
<tr>
<td>HDL-cholesterol &gt;65 mg/dl</td>
<td>0.07 (0.01 to 0.33)</td>
</tr>
</tbody>
</table>

AGT = angiotensinogen; chol. = cholesterol; CI = confidence interval; HDL = high-density lipoprotein; Lp(a) = lipoprotein (a); OR = odds ratio.

Conclusions. Our data provide evidence of a two-fold increased risk for CHD in subjects with the AGT-235T genotype, while AGT-M235 is associated with a reduction in risk, independently of BP. Whether AGT (M235T) polymorphism mediates CHD related effects or acts as a marker of other gene polymorphisms as AGT A(-6)G needs further evaluation.

APPENDIX

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