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Genotype – phenotype analysis of angiotensinogen polymorphisms and essential hypertension: the importance of haplotypes

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Abstract

Objectives—To better understand the relationship between angiotensinogen (*AGT*) genetic variation and essential hypertension, *AGT* genotypes and haplotypes were tested for association with hypertensive endophenotypes and essential hypertension.

Methods—256 HyperPATH/SCOR cases and 126 controls were genotyped for 24 SNPs in the *AGT* gene. SNPs and *AGT* haplotypes were tested for association with plasma *AGT*, renal plasma flow, and essential hypertension.

Results—New associations between essential hypertension, plasma *AGT*, and renal plasma flow (RPF) are reported for alleles –1178G, 6066A, 6152A, 6233C, and 12822C. The maximum odds ratio for association of hypertension and *AGT* genetic variation was 2.3 (95% CI 1.5 – 3.8; $p < 0.0003$) for allele 6233C. Previous associations for –1074T, –532T, –217A, –6A, and 4072C are confirmed ($p < 0.05$). Sodium depletion enhances associations between *AGT* SNPs and plasma *AGT*. Most individually associated SNPs, including –6A and 4072C, are found on a common complete *AGT* haplotype, H4 (frequency = 0.09). Individuals with haplotype H4 have significantly higher plasma *AGT* and reduced renal plasma flow ($p < 0.003$ and $p < 0.0002$, respectively). Other common haplotypes are not associated with plasma *AGT* levels in this data set despite the presence of the –6A and 4072C alleles, suggesting that *AGT* haplotype H4 is more predictive of elevated plasma *AGT* than is –6A or 4072C.

Conclusions—This study demonstrates the importance of analyzing haplotypes in addition to single genotypes in association studies. By demonstrating the dependence of *AGT* associations on sodium depletion status, it helps to explain previous conflicting association results.

Keywords

Angiotensinogen; essential hypertension; association study; haplotype

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Conflict of Interests The authors have no conflicts of interest to declare.

Supplemental files Genotype and phenotype data are provided in Supplementary File 1.

Introduction

Linkage studies, animal models, and pharmacological data show that the Renin-Angiotensin-Aldosterone System (RAAS) plays a key role in the regulation of blood pressure [1–3]. Angiotensinogen (AGT) is initially cleaved by renin to produce angiotensin I, which is rapidly processed by angiotensin converting enzyme (ACE) to produce the powerful octapeptide vasopressor, angiotensin II. Blood pressure and volume are normally regulated through changes in circulating renin concentrations [4]. Plasma AGT concentrations, however, are lower than the K_m for renin, implying that increases in plasma AGT can increase plasma angiotensin under physiological conditions [5]. Factors, including genetic variation, that elevate plasma AGT may contribute to elevated blood pressure, while blood pressure can be reduced and renal blood flow increased by blocking components of the RAAS pathway with ACE inhibitors and angiotensin II (type-1) receptor blockers [6–8].

Linkage between essential hypertension and the *AGT* locus was initially demonstrated in European sib-pairs, and affected individuals homozygous for amino acid 235T had, on average, ~20% higher plasma AGT than did 235M homozygotes [1]. In transgenic mice, adding up to four additional copies of the *AGT* gene can increase plasma AGT levels and blood pressure by ~8 mmHg per copy [2]. A meta-analysis of more than 45,000 individuals has provided summary evidence that the *AGT* locus (marker T235M) is associated with plasma AGT levels in Europeans and with hypertension in Europeans and Asians in a dose-dependent manner [9]. Although many studies show a critical role for *AGT* in essential hypertension, linkage to this gene has not been replicated in some studies [10,11]. The –6A promoter allele is in linkage disequilibrium with 235T, and the presence of the –6A leads to increased transcription [12]. Other alleles in the *AGT* promoter, including –20A, –217A, –532T and –793A, also affect plasma AGT levels and blood pressure [12–19], but these effects can vary by ethnicity and sex [17,19,20].

To provide further resolution of the contribution of *AGT* genetic variation to hypertension, we have performed high-density genotyping and created haplotypes across the *AGT* promoter and gene region in 256 cases and 126 controls from the HyperPATH / Specialized Center of Research (SCOR) study group. New associations between *AGT* haplotypes and plasma AGT levels, renal plasma flow, and hypertension provide insight into the complex contribution of angiotensinogen to blood pressure regulation. Our results show that haplotypes can provide more robust associations with angiotensinogen levels and hypertension than individual SNPs (e.g., A-6G and T235M), and we show that these associations are strongly influenced by physiological sodium status. These findings help to explain previous inconsistencies in linkage and association results between *AGT* and essential hypertension.

Methods

Study participants

Hypertensive patients and controls were recruited and phenotyped using inpatient procedures at the General Clinical Research Centers at Brigham and Women's Hospital (Boston, MA), University of Utah (Salt Lake City, UT), Institut National de la Santé et de la Recherche Médicale (Paris, France), Vanderbilt University (Nashville, TN), and Sapienza-Università di Roma (Rome, Italy). All individuals were of European or European-American ancestry and were participants in the HyperPATH / Specialized Center of Research (SCOR) collaborative studies. Recruitment details have been previously described [21–23]. Individuals with evidence of secondary hypertension, diabetes, ethanol consumption exceeding 250g per week, or renal insufficiency (serum creatinine > 1.9 mg/dL) were

excluded from the analyses. All study subjects had BMI values $< 40 \text{ kg/m}^2$. Hypertensives were withdrawn from medications over a 2-week period prior to phenotype evaluation. The final study set was created from the multicenter samples by selecting unrelated cases and controls (from independent pedigrees) for which DNA samples were also available for genotyping (Boston (145), Italy (24), Paris (47), Utah (136), Vanderbilt (30)). All patient recruitment and protocols were performed with Institutional Review Board approval, and all participants provided informed written consent.

Genotyping

Genomic regions containing each polymorphic variant were amplified by PCR (20 mM Tris pH 8.8, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 50 μM each dNTP, 1 μM each primer, and 1 U Taq DNA polymerase). Primers for PCR were selected using the Primer3 software package. PCR amplicons for multiplex SNaPshot reactions were combined in roughly equal molar amounts. Primers and unincorporated dNTPs were removed from the PCR reactions by adding shrimp alkaline phosphatase (SAP) (2U) and ExoI nuclease (1U) to the pooled amplicons and incubating 90 minutes at 37°C followed by heat inactivation at 75°C for 15 minutes. A detection primer adjacent to each SNP was annealed and extended by 1 base with fluorescently labeled ddNTPs using SNaPshot reagents and conditions recommended by the manufacturer (Applied Biosystems (ABI)). Multiple SNPs were assayed in a single reaction by using detection primers of varying length (20 – 62 bp with 8 bp separation). SNaPshot extension reactions were dephosphorylated with 1U of SAP. Extension products were assayed by capillary electrophoresis on an ABI 3100 genetic analyzer. Genotypes were called using the Genescan and Genotyper software packages (ABI). All SNPs conformed to Hardy-Weinberg expectations in the study populations.

Phenotype evaluation

Phenotypic data were obtained through the HyperPATH / SCOR collaborative research group. Variables, including blood pressure, body mass index (BMI), plasma AGT, renal plasma flow (RPF), and infusion of angiotensin II, have been previously described [21,22,24,25]. Briefly, blood pressure was measured by a standard auscultatory method using a mercury sphygmomanometer with patients in the seated position. Hypertensive status was assigned for patients with blood pressure exceeding 140 mmHg systolic and 90 mmHg diastolic on three separate visits. The average blood pressure from the three measurements was used for quantitative analyses. Plasma AGT was measured by radioimmunoassay of angiotensin I after complete cleavage by renin using standard methods [26]. Angiotensin II was administered by intravenous injection at 3ng/kg/min for 45 minutes. Following a loading dose, steady-state plasma PAH was 2 mg/dl prior to experimental protocol. PAH clearance, measured by spectrophotometry, was then used to calculate baseline and angiotensin-stimulated effective renal plasma flow under low-salt (10 mEq) and high-salt (200 mEq) daily dietary conditions [22]. Average trait values for the study population are shown in Table 1.

Statistical Analysis

Associations between single SNPs and hypertension as a categorical variable were tested by chi-square analysis. We calculated the power to detect associations between variation at the *AGT* locus and hypertension as a discrete trait in our case-control data set [27]. Assuming a prevalence of hypertension of 30% in the general population, relative risk for the risk allele of 1.5, and a risk allele frequency of 0.1, this data set achieves 78% power at $\alpha = 0.05$ for an additive genetic model. *AGT* haplotypes were tested for association with hypertension using a randomization test (10,000 permutations).

Each of the 24 SNPs was tested for association with plasma AGT and renal plasma flow under high- and low-sodium conditions. Because standard association and regression analyses are sensitive to phenotypic outliers, we identified and removed outlier values that exceeded the mean $\pm$ three times the inter-quartile range in the raw data set for each quantitative trait. Genotype-phenotype associations for quantitative variables were tested using linear regression as implemented in the PLINK software package [28]. Specifically, we tested whether the response variable (e.g., plasma AGT, renal plasma flow) was correlated with *AGT* genotype. All single-SNP regression analyses were performed with age, sex, BMI, and sampling location as covariates. Plasma AGT and renal plasma flow were not adjusted by blood pressure status because such an adjustment could reduce or eliminate any potential association between *AGT* genotype and the hypertensive phenotypes. Using the SNPs individually for the regression analyses maximized the number of samples available for testing. Significant *p*-values were obtained for the additive and dominant models but, as the quantitative trait means suggested an allele-dosage effect on plasma AGT, *p*-values are reported for the additive model. We report *p*-values at the 0.05 level. Due to linkage disequilibrium in the *AGT* region, test results for individual SNPs are correlated, making the Bonferroni correction for multiple comparisons very conservative. Nonetheless, we also indicate which associations are significant at the Bonferroni-corrected level of 0.002.

AGT haplotypes across the entire ~14 kb *AGT* region were constructed from genotype data using the FAST_PHASE program [29]. Six major haplotypes, labeled H1 through H6, were inferred. Haplotype frequencies were evaluated for stratification by sampling location using a permutation test. Only haplotype H5, in one population, showed a significant difference due to location (frequency of 0.100 in Utah vs. 0.044 in all other locations, $p = 0.003$). ANOVA was used to test for significant differences in average plasma AGT among haplotype groups. Assuming equal group weighting, this data set has 80%, 50%, or 25% power to detect an effect size of ~25%, ~18%, or ~13%, respectively, at $\alpha = 0.025$. A linear regression analysis was performed in which the dependent variable was plasma AGT and the independent variables were haplotype groups H4 and H1/H2 and sex. Genetic distances between haplotypes were estimated, and a neighbor-joining network was constructed using the PHYLIP package [30].

Results

Association of *AGT* SNPs and haplotypes with essential hypertension

Twenty-four SNPs spanning the *AGT* locus and selected for allele frequencies of $>5\%$ in the general population were genotyped and tested for association with hypertension. Eleven of the 24 SNP alleles, including -6A and 4072C (aa. 235T), showed positive associations with hypertensive status ($p < 0.05$) (Figure 1). Four SNP alleles (1164A, 6066A, 6233C, and 12822C) had significance levels < 0.01 , and alleles 1164A and 6233C remained significantly associated with hypertension after a Bonferroni correction for 24 comparisons. Allele 6233C (rs3789669), which was found in 21.8% of hypertensives and 10.6% of normotensive controls, produced the maximum odds ratio (2.3, 95% CI 1.5 – 3.8, $p < 0.0003$).

Association of *AGT* genotypes with plasma AGT levels

We tested the hypothesis that *AGT* genotype is correlated with plasma AGT levels using the 113 subjects for whom plasma AGT values were available (Paris (46), Boston (36) and Utah (31)). Under low-salt conditions (10 mEq), 16 of 24 SNPs are associated with elevated plasma AGT after controlling for age, sex, BMI, and sampling location as covariates in a linear regression analysis (Table 2). Notably, all but one of the SNPs that showed significant association with the trait of essential hypertension were also significantly associated with

elevated plasma AGT under physiological conditions of low-sodium. All 16 SNPs remained associated with elevated low-salt plasma AGT using the Paris samples alone, and 7 SNPs (including 4 promoter SNPs) were associated in the Utah sample alone ($p < 0.05$), demonstrating that the observed associations are not due to stratification among collection sites. As plasma AGT levels increased, there was a modest, but non-significant, increase in low-salt diastolic and systolic blood pressure ($r = 0.08$, $p > 0.4$; $r = 0.09$, $p > 0.3$, respectively).

Under high-sodium conditions (200 mEq), only 7 of the 16 SNPs remained associated with plasma AGT ($p < 0.05$), none of the 16 were associated at $p < 0.01$, and most associations were much less significant than for the low-sodium conditions (Figure 2). This result demonstrates that the association between *AGT* SNP variation and elevated plasma AGT is enhanced under physiological conditions of sodium depletion.

Association of *AGT* haplotypes with plasma AGT levels and hypertension

56 unique haplotypes were inferred in the entire case-control set, including 33 singletons and 17 low-frequency (< 0.05) haplotypes. Six major haplotypes each exceeded a population frequency of 0.05 and account for 84% of all haplotypes in the study population (Table 3). Among the six major haplotypes, the configuration of haplotype H4 is unusual: it contains 15 of the 16 SNP alleles associated with elevated plasma AGT and, uniquely among the common haplotypes, contains 4 promoter alleles associated with elevated plasma AGT. Haplotype H4 contains the -6A promoter allele, and it is uniquely identified by SNP alleles -1178G, -1074T, -532T, -217A, and 6152A. These five markers are in high LD over all samples ($r^2 \geq 0.85$), and each effectively tags the H4 haplotype. However, r^2 values between H4 tagging SNPs and the A-6G polymorphism are low ($r^2 < 0.16$) (see Figure 2). In a comparison of the four major haplotypes that contain the -6A promoter allele, 78% of those who have the H4 haplotype are hypertensive, while only 69% of those who have any of the other three haplotypes are hypertensive. Over all cases and controls, H4 is the only haplotype positively associated with hypertension at a level that approaches statistical significance ($p = 0.053$).

For the 113 individuals in whom plasma AGT was measured, we divided the sample into those that carry H4 and those without H4. Under low-sodium conditions, the mean plasma AGT level for the H4 group (1304 ng/ml, $n = 26$) is 18% higher than for the group containing all other haplotypes (1101 ng/ml, $n = 87$; $p < 0.002$) and 15% higher than the group containing one or more copies of non-H4 -6A-bearing haplotypes (1136 ng/ml, $n = 42$; $p < 0.036$). Plasma AGT was also significantly higher in the H4 group for the Paris samples alone ($p < 0.003$). Under low-salt conditions, plasma renin activity was higher in the H4 group than in non-H4 group (2.54 vs. 2.32 ng/mL/hr), but this difference was not statistically significant. Under high-salt conditions and consistent with the single-marker results, the mean value for plasma AGT in the H4-bearing group is nearly identical to that of the non-H4 bearing group (1160 ng/ml vs. 1162 ng/ml).

The association results suggest three primary *AGT* haplotype classes. H4 is associated with hypertension and elevated plasma AGT, H1 and H2 are protective, and others haplotypes have minimal effects. To test this hypothesis formally, we estimated the additive effects of H4 and H1/H2 haplotype copies on plasma AGT values. In the best-model linear regression, with sex as a significant covariate ($p < 0.027$), low-salt plasma AGT values were significantly predicted by these haplotype classes. H4 was positively associated with plasma AGT ($p < 0.013$), while H1/H2 was negatively associated ($p < 0.025$; overall $p < 0.001$, adjusted $R^2 = 0.14$).

Association of AGT genotype and renal function

Because previous studies have demonstrated an association of the promoter variant rs5051 (-6A) with a renal vascular response [21], we tested whether this association could be further refined with additional *AGT* SNPs and haplotypes. Association statistics were calculated for each of the 24 SNPs and renal plasma flow (RPF) under high- and low-sodium conditions, using sex, age, and BMI as covariates in those samples measured for RPF. Study location was also added to the regression to control for possible between-location differences. Two patterns are apparent: promoter alleles -1178G, -1074T, -532T, -217A and several intronic SNP alleles are associated with reduced renal plasma flow. The associations are present under low-salt conditions and are less significant under high-salt conditions unless the patient was infused with angiotensin II (Table 4). SNP alleles -1178G, -1074T, -217A, and 6152A remained associated with RPF when hypertensive and normotensive groups were analyzed separately ($p < 0.05$). However, the difference between baseline RPF and angiotensin II stimulated RPF was not significantly associated with *AGT* genetic variation under either high- or low-salt conditions. These findings suggest that associations between *AGT* genotype and RPF are increased by conditions that systemically activate the RAAS.

The *AGT* alleles showing significant associations with reduced RPF are found on haplotype H4, and the associated promoter alleles occur uniquely on haplotype H4. An approximate 11% reduction in high-salt RPF following angiotensinogen II infusion occurs in heterozygotes for those SNPs showing an association (Table 5). Reduced RPF for associated alleles was also observed in hypertensives or normotensives separately. As expected, there is a significant negative correlation between plasma AGT and low-sodium RPF in the subset of samples having both measurements ($r = -0.38$, $p < 0.02$; $n = 37$).

To refine the relationship between *AGT* and RPF, we again split the data set into those samples with H4 ($n = 44$) and all others ($n = 201$). The average high-salt RPF after angiotensin II infusion is significantly lower in the H4 group (388.9 pmol/L vs. 440.6 pmol/L; $p < 0.00017$). To further evaluate this result, all 24 SNPs were retested for association in individuals lacking the H4 haplotype. The removal of the 44 H4 samples completely eliminates significant associations between *AGT* SNP genotypes and RPF. These results suggest that haplotype H4 is the major contributor to the association between *AGT* genotype and renal plasma flow in this study cohort.

Association of AGT variation and blood pressure

We tested each of the 24 *AGT* SNPs for association with diastolic and systolic blood pressure. No significant associations were found after correcting for the effects of age, sex, and BMI. Thus, although significant associations are found between *AGT* variation and the categorical variable of hypertension, associations between *AGT* genotypes and the continuous variables of diastolic and systolic blood pressure did not reach significance in this data set ($p > 0.05$).

Worldwide distribution of haplotype H4

Previous assessments of the contribution of the *AGT* gene to essential hypertension have obtained variable results, depending in part on the geographic origin of the study population. Since haplotype H4 captures much of the association signal for the hypertension-related traits of increased plasma AGT and reduced renal plasma flow in Europeans, we analyzed the distribution of haplotype H4 relative to the A-6G SNP in worldwide populations using previously published *AGT* sequence data [31]. Consistent with ascertainment on hypertensive status, haplotype H4 occurs at a higher frequency in our European hypertensive study cohort than in randomly selected western Europeans (9% vs. 6%).

The six major haplotypes identified and characterized by 24 SNPs in this study cohort make up at least 74% of all *AGT* haplotypes in major Eurasian population groups (Figure 3). In contrast, these six haplotypes represent only 17% of all haplotypes in sub-Saharan Africans ($n = 74$), where *AGT* haplotype diversity is high [31]. The frequency of H4 in Africans is 7%. Another haplotype, nearly identical to H4 but having cytosine at position -532, has a frequency of 6% in Africans, making H4 and this nearly identical haplotype frequent in Africans relative to other haplotypes.

Haplotype H4 harbors the -6A and 4072C alleles often used in association studies. Due to the relatively low frequency of H4 and the high frequency of the -6A promoter allele, haplotype H4 captures only a portion of the -6A alleles in a given population. Worldwide, the frequency of haplotype H4 is not well-correlated with the population frequency of the -6A promoter variant ($r = 0.36$, $p > 0.25$) (Table 6).

Discussion

Many previous studies have provided evidence that common SNPs at the *AGT* locus influence transcription rates, plasma AGT levels, or blood pressure [9,12,13,18–20,31–34]. Variants -1074T, -532T, -217A, -20C, and -6A within the promoter have been implicated in essential hypertension, individually or in combination, and replicated in a number of recent functional assays or association studies [13,15–17]. Consistent with these studies, our analysis detects significant associations between these *AGT* alleles and increased plasma AGT levels and essential hypertension.

Our analysis identifies a complete *AGT* haplotype, H4, which contains the SNP alleles individually associated with essential hypertension and elevated plasma AGT. This is the fourth most common haplotype in the analysis cohort and has a frequency of 6–13% in European populations. Individuals with H4, primarily heterozygotes (e.g., H4/H1), account for a large proportion of the association signal in the data even though most copies of the -6A and 4072C are found on other haplotypes. Of the common Eurasian *AGT* haplotypes, H4 uniquely contains promoter alleles -1178G, -1074T, -532T and -217A. Consistent with these results, Brand-Herrmann *et al.* showed that the -532T/-6A *AGT* haplotype is associated with elevated blood pressure (systolic, diastolic, ambulatory) in untreated hypertensive subjects [13]. In addition, Jain *et al.* [35] have recently shown that a human promoter haplotype containing -217A confers an allele-dose-specific increase in *AGT* transcription in liver and kidney tissues compared to the -217G-containing promoter haplotype in transgenic mice.

A key factor affecting the strength of the association between *AGT* genotype and plasma AGT levels was sodium intake. Associations were more numerous and more significant under physiological conditions of sodium depletion. It is well-established that sodium loading has an inhibitory effect on the RAAS system and renin expression in humans and model organisms [36–39]. Our results suggest that differences in plasma AGT levels associated with *AGT* SNP variation are detectable under low-sodium conditions, but that RAAS down-regulation under sodium loading reduces these allelic differences and associations. Thus, these results suggest that future *AGT*-blood pressure association studies should account for physiological sodium status.

Several previous studies have found an influence of *AGT* genetic variation on renal traits. Individuals homozygous for the -6A allele are over-represented among hypertensive patients with low renal plasma flow on high-salt diets [21]. Younger hypertensive 235T homozygotes are characterized by a prematurely blunted renal vascular response to increased salt intake [40]. The non-modulator hypertensive phenotype, characterized by

blunted renal response to angiotensin II infusion under a high-sodium diet, is correlated with genotype 235TT, but this effect is enhanced by adding other correlated genotypes from *ACE* and *CYP11B2* [41,42].

In our study, baseline associations *AGT* variation and renal plasma flow (RPF) were stronger under low-salt than high-salt conditions. More significant associations, however, occurred following angiotensin II infusion under high-salt conditions. While several interpretations are possible, we suggest that allelic variants found on the H4 haplotype background lead to increased responsiveness of *AGT* expression, and that H4 identifies a subset of the -6A-bearing individuals with an enhanced angiotensin II response and lower RPF. Conditions that activate RAAS systemically, such as sodium depletion and angiotensin II infusion, may have a greater effect on *AGT* H4 than on other haplotypes, thus producing detectable differences among haplotype classes. This model permits H4 to maintain higher angiotensinogen levels under RAAS-activating conditions, possibly leading to a higher set point for pressure natriuresis and blood pressure over time.

Increasing molecular evidence now suggests that several common diseases involving metabolism, sodium homeostasis, and hypertension may be the result of evolutionarily adapted alleles that are deleterious in modern environments [3,43,44]. The ancestral *AGT* -6A allele frequency decreases with latitude and, along with -217A, may confer positive heat adaptation [45]. The derived -6G allele appears to be under positive selection in some non-African populations [31]. Additionally, the frequency of the *AGT* 4072C allele (aa. 235T) is correlated with the unlinked *CYP3A5**3 allele, which is also associated with salt and water retention [46]. At these loci, alleles favoring salt retention, vasoconstriction, and a predisposition to hypertension are more common in African populations [44].

A comparison of the common Eurasian *AGT* haplotypes in this study shows that H4 (and H5) are more divergent from the ancestral form of *AGT* than are the H3 and H6 haplotypes, even though the latter also contain the ancestral -6A promoter allele (Figure 4). *AGT* haplotype H4 contains several human-specific alleles associated with elevated *AGT* levels (-1178G, -217A, 1164A, 6066A, 9597C). As humans underwent range expansion into more arid environments within Africa, specific mutations and recombination events may have produced *AGT* haplotype configurations (i.e., H4) that favored vasoconstriction, sodium retention, and limited volume depletion via RAAS activation and the effect of this activation in the kidney.

Haplotype H4, along with a closely related haplotype that differs from H4 at position -532T only, accounts for 13% of our sample of sub-Saharan haplotypes. These two haplotypes are common in Africans, relative to other haplotypes. An independent assessment of 57 Nigerians found that one of seven common *AGT* haplotypes was significantly associated with elevated plasma *AGT* levels [47]. For nine shared markers located between -1074 and 4072, all alleles reported for this Nigerian haplotype are identical to haplotype H4. Consistent with the potential adaptive features described above, the frequency of haplotype H4 is high (23%) in tropical South Indians.

We suggest that haplotype H4 may be a predisposing factor for essential hypertension via elevated *AGT* levels. In this study, activation of the RAAS system, either through sodium depletion or angiotensin II infusion, produced stronger associations between haplotype variation and hypertension-associated phenotypes. Replication of these results in larger cohorts is feasible and necessary. In particular, the relatively low frequency of the H4 haplotype limited the present study to those effects seen in heterozygotes. Larger studies can assess whether haplotype H4 has significant additive effects in homozygotes. Additional analyses of the molecular mechanisms influencing transcriptional regulation of the human

AGT gene by SNP and haplotype class are also needed. The distributions of hypertensive trait values for H4 and non-H4 groups overlap extensively, so caution is warranted in using this haplotype to predict plasma AGT, RPF, or hypertensive predisposition of a single individual. Nevertheless, identification of hypertensive patients with *AGT* haplotypes that increase plasma AGT levels could help to optimize pharmacological approaches to hypertension management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The location and association of *AGT* SNPs and haplotypes with the categorical trait of essential hypertension. Marker locations and SNP alleles associated with hypertension in 256 cases and 126 controls are shown above the gene. Haplotype block structure and those blocks associated with hypertension in cases and controls are shown below the gene. The three predisposing blocks are components of complete haplotypes H4 and H5 while the two protective blocks are components of haplotypes H1 and H2.

Figure 2.

Association of *AGT* SNPs and plasma AGT for high- and low-sodium conditions. For each *AGT* SNP, the high- and low-sodium *p*-values for association with plasma AGT are plotted by SNP position. The dotted lines indicate a *p*-value of 0.01 (lower) and a Bonferroni-corrected *p*-value of 0.002 (upper). A plot showing the pairwise linkage disequilibrium (r^2) between the *AGT* SNPs is shown below.

Figure 3.

The distribution of common *AGT* haplotypes in world populations. Circle diagrams show the frequency of the six major *AGT* haplotypes identified in the study cohort in populations from Africa, northern Europe, the Mideast, South India, Central Asia, China, and East Asia (Japan/Korea). The frequencies of the six study cohort haplotypes range from 17% in Africans to 89% in Central Asians.

Figure 4.

A neighbor-joining network for the six common Eurasian *AGT* haplotypes. The network is rooted by a hypothetical ancestral *AGT* haplotype based on the nucleotide present at the orthologous position in non-human primates for each of the 24 human *AGT* SNPs. *AGT* haplotypes H3, H4, H5, and H6 contain the ancestral alleles –6A and 4072C (aa. 235T). Haplotype H4 is predisposing to hypertensive phenotypic traits and has diverged from H3, H6, and the ancestral *AGT* haplotype.

Table 1

Average trait values and standard deviation for the study population groups.

n	Age (years)	BMI (kg/m ²)	Plasma AGT (ng/mL) [*]		RPF (mL/min) [§]		Diastolic (mmHg)		Systolic (mmHg)	
			Low-Na ⁺	High-Na ⁺	Low-Na ⁺	High-Na ⁺	Low-Na ⁺	High-Na ⁺	Low-Na ⁺	High-Na ⁺
Cases	256	48.05 (± 8.56)	1156.04 (± 300.81)	1199.64 (± 315.44)	476.25 (± 86.50)	512.27 (± 98.60)	80.68 (± 10.50)	89.51 (± 11.34)	131.19 (± 16.64)	146.80 (± 18.19)
Males	164	47.91 (± 8.75)	1103.15 (± 235.98)	1109.24 (± 240.11)	480.82 (± 88.47)	513.94 (± 98.26)	81.38 (± 9.75)	90.39 (± 11.66)	131.01 (± 15.88)	144.96 (± 16.39)
Females	92	48.29 (± 8.27)	1230.57 (± 363.65)	1326.60 (± 364.51)	465.43 (± 81.86)	508.68 (± 100.11)	79.50 (± 11.61)	88.06 (± 10.71)	131.49 (± 17.94)	149.84 (± 20.58)
Controls	126	39.43 (± 11.34)	— [‡]	— [‡]	557.63 (± 128.63)	584.90 (± 116.78)	63.69 (± 7.04)	66.63 (± 8.18)	107.08 (± 12.00)	111.08 (± 13.41)
Males	59	38.39 (± 11.99)	— [‡]	— [‡]	560.58 (± 133.66)	595.63 (± 134.78)	66.60 (± 6.60)	68.84 (± 7.60)	112.61 (± 12.14)	115.16 (± 13.29)
Females	67	40.34 (± 10.73)	— [‡]	— [‡]	555.50 (± 126.21)	576.89 (± 101.91)	61.02 (± 6.39)	64.62 (± 8.22)	101.98 (± 9.39)	107.40 (± 12.50)
All individuals	382	45.20 (± 10.38)	1147.87 (± 300.38)	1185.50 (± 314.96)	510.56 (± 113.42)	537.90 (± 110.76)	74.82 (± 12.43)	81.44 (± 15.05)	122.83 (± 19.04)	134.20 (± 23.86)

^{*} For plasma AGT, *n* = 113 (106 cases, 7 controls; 62 male cases, 44 female cases)

[‡] *n* < 10.

[§] For RPF averages (baseline), low-sodium *n* = 204 (118 cases, 86 controls), and high-sodium *n* = 272 (176 cases, 96 controls).

Table 2

Mean low-sodium plasma AGT values by genotype class.

SNP	Location	Ref. allele	Plasma AGT (ng/ml)			p-value
			1/1	1/2	2/2	
rs11122580	-1178	G	-	1291	1092	0.0013*
rs2071404	-1074	T	-	1279	1098	0.0042
rs2071406	-775	C	-	1243	1139	-
rs5046	-532	T	-	1310	1092	0.0006*
rs5049	-217	A	-	1319	1087	0.0008*
rs5050	-20	C	-	1113	1166	-
rs5051	-6	A	1268	1162	1057	0.0045
rs2493134	507	G	1268	1158	1059	0.0055
rs7539013	698	A	1300	1166	1066	0.0041
rs2004776	1164	A	1450	1235	1049	<10 ⁻⁴ *
rs4762	3889	T	-	1069	1176	-
rs699	4072	C	1268	1158	1059	0.0055
rs6687360	4874	A	1300	1161	1070	0.0062
rs2478543	5556	G	1264	1158	1062	0.0100
rs3789671	6066	A	1502 [†]	1293	1054	<10 ⁻⁴ *
rs3789670	6152	A	-	1276	1106	0.0073
rs3789669	6233	C	1502 [†]	1284	1055	<10 ⁻⁴ *
rs2493132	6309	G	1188	1146	1142	-
rs3789667	6420	T	1465	1108	1168	-
rs3827749	8307	T	1372	1094	1165	-
rs2478523	8357	C	1302	1145	1113	-
rs11122575	9597	C	-	1334	1120	0.0082
rs7079	11525	A	1008	1140	1173	-
rs943580	12822	C	1291	1142	1064	0.0029

Significant associations with plasma AGT levels by linear regression with adjustment for covariates of age, sex, BMI, and sampling location are shown ($p < 0.05$). The reference allele is 1.

* $p < 0.002$ (Bonferroni correction)

[†]Class size is 2 and p -values are shown for regression with the 1/1 class omitted. Counts in the 1/1 classes for other associated SNPs range from 7 to 23; n range by SNP was 98 to 113 individuals.

Common AGT haplotypes and frequencies in European cases and controls.

Table 3

	Haplotypes																								Count	Cases/Controls	p-value
	-1178	-1074	-775	-532	-217	-20	-6	507	698	1164	3889	4072	4874	5556	6066	6152	6233	6309	6420	8307	8357	9597	11525	12822			
H1	A	G	T	C	G	A	G	A	G	G	C	T	G	A	C	G	T	A	C	C	T	T	A	T	230	0.486/0.581	0.029*
H2	A	G	T	C	G	A	G	A	G	G	C	T	G	A	C	G	T	A	C	C	T	T	C	T	162	0.103/0.128	0.844
H3	A	G	T	C	G	A	G	A	G	G	T	C	A	G	C	G	T	G	T	T	C	T	C	C	84	0.108/0.062	0.053 [†]
H4	G	T	T	T	A	A	<u>G</u>	<u>A</u>	<u>A</u>	<u>A</u>	C	<u>C</u>	<u>A</u>	<u>G</u>	<u>A</u>	A	<u>C</u>	A	C	C	C	T	C	<u>C</u>	70	0.075/0.040	0.368
H5	A	G	T	C	G	A	A	G	A	A	C	C	A	G	A	G	C	G	C	C	T	C	C	C	50	0.065/0.044	0.856
H6	A	G	T	C	G	C	A	G	A	A	C	C	A	G	C	G	T	G	T	T	C	T	C	C	43	-	-
Others																									125		

SNPs are shown 5' to 3' with respect to the AGT minus strand transcript. Fifteen of 16 SNP alleles individually associated with elevated plasma AGT occur on haplotype H4 and are underlined. SNPs unique to H4 are shown in bold. Permutation-based p-values for the association of each common haplotype with "hypertension" as a categorical trait in 256 cases and 126 controls are indicated.

Because H1 and H2 account for all common -6G/4072T bearing haplotypes and differ at C11525A only, SNP 11525 was omitted from the association test allowing H1 and H2 to be combined. The G-6A promoter and C4072T (aa. T235M) SNPs are shaded. Permutation-based p-values for the 2-marker haplotypes -6G/4072T and -6A/4072C are 0.035 (protective) and 0.044 (predisposing), respectively.

* protective

[†] predisposing

Table 4

Association of *AGT* genotype and renal plasma flow.

SNP	Location	Ref. allele	Low Salt	Low Salt + All	High Salt	High Salt + All
rs11122580	-1178	G	0.019	0.013	-	0.003
rs2071404	-1074	T	0.019	0.013	0.050	0.003
rs2071406	-775	C	-	-	-	-
rs5046	-532	T	-	-	-	0.003
rs5049	-217	A	0.047	0.042	-	0.003
rs5050	-20	C	-	-	-	-
rs5051	-6	A	-	-	-	-
rs2493134	507	G	-	-	-	-
rs7539013	698	A	-	-	-	-
rs2004776	1164	A	0.019	0.033	-	0.039
rs4762	3889	T	-	-	-	-
rs699	4072	C	-	-	-	-
rs6687360	4874	A	-	-	-	-
rs2478543	5556	G	-	-	-	-
rs3789671	6066	A	0.041	-	-	0.028
rs3789670	6152	A	0.022	0.018	0.036	<10 ⁻⁴ *
rs3789669	6233	C	0.048	-	-	-
rs2493132	6309	G	-	-	-	-
rs3789667	6420	T	-	-	-	-
rs3827749	8307	T	-	-	-	-
rs2478523	8357	C	-	-	-	-
rs11122575	9597	C	-	-	-	-
rs7079	11525	A	-	-	-	-
rs943580	12822	C	-	-	-	-

All *p*-values are for linear regression of measured renal plasma flow (mL/min) and *AGT* genotype with age, sex, BMI, and collection location as covariates; only *p*-values <= 0.05 are shown. Minimum individuals were *n* = 180 low-sodium and *n* = 219 high-sodium; All, infused-angiotensin II.

* Value is significant with Bonferroni correction for multiple comparisons.

Table 5

Quantitative trait means by genotype class for high-salt renal plasma flow following angiotensin II infusion.

SNP	Location	Ref. allele	RPF(mL/min)			p-value
			1/1	1/2	2/2	
rs11122580	-1178	G	NA	392.3	440.8	0.00277
rs2071404	-1074	T	NA	392.3	441.1	0.00239
rs2071406	-775	C	408.7	434.5	430	-
rs5046	-532	T	NA	397.6	440.9	0.00256
rs5049	-217	A	NA	393.5	441.9	0.00251
rs5050	-20	C	470	432.7	428.3	-
rs5051	-6	A	422.9	430	440.5	-
rs2493134	507	G	424	430.8	438.5	-
rs7539013	698	A	427.3	424.3	441.8	-
rs2004776	1164	A	412.2	413.9	445.8	0.03647
rs4762	3889	T	502.9	439.8	429.4	-
rs699	4072	C	424	430.4	439.7	-
rs6687360	4874	A	425.7	423.5	442	-
rs2478543	5556	G	419.7	431.9	438.1	-
rs3789671	6066	A	429.1	401.8	446.1	0.02477
rs3789670	6152	A	NA	389.5	441.3	0.00091*
rs3789669	6233	C	432.6	402	447.2	0.04909
rs2493132	6309	G	437	432.5	428.1	-
rs3789667	6420	T	433.7	437.1	428	-
rs3827749	8307	T	429.7	437.2	429.6	-
rs2478523	8357	C	415.1	422.9	438.9	-
rs11122575	9597	C	508.7	406.7	434.5	-
rs7079	11525	A	460.4	425	429.2	-
rs943580	12822	C	416.4	430	442.8	-

Regression p-values are shown for significant associations for RPF under high-salt and angiotensin II infusion ($p < 0.05$); $n = 237 - 245$ individuals; All, infused-angiotensin II.

* $p < 0.002$ (Bonferroni correction)

Table 6

Frequency of haplotype H4 and promoter allele -6A in world populations.

Population	n	H4	-6A
S. Indians	30	0.233	0.750
E. Asians (Japanese/Koreans)	48	0.208	0.823
Mideast (Druze)	24	0.187	0.604
S.E. Asians	24	0.146	0.812
S. Chinese	24	0.146	0.854
E. Europeans	24	0.125	0.500
Ashkenazi	24	0.104	0.542
Mideast (Palestinians)	24	0.083	0.458
Africans	74	0.074	0.899
Europeans	24	0.062	0.292
C. Asians	24	0.042	0.729
N. Chinese	24	0.021	0.604

(Correlation: $r = 0.36$, $p > 0.25$; SNP genotypes based on data from Nakajima *et al.*, 2004. The ancestral -6A allele is in linkage disequilibrium with amino acid 235T. The frequency of 235T is within 2% of the estimate of -6A in each population.)