Threonines at position 174 and 235 of the angiotensinogen polypeptide chain are related to familial history of hypertension in a Spanish-Mediterranean population

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ABSTRACT

This study investigates the association between the allelic distribution of two polymorphisms of the angiotensinogen (AGT) gene (T174M and M235T in the polypeptide chain) and blood pressure (BP) in a Mediterranean population in the south-west of Europe. The sample consists of 1322 participants from urban and rural areas, from the province of Albacete (218 462 inhabitants), located in the south-east of Spain. The subsample of this study, adjusted by age (over 18 years old) and sex, consists of 401 individuals. A case-control study is conducted which analyses 205 individuals from the group with the highest BP (fifth quintile) and 196 from the group with the lowest BP (first quintile). In addition, a comparative and associated analysis of these polymorphisms with BP level and family history of hypertension is carried out. The T174 allele proved to be more common in the fifth quintile group, although not statistically so. When the presence of threonine was analysed in both polymorphism positions (174 and 235), the TTTT genotype was found to be more common in the fifth quintile than in the first quintile. Moreover, the TTTT genotype was significantly more common in individuals with a family history of hypertension, indicating that it could be considered a predisposing factor to high BP in individuals from such families. In addition, the T174M-T235T genotype was more common in the first quintile group, and showed significant association ($P=0.05$) with the group that had no family history of hypertension.

KEY WORDS: Angiotensinogen. Blood pressure. Hypertension. Polymorphism (genetics).

Introduction

Blood pressure is regulated or at least influenced by many genetic, gene-gene, gene-environment and behavioural factors. Elevated arterial blood pressure (hypertension) is one of the principal risk factors for cardiovascular disease. The genetic basis of hypertension is complex. Certain monogenic hypertensive syndromes have been described (glucocorticoid-remediable aldosteronism, apparent mineralocorticoid excess and Liddle syndrome); however, essential hypertension, in which no underlying cause has been identified, is a polygenic disease that has not been solved at the genetic level so far.[1-3] More than 50 different genes have been implicated as important for the regulation of blood pressure (BP), coding for ion channels, enzymes (e.g. nitric oxide synthase) and diverse peptides.[4]

The renin-angiotensin system (RAS) is the principal mediator of vasoconstriction, sodium retention and cellular proliferation, and is thought to play an important role in the regulation of BP. Angiotensinogen (AGT), the first step of the cascade, serves as the substrate for the generation of angiotensin I (AI) by renin, a carboxyl-protease that cleaves the Leu[10]-Leu[11] bond in AGT. Then, AI is converted to angiotensin II (AII) by angiotensin I-converting enzyme (ACE). AII stimulates the secretion of aldosterone and exerts a profound pressor and antinatriuretic effect. As the plasma concentration of AGT is close to the Km of renin for this reaction, it has been suggested that AGT might be as important as renin in the determination of the rate of generation of AI.[5] Epidemiological surveys have shown a correlation between plasma AGT level and BP.[6]

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The *AGT* gene is located on chromosome arm 1q42-43 and comprises five exons and four introns.[7,8] A C ⇐ T transition at nucleotide 521 in exon 2 causes a sense mutation at amino acid position 174, changing threonine to methionine, and a T ⇐ C transition at nucleotide 704 causes a sense mutation at amino acid 235, changing methionine to threonine.[9]

In 1992, a large study of French and US (Utah) families showed a significant allele frequency difference between hypertensive subjects and unrelated controls for the two substitutive polymorphisms of the angiotensinogen gene (174 [T174M] and 235 [M235T]).[9] Subsequently, a number of

Table 1. Age, sex, systolic blood pressure, diastolic blood pressure, body mass index and waist/hip ratio: mean (standard deviation) and percentages of the two quintiles of the study

	Age (years)	Sex (% male)	SBP (mm Hg)	DBP (mm Hg)	BMI	W/H ratio
1st quintile	50.4 (17.8)	44.7	117.3 (16.5)	72.1 (10)	26.3 (4.3)	0.85 (0.09)
5th quintile	51.6 (17.9)	47.9	138* (21.4)	79.8* (12.1)	29.1* (5)	0.89* (0.08)

* $P<0.001$ between groups.

SBP: systolic BP; DBP: diastolic BP; BMI: body mass index and W/H ratio: waist/hip ratio.

Table 2. AGT genotype distribution and allele frequencies of the M235T and T174M polymorphisms in the first and fifth quintiles of the studied population

	Genotypes – No (%)						Alleles – No (Freq)			
	M235M	M235T	T235T	M174M	M174T	T174T	M235	235T	174M	T174
1st quintile (n=196)	53 (27)	102 (52)	41 (21)	3 (1.5)	51 (26.1)	142 (72.4)	208 (0.531)	184 (0.469)	57 (0.145)	335 (0.855)
5th quintile (n=205)	64 (31)	104 (51)	37 (18)	1 (0.5)	40 (19.5)	164 (80)	232 (0.566)	178 (0.434)	42 (0.102)	368 (0.898)
	P=0.56 (NS)						P=0.316 (NS)			

NS: No significant difference.

case-control studies have been performed around the world, the results of which have been contradictory. Some report negative results[10-19] but most reveal a significant relationship between hypertension and the M235T variant of the angiotensinogen gene.[9,20-27] Other studies have found a significant association between the 235T allele and a higher plasma concentration of angiotensinogen.[9,28-31]

Such contradictory reports emphasise the importance of using homologous populations. Environmental and behavioural factors affecting individual, family, population or species-wide genetic variants play an important role in quantitative traits, such as BP, and they must be taken into account in order to understand how molecular biology influences hypertension.

In the present study, we investigate the distribution of the two polymorphisms of the angiotensinogen gene (T174M and M235T) in a Mediterranean population, with a characteristic diet and well-defined social habits,[32] and whether or not they were associated with high BP. The predicted secondary structures of AGT polypeptide polymorphisms reveal changes in the proportion of β-sheet conformation, and we hypothesise that secondary structure of AGT influences processing efficiency, plasma levels of AGT and thus BP.

Materials and methods

Study subjects

A cross-sectional study of the general population of the province of Albacete, south-east Spain (population: 218 462 over 18 years of age), with similar demographic characteristics to the rest of the country, was carried out in order to establish the prevalence of different cardiovascular risk factors. All those included in the sample were over 18 years of age.

The sample size was calculated on the basis of a previous survey in which the risk factor with minor expected prevalence was peripheral artery disease (1.4%).[33] To obtain a confidence interval of 0.9% to 1.9% for the expected prevalence of peripheral artery disease, a total of 2121 participants was required; however, the final sample comprised 1322 persons due to the fact that the study looked at the general population and not patients. Nonetheless, the reduction in numbers did not imply a loss of statistical significance.

The random stratified sample was examined in two phases with sample sizes proportional to the sizes of the local population (i.e., 40% participants were from the capital [urban area], 23.1% from towns of more than 10 000 inhabitants [small urban areas], and 36,9% from towns and villages of less then 10 000 inhabitants [rural areas]). During the first phase of the study, participants in each group were selected at random from 22 population zones; in the second phase, participants were selected by systematic random sampling and then contacted.

Baseline information was collected by previously designed questionnaires,[34] and included personal and family history of cardiovascular disease (family history defined as a father, mother or siblings with hypertension), and risk factors such as tobacco use, stress, sedentary life, etc.

After completion of the questionnaires, the patient's weight, height and BP were recorded. Blood pressure was measured following the recommendations of the British Society of Hypertension, in the sitting position after 15 min rest using a conventional mercury sphygmomanometer. The

Table 3. AGT genotype distribution of the T174T-T235T and T174M-T235T polymorphisms in the first and fifth quintiles of the studied population

	T174T-T235T	Other genotypes*	T174M-T235T	Other genotypes**
1st quintile (n=196)	17 (8.7)	179 (91.3)	21 (10.7)	175 (89.3)
5th quintile (n=205)	24 (11.7)	181 (88.3)	12 (5.9)	193 (94.1)
	P=316 (NS)		P=0.148 (NS)	

NS: No significant difference.

*T174T-M235M, T174T-M235T, T174M-M235T, T174M-T235T and M174M-T235T.

**T174T-T235T, T174T-M235M, T174T-M235T, T174M-M235T and M174M-T235T.

Genotypes M174M-M235M, T174M-M235M and M174M-M235T were not found in the studied population.

Table 4. AGT genotype distribution of the T174T-T235T and T174M-T235T polymorphisms according to familial history of hypertension

	T174T-T235T	Other genotypes*	T174M-T235T	Other genotypes**
No familial history of HTN (n=294)	24 (8.2)	270 (91.8)	27 (9.2)	267 (90.9)
No familial history of HTN (n=107)	17 (15.9)	90 (84.1)	6 (5.6)	101 (94.4)
	P=0.024 (SD)		P=0.05072 (SD)	

SD: Significant difference.

HTN: hypertension.

*T174T-M235M, T174T-M235T, T174M-M235T, T174M-T235T and M174M-T235T.

**T174T-T235T, T174T-M235M, T174T-M235T, T174M-M235T and M174M-T235T.

Genotypes M174M-M235M, T174M-M235M and M174M-M235T were not found in the studied population.

lower of two measurements taken 5 min apart was selected for the study. Systolic and diastolic BP were defined as the first and fifth Korotkoff sounds, respectively. Seven trained observers who had passed both a certification test with a double-headed stethoscope and a video test conducted the study. Certification was repeated every three months.

For this study, a subsample, adjusted by age (decades) and sex, of 205 participants with the highest BP values (fifth quintile group: 138/79.8 mm Hg) were compared with 196 participants with the lowest BP values (first quintile group: 117.3/72.1 mm Hg), establishing a case-control study from the cross-sectional survey described previously. The decrease in the theoretical number of individuals required (264) for each quintile was due to the nature of the population studied (Table 1).

In the two subsample groups, two genetic variants (T174M and M235T) of the angiotensinogen gene were studied by polymerase chain reaction (PCR), combined with restriction analysis of the PCR product.

Experimental procedures

For genotype analysis, genomic DNA was extracted using a technique previously reported.[35] The primers used for AGT amplification were as follows:

Primer A (sense): 5′-GATGCGCACAAGGTCCTGTC-3′; and,

Primer B (antisense): a 40-bp GC-clamp attached 5′ -CGCCCGCCCCGCCCGCCGCCCGCCCCGCCCGCCGC CGCTGCTGTCCACACTGGCTCGC-3′, as described by Rutledge *et al*.[36]

The PCR product was 338 bp long. The 60-bp length of primer B was designed to ensure visualisation of the fragments by agarose electrophoresis, after digestion with the NcoI and BstUI restriction enzymes.

The two point mutations in nucleotides +521 (T174M) and +704 (M235T), both in the second exon of the gene, were detected using restriction analysis of a mispairing PCR product.[36]

A naturally occurring NcoI restriction site was present in codon 174 only in the mutated genotype (M). The M235T mutation was detected using a BstUI restriction site that was created by the mispairing primer method used in the PCR reaction. The digested fragments were visualised by electrophoresis on a 2% agarose gel, stained with ethidium bromide.

The PCR reaction was performed using 2.5 units *Thermus aquaticus* (*Taq*) polymerase (Perking-Elmer Cetus), 200-400 ng DNA template, 40 pmol of each primer, 125 µmol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L Tris HCL (pH 8,3), 1.5 mmol/L $MgCl_2$ and 0.01% (w/v) gelatine. The reaction comprised 35 cycles (94°C denaturation for 30 sec, 64°C annealing for 30 sec and a 72°C extension for 2 min). Finally, the reaction was maintained at 72°C for 10 min to allow the

Taq polymerase to complete any extension.

Samples (30 µL) of the PCR reaction product were used for the NcoI and BstUI (New England Biolabs) digestions, performed at 37°C for NcoI and 60°C for BstUI, for 2 h. The mispairing primer (primer B) created the BstUI restriction site only in the mutated form of position 235 (235T).

Protein secondary structure prediction was performed using the Frishman multi-layered method programme from the Skirball Institute of Biomolecular Medicine (http://saturn.med.nyu.edu/searching/Sspred/queryss.html).

Statistical analysis

Differences between variables in the first and fifth quintiles were examined by Student's *t*-test and χ^2 statistics. Statistical significance was indicated if computed two-tailed probability value was less than 5% ($P<0.05$). The SPSS PC+ computer programme (Statcalc/Epinfo 5.1) was used for statistical analysis. The Kolmogorov-Smirnov test was used to verify that the data were normally distributed.

Results

In this study, allelic frequencies were in Hardy-Weinberg equilibrium for the AGT polymorphisms. When compared individually, distribution of each genotype polymorphism in the two groups did not show a significant relationship between M235T variant and BP (Table 2).

The presence of both polymorphisms (174 and 235) in each individual was studied in order to analyse the relationship between the amino acid substitutions in the preangiotensinogen polypeptide and BP value. The frequency of the T174T-T235T genotype in the fifth quintile was higher than in the group with lower BP (Table 3), although the difference was not statistically significant. The frequency of the T174M-T235T genotype was higher in the first quintile group, but not statistically significant (Table 3). Of the nine genotype combinations possible, M174M-M235M, T174M-M235M and M174M-M235T were not found in the population studied.

The relationship between angiotensinogen genotype and family history of hypertension was also analysed, and a significant association was found with the T174T-T235T genotype (Table 4), indicating that this could be considered a predisposing factor for the development of high BP in individuals with a family history of hypertension.

The prevalence of the T174M-T235T genotype in the first quintile group was near significance in the group with a family history of hypertension (Table 4) and significantly associated ($P=0.05$) with the group of individuals with no familial history of hypertension.

A significant difference between body mass index (BMI) and waist to hip (W/H) ratio and the first and fifth quintiles ($P<0.001$) was observed (Table 1).

Discussion

Angiotensinogen is the first step of the renin-angiotensin cascade system and therefore study of genetic variation in this polypeptide is a subject of interest. Of the genes associated with the renin-angiotensin system studied, researchers have found a frequent relationship between the *AGT* gene structure and BP.

In 1992, a pioneering investigation of *AGT* gene variants in 215 sibships from American and French populations reported an association between high BP and two of the 15 polymorphisms studied. These two polymorphisms corresponded to sequences present in the second exon of the gene and were named T174M and M235T.[9] Subsequently, a number of population groups have been tested for these two variants, most of which showed a positive relationship between the 235T variant and hypertension.[20-27,30] However, other studies performed in different groups did not find any relationship between these two variables.[10-19] In the case of variants in position174, the association with BP is not so high.[9,37,38]

To our knowledge, this is the first analysis of the relationship between the two AGT polymorphisms and BP, and this approach was taken because we estimate that it has biological significance to the structure-function relationship. The deduced amino acid sequence from the cloned complementary DNA (cDNA) showed that the angiotensinogen molecule consists of 452 amino acid residues with the angiotensin II sequence at its amino terminal portion.[7,39] Comparison of the predicted secondary structures of the angiotensinogen polypeptide polymorphisms shows that the region containing amino acid position 174 increased the proportion of β-sheet conformation in the T174 variant. In the case of position 235, a β region characterises the 235T variant (Figure 1) but is not present in the M235 variant.

Although speculative, we propose that this change in the protein conformation of angiotensinogen (452 amino acids) could influence its cleavage by renin to produce the decapeptide angiotensin I. The remaining 442 amino acids in AGT have no known function;[4] however, this secondary structure could influence processing efficiency and thus plasma levels of AGT.

In support of this hypothesis, a strong association between the 235T variant and high plasma AGT level has been reported.[9,28-31] In addition, Cohen and co-workers[40] suggested that the 235T variant triggers a conformational modification of the angiotensinogen molecule that can be detected by specific monoclonal antibodies.

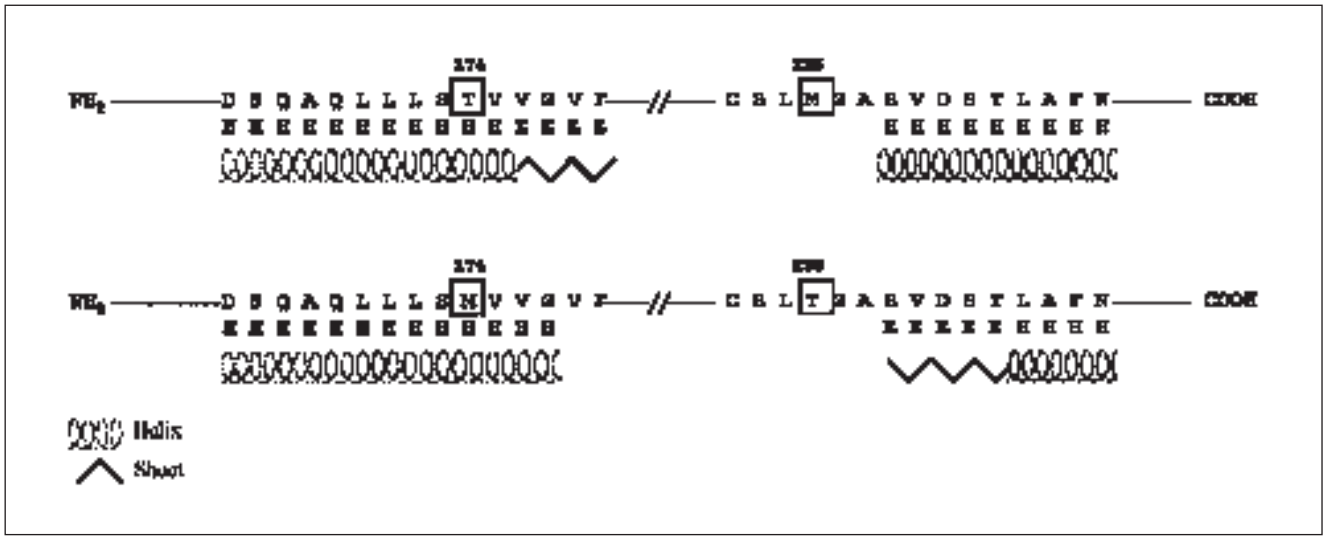
The association between TTTT genotype and a family history of hypertension suggests that this genotype could predispose to high BP values. The presence of threonine at position 174 of the AGT polypeptide chain is associated with a family history of hypertension, whereas the presence of methionine is not.

It is not unexpected that a population with higher BMI and W/H ratio should also have a higher BP. Aspects such as weight, skinfold thickness, BMI and W/H ratio frequently are found to be positively associated with BP.[41-42]

A variety of genetic and environmental factors contribute to variation in BP, even in an individual. We believe that body characteristics could have had a significant impact upon our study, perhaps even more so than the genetic determinants studied. It must be remembered that BP is likely to be a polygenic character that results from the inheritance of a number of genes, including those that influence phenotypic quantitative traits.

It has been reported that age could modulate the influence of AGT genetic variation on BP levels; however, no significant association between BP and the AGT genotypes or allele frequencies studied was found in the present study

Fig. 1. Secondary structure prediction of AGT 174 and 235 polymorphisms produced by the Frishman multi-layered method.



in participants who were less than 50 years of age.

Disagreements with the results of other Caucasian population studies emphasises the importance of using homologous populations when comparing AGT gene variants. Such studies could contribute to the production of a map of the distribution of AGT allelic frequencies and the plausible influence of genetic and epigenetic factors on BP.

Finally, one can speculate, for example, that a Mediterranean-type diet and social habits could play a role in minimising any possible genetic effect on BP. As Willett and co-workers wrote: 'The Mediterranean diet constitutes a centuries-old tradition that contributes to excellent health, provides a sense of pleasure and well-being'.[32] □

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