Original Article

Genetic Polymorphisms of Angiotensinogen and Essential Hypertension in a Tibetan Population

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The human angiotensinogen gene (*AGT*) is a promising candidate for an essential hypertension-susceptibility gene. We aimed to explore the single-locus, haplotype and epistasis patterns of three polymorphisms of *AGT* (A−20C, A−6G and M235T) and their relation to the risk of essential hypertension in a Tibetan population. The three polymorphisms were genotyped in 333 essential hypertension patients and 235 healthy controls on the basis of a door-to-door cross-sectional study. Genotyping was performed using polymerase chain reaction (PCR)–restriction fragment length polymerase (RFLP) and direct sequencing techniques. The data were analyzed using the EH/EH+ program and the multifactor dimensionality reduction (MDR) method. Our single-locus analysis revealed that except for a marginal, significant association of A−20C allele distribution, no significant association between genotype and allele distributions of the A−20C, A−6G, or M235T polymorphism of *AGT* and essential hypertension was found. In haplotype analysis, we found that the H_1 haplotype may be the risk-conferring factor for hypertension, even after the Bonferroni correction. In epistasis analysis, we selected the final best model, which included the A−20C and A−6G polymorphisms with a strong synergistic effect. This model had a maximum testing accuracy of 0.564 and a maximum cross validation consistency of 10 out of 10 (p=0.001). The present study thus provides evidence of a strong synergistic effect of the A−20C and A−6G polymorphisms of *AGT*, which were not found to be associated with essential hypertension in the single-locus analysis. Moreover, we have proposed a promising data–mining analytical method using the open-source MDR software package for detecting and characterizing gene-gene interactions. (*Hypertens Res* 2007; 30: 1129–1137)

***Key Words*: essential hypertension, angiotensinogen, single-locus, haplotype, epistasis**

Introduction

Human essential hypertension, which accounts for 90% of the hypertensive population, is a complex multifactorial and polygenic disorder that is thought to result from an interaction between an individual's genetic makeup and various environmental factors (*1*, *2*). Given that hypertension is a major risk factor for cardiovascular diseases and prevails globally, there has been growing emphasis on the importance of preventing hypertension to lessen the public health burden (*3*, *4*). One preventive approach is to identify disease-susceptibility genes

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that involve a specific physiological or cellular function.

Because of the many biochemical and physiological mechanisms involved in regulating blood pressure, many candidate genes for essential hypertension have been identified. The angiotensinogen gene (gene: *AGT* and protein: AGT) is one of the most promising. The gene encoding AGT is assigned to chromosome 1q42 and comprises five exons and four introns distributing over 13 kb (*5*, *6*). A previous study regarding the activation or duplication of *AGT* in transgenic mice demonstrated a correlation among *AGT* expression, plasma AGT concentrations and blood pressure (*7*). As early as 1992, Jeunemaitre *et al*. examined the association between 15 genetic polymorphisms of human *AGT* and essential hypertension, and found that an amino acid substitution (methionine to threonine) at codon 235 (M235T) in exon 2 of *AGT* increased not only the risk of essential hypertension but also the plasma AGT concentration in white subjects (*8*). Following this initial report, a large number of association studies regarding the M235T polymorphism and essential hypertension have been conducted and have yielded inconsistent results. Several major meta-analyses, however, have confirmably demonstrated a significant association between *AGT* M235T polymorphism and hypertension, with the 235T allele conferring a combined relative risk of ~1.2 (*9*–*11*). Thus clarifying the role of *AGT* may help to elucidate the genetic makeup of essential hypertension.

Given the multifactorial nature of essential hypertension, it is assumed that multiple genes/loci contribute to the etiology of this condition, independently or synergistically with other genes/loci. To fully unravel the genetic architecture of essential hypertension, it may be more useful to focus on the transmission of multilocus haplotypes and the synergistic effects within candidate genes, that is, the interactions of genetic variants with one another (*12*, *13*). This has been proposed as the reason that traditional single-locus association analyses are ineffective in explaining the majority of the genetic contribution to complex traits (*14*), such as blood pressure. Also, it is clear that the occurrence of genetic variants of *AGT* is heterogeneous across races and ethnicities (*15*). To address these issues and to extend association studies of *AGT* to other populations, we explored the single-locus, haplotype and epistasis patterns of three polymorphisms of *AGT* (two in the promoter region: A−20C and A−6G; and one in exon 2: M235T) and determined their relation to the risk of essential hypertension in a stable community–based population in the city of Lhasa, China. The genetic homogeneity and geographic stability of this population, along with their shared exposure to common environmental conditions, provided an excellent opportunity for studying the genetic predisposition to essential hypertension.

Table 1. The Characteristics of the Study Population

	Hypertensives (n=333)	Normotensives (n=235)	p
Sex (M/F)	172/161	126/109	
Age (years)	52.27±11.89	49.63±10.02	0.1250
BMI (kg/m^2)	25.76±2.96	22.35±2.21	<0.0001
SBP (mmHg)	176.89±15.52	112.60±12.38	<0.0001
DBP (mmHg)	106.97±11.06	75.03±10.25	<0.0001
TG (mmol/L)	1.62±1.53	1.44±1.15	0.1220
TC (mmol/L)	5.51±1.45	5.22±1.58	0.0051
HDLC (mmol/L)	1.64±1.06	1.66±0.97	0.8231
BUN (mmol/L)	6.20±1.81	5.11±1.35	<0.0001
Scr (μmol/L)	97.16±19.65	83.56±15.66	<0.0001

M/F, males/females; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; BUN, blood urea nitrogen; Scr, serum creatinine. Values are expressed as mean±SD. p values were calculated using an unpaired t-test. p was calculated using χ^2 test 2×2 contingency table with degree of freedom of 2.

Methods

Selection of the Study Population

Our study was based on the results of a door-to-door cross-sectional investigation conducted from 1997 to 1998 in stable residential communities in the urban district of Lhasa, the capital of the Tibet Autonomous Region, as previously described (*16*, *17*). All participants of the study were unrelated and of Tibetan origin without a history of intermarriage, and they were all permanent residents of the city of Lhasa, a remote mountainous region at an elevation of 3,700 m above sea level. This study was approved by the Ethics Committee of the Chinese Academy of Medical Sciences/Peking Union Medical College. A total of 1,322 subjects in Lhasa were examined, of whom only 568 were enrolled in this study after receiving a full explanation and providing informed consent. Of 568 subjects, 333 (58.6%) were found to have essential hypertension according to the following criteria: 1) absence of consanguinity at enrollment; 2) onset of hypertension after age 20 and before age 60 years; 3) systolic blood pressure (SBP) ≥160 mmHg or diastolic blood pressure (DBP) ≥95 mmHg; 4) absence of secondary causes of hypertension based on extensive biochemical and clinical examination; 5) absence of pharmacological treatment for hypertension. The remaining 235 (41.4%) subjects were chosen as healthy controls based on their comparable age, gender, and geographical locations compared with their hypertensive counterparts. All of the normotensives had SBP and DBP less than 130 and 85 mmHg, respectively. To simplify the analyses and the interpretation of the results, subjects with a clinical history of sec-

Table 2. The Distributions of Genotypes and Alleles for Each of the Three *AGT* Polymorphisms Studied in Both Groups and Odds Ratios for Essential Hypertension

Locus	Variants	Hypertensives (n=333)	Normotensives (n=235)	χ^2	p*	OR (95% CI)	$p^{\dagger}$
A−20C	AA	216 (64.86)	132 (56.17)			0.70 (0.51–0.96)	0.0254
	AC	113 (33.93)	97 (41.28)	5.14	0.0766	0.69 (0.49–0.98)	0.0365
	CC	4 (1.20)	6 (2.55)			0.46 (0.13–1.66)	0.2384
	A/C	81.83/18.17	76.81/23.19	4.31	0.0380		
A−6G	AA	222 (66.67)	147 (62.55)			0.80 (0.58–1.01)	0.1609
	AG	108 (32.43)	81 (34.47)	3.91	0.1416	0.84 (0.59–1.18)	0.3117
	GG	3 (0.90)	7 (2.98)			0.30 (0.08–1.16)	0.0801
	A/G	82.88/17.12	79.79/20.21	1.76	0.1848		
M235T	TT	203 (60.96)	145 (61.70)			0.97 (0.71–1.32)	0.8513
	TM	124 (37.24)	82 (34.89)	1.66	0.4368	1.03 (0.73–1.45)	0.8584
	MM	6 (1.80)	8 (3.40)			0.52 (0.18–1.52)	0.2327
	T/M	79.58/20.42	79.15/20.85	0.03	0.8597		

Genotype data are expressed as number (%) and allele data as %. *p was calculated by χ^2 test 3×2 contingency table for genotype distribution and 2×2 contingency table for allele distribution. †p was calculated using the logistic regression analysis, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated accordingly with the top for additive model, the middle for dominant model and the bottom for recessive model.

ondary hypertension, coronary heart disease, diabetes and renal insufficiency were excluded from the study.

DNA Extraction

Venous blood (5 mL) was collected from each subject in tubes containing 50 mmol/L Na_2-EDTA. The serum was simultaneously isolated and frozen for biochemical assay. Genomic DNA was extracted from peripheral leukocytes using proteinase K/phenol/chloroform purification, followed by ethanol precipitation, and stored in 10 mmol/L Tris-HCl, 1 mmol/L Na_2-EDTA, pH 8.0.

Anthropometric and Biochemical Measurement

Blood pressure was measured in the seated position after 10 min of rest using a mercury sphygmomanometer by experienced and certified examiners. Body weight and height were measured with subjects dressed in light indoor clothing and without footwear. Body mass index (BMI) was calculated as (weight in kg)/(height in m)^2. Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLC), blood urea nitrogen (BUN) and serum creatinine (Scr) concentrations were determined enzymatically using commercially available kits and an auto-analyzer (The First Hospital of Fangshan District, Beijing, China).

Genotyping Analysis

The genotypes of *AGT* polymorphisms were detected by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). With respect to the M235T variant, the sequence of the forward primer was 5′-GATGCGCAC AAGGTCCTG-3′, and the sequence of the reverse primer was 5′-CAGGGTGCTGTCCACACTGGCTCGC-3′. PCR was carried out in 25-μL reactions including 10 pmol of each primer, 200 μmol/L deoxynucleoside triphosphates (dNTPs), 2.0 mmol/L $MgCl_2$, 2.5 mmol/L KCl, and 0.5 units *Taq* DNA polymerase. Amplifications were done in a PTC-200 MJ Research Peltier Thermal Cycler (Perkin-Elmer Corp., Foster City, USA) for 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The restriction enzyme Sfa NI (Promega, Beijing, China) recognized the amplified fragment (303 bp) when M235 was present (266 bp and 37 bp), and not when T235 was present. After restriction enzyme treatment, the reaction mixture was separated on 2% agarose gels. The A−20C and A−6G polymorphisms were genotyped according to a previous report (*18*). Moreover, the accuracy of our screening method was confirmed by direct sequencing (BGI LifeTech Co., Ltd., Beijing, China) of amplified DNA from randomly selected samples (10%), and no difference in results was found between the two methods (data not shown).

Statistical Analysis

Simple statistical analysis was performed using SAS version 9.1.3 (SAS Institute Inc., Cary, USA). Means of quantitative variables were compared by unpaired *t*-test. The χ^2 test or Fisher's exact test was used to assess the goodness-of-fit between the observed allele frequencies and the expected counterparts by Hardy-Weinberg equilibrium and to evaluate the differences in genotype and allele distributions between hypertensives and normotensives. Each genotype was

Table 3. The Pairwise Linkage Disequilibrium Analysis

D'/r^2	A−20C	A−6G	M235T
A−20C	1/1	0.691/0.444	0.731/0.463
A−6G	0.552/0.256	1/1	0.551/0.244
M235T	0.762/0.455	0.607/0.344	1/1

The upper triangular data denoted the pairwise linkage disequilibrium coefficients in hypertensives and the lower triangular data in normotensives.

assessed by logistic regression analysis under assumption of additive (major homozygotes *vs*. heterozygotes *vs*. minor homozygotes), dominant (major homozygotes *vs*. heterozygotes/minor homozygotes) and recessive (major homozygotes/heterozygotes *vs*. minor homozygotes) genetic models. For each odds ratio (OR), we calculated the 2-tailed probability value and 95% confidence interval (CI). Quantitative variables were expressed as the mean±SD, and two-sided p values <0.05 were considered statistically significant.

Haplotype frequencies were estimated using the EH/EH+ program (*19*). We compared haplotype frequencies between hypertensives and normotensives by χ^2-test from a series of 2×2 contingency tables by combining other haplotypes. A probability value less than 0.00625 (0.05/8) was considered statistically significant after Bonferroni correction in haplotype-based multiple testing. The pairwise linkage disequilibrium coefficient was calculated with estimated haplotype frequencies using the 2LD program. The extent of disequilibrium was expressed in terms of D' and r^2.

The evaluation of epistasis or gene-gene interactions was performed using the open-source multifactor dimensionality reduction (MDR) software package (v.1.0.0) (*20*, *21*) available from http://www.epistasis.org/ With MDR, multilocus genotypes are pooled into high risk and low risk groups, effectively reducing the dimensionality of the genotype predictors from N dimensions to one dimension. The new one-dimensional multilocus genotype variable was evaluated for its ability to classify and predict disease status using cross-validation and permutation testing. A naïve Bayes classifier in the context of 10-fold cross-validation was used to estimate the testing accuracy of each best one-, two-, and three-factor model. The model with maximum testing accuracy and cross-validation consistency was chosen as the best model. Statistical significance was evaluated by comparing the average testing accuracy from the observed data with the distribution of average testing accuracies under the null hypothesis of no association derived empirically from 1,000 permutations. The null hypothesis was rejected when the Monte Carlo probability value derived from the permutation test was 0.05 or lower. The combination of cross-validation and permutation testing corrects for multiple testing. Furthermore, measures of interaction information were used to provide a statistical interpretation of gene-gene interaction models and interaction dendrograms were used to visualize the nature of the dependencies.

Results

The demographic and clinical characteristics of the study population are shown in Table 1. The BMI, SBP, DBP, TC, BUN and Scr levels were significantly higher in hypertensives than in normotensives (all $p<0.01$). No differences were found in age and plasma TG and HDLC levels between the two groups.

Table 2 showed the distributions of genotypes and alleles for each of the three *AGT* polymorphisms studied, along with the OR for essential hypertension. There was no detectable deviation from Hardy-Weinberg equilibrium for the three studied polymorphisms in either group. In single-locus analysis, except for a marginal, significant trend of an association between the A−20C allele distribution and hypertension, no significant association in relation to genotype or allele distributions was found between hypertensive and normotensives. The frequency of the −20A allele was significantly higher in hypertensives than in normotensives ($p=0.0380$). In logistic regression analysis, there was a significant decrease in the OR (OR=0.70, 95% CI 0.51–0.96, $p=0.0254$ for the additive model; OR=0.69, 95% CI 0.49–0.98, $p=0.0365$ for the dominant model and OR=0.46, 95% CI 0.13–1.66, $p=0.2384$ for the recessive model) for essential hypertension under the assumption of additive, dominant, or recessive mode of inheritance.

To understand the relationships among the three polymorphisms genotyped in *AGT*, we first assessed the pairwise linkage disequilibrium measured by D'. Note that in Table 3, there was moderate linkage disequilibrium between A−20C and M235T polymorphisms ($D'=0.731$ in hypertensives and $D'=0.762$ in normotensives). The degree of linkage disequilibrium for the A−20C and A−6G polymorphisms was stronger in hypertensives than in normotensives (D': 0.691 *vs*. 0.552), and the reverse was found for the A−6G and M235T polymorphisms (D': 0.551 *vs*. 0.607).

Table 4 shows the results of the haplotype analysis for the three polymorphism combinations. The omnibus haplotype test showed significant association with essential hypertension ($\chi^2=16.27$, $p=0.0227$), indicating that the overall haplotype frequency difference between hypertensives and normotensives was significant, and thus there might exist some disease-predisposing or disease-protective haplotypes. Accordingly, the frequency of haplotype H_1 (A-A-T) ($p=0.0056$) was significantly higher, while the frequencies of H_4 (A-G-M) ($p=0.0381$) and H_5 (C-A-T) ($p=0.0063$) were significantly lower in hypertensives than that in normotensives. However, the results were not statistically significant after the stringent Bonferroni correction for probability value was executed ($p>0.05/8$ [eight individual haplotype analyses were performed]), except in the case of the H_1 haplotype.

Table 5 summarizes the results of the exhaustive MDR analysis that evaluated all possible combinations of the studied polymorphisms. The best model of each order is shown

Table 4. Haplotype Structure and Frequencies of the *AGT* Gene in Both Groups

Haplotype	A−20C, A−6G, M235T	Hypertensives (%)	Normotensives (%)	df	χ^2	p*
H_1	A-A-T	71.89	64.15	1	7.67	0.0056
H_2	A-A-M	4.61	5.71	1	0.69	0.4053
H_3	A-G-T	3.81	5.15	1	1.19	0.2764
H_4	A-G-M	0.52	1.80	1	4.30	0.0381
H_5	C-A-T	2.51	5.66	1	7.48	0.0063
H_6	C-A-M	3.88	4.27	1	0.11	0.7425
H_7	C-G-T	2.38	1.89	1	0.30	0.5825
H_8	C-G-M	10.41	11.37	1	0.27	0.6063
	ln (L)‡	−568.83	−440.65	7	16.27	0.0227†

*p was calculated using χ^2-test from a serious of 2×2 contingency tables by combining other haplotypes. †p was calculated using χ^2-test 8×2 contingency table with degree of freedom (df) of 7. ‡Likelihood ratio statistic for omnibus test in the EH/EH+ program.

Table 5. The MDR Analysis Summary

Polymorphisms included in the best combination	Testing accuracy	Cross-validation consistency	p-value
A−20C	0.543	8	0.0547
A−20C, A−6G	0.564	10	0.0010*
M235T, A−20C, A−6G	0.533	9	0.0107

*Overall best MDR model.

along with its testing accuracy, cross-validation consistency and significance level as determined by permutation testing. The overall best MDR model included the A−20C and A−6G polymorphisms. This model had a maximum testing accuracy of 0.564 and a maximum cross validation consistency of 10 out of 10. This model was significant at the 0.001 level, which indicates that a model this good or better was observed only one time out of 1,000 permutations and is thus unlikely under the null hypothesis of no association.

Figure 1 summarizes the interaction information analysis for the studied polymorphisms. Shown is an interaction dendrogram (left panel) highlighting the amount of information gained about the case-control status by putting all polymorphisms together using the MDR method. A red or orange line connecting two polymorphisms suggests a positive information gain that can be interpreted as a synergistic or nonadditive relationship, while a blue or green line suggests a loss of information that can be interpreted as a redundancy or correlation (*e.g.*, linkage disequilibrium). A yellow line indicates independence or additivity. The interaction information analysis indicated that polymorphisms A−20C and A−6G had a strong synergistic effect. The set including the M235 polymorphism and the set including the A−20C and A−6G polymorphisms were connected by an orange line, suggesting the effects of each set were also synergistic, although the synergism was somewhat weaker than that between the A−20C and A−6G polymorphisms.

The distribution of hypertensives and normotensives for the set comprising the A−20C and A−6G polymorphisms is depicted in Fig. 1, right. Note that the pattern of high-risk (dark gray) and low-risk (light gray) genotype combinations is nonlinear across the nine two-locus genotype cells. This constitutes evidence of an interaction, as suggested by the interaction information analysis.

Discussion

Numerous epidemiological studies have suggested that several genetic variants increase the risk for essential hypertension (*3*), but the genes underlying the genetic susceptibility to this condition remain to be identified definitively. Previous studies in the field of transcriptomics have elucidated that transgenic animals carrying *AGT* develop hypertension and the more copies of *AGT* the higher the blood pressure, suggesting a correlation among *AGT* expression, plasma AGT concentrations and blood pressure (*7*, *22*). In addition, large numbers of linkage analyses and association studies have demonstrated that variations in *AGT* are correlated with variations in plasma AGT levels and with risk of hypertension (*8*, *23–25*). However, the results of these studies have been inconsistent, with no consensus on their implications, mainly because most studies have focused on the single-locus effect and disregarded epistasis, a phenomenon whereby the effects of a given gene/locus on a biological trait are masked or enhanced by one or more other genes/loci (*26*). Indeed, there is increasing evidence that epistasis or gene-gene interactions play an important role in determining an individual's risk of complex diseases (*27*, *28*). Because of the multifactorial nature of essential hypertension, the synergistic effects of candidate genes—that is, the interactions of genetic variants with one another—are the rule rather than the exception in determining the observed phenotype (*12*).

Bearing this in mind, in the present study, we hypothesized that interactions between polymorphisms of the *AGT* may have an independent or synergistic effect on the pathogenesis

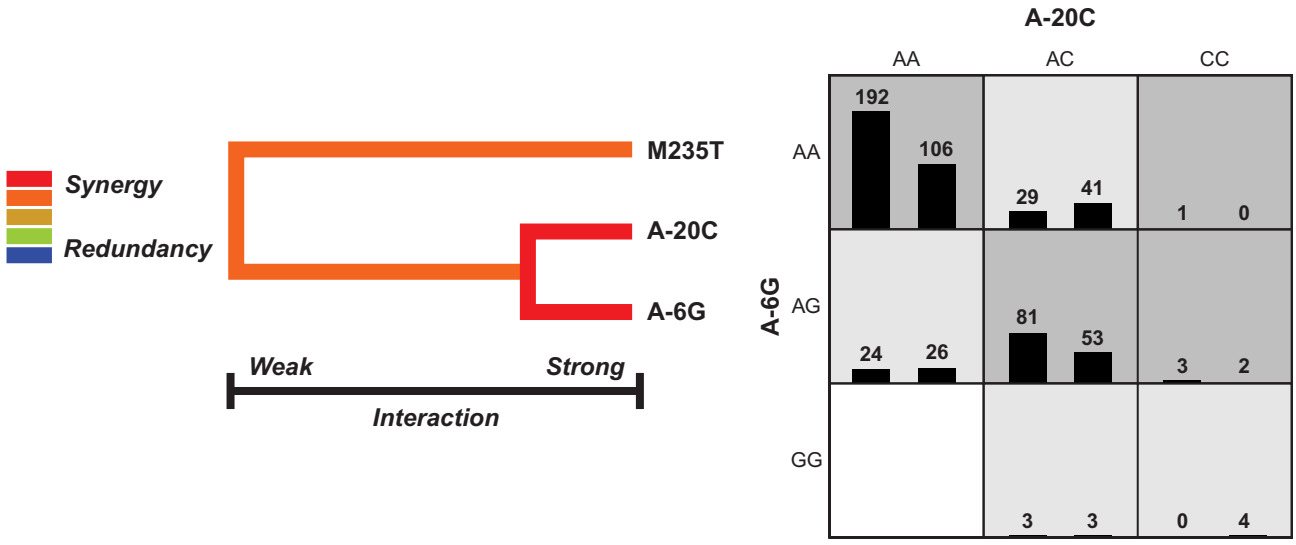

Fig. 1. *Interaction dendrogram for the three polymorphisms modeled by MDR (at left). Note the strong synergistic (i.e., nonadditive) effects of the A−20C and A−6G. These are the two polymorphisms that comprise the overall best MDR model. Also shown are the distribution of cases (left bars) and controls (right bars) for each genotype combination in each pair of interacting polymorphisms (at right). Boxes were labeled as high-risk if the ratio of the number of hypertensives to normotensives met or exceeded the threshold of 1.42 (333/235), and boxes were labeled as low-risk if the threshold was not exceeded. Note the nonlinear patterns of high-risk (dark gray) and low-risk (light gray) genotype combinations indicative of interaction.*

of hypertension, and therefore may explain differences in an individual's genetic susceptibility. We explored the contributions of single-locus, haplotype and epistasis patterns of three *AGT* polymorphisms to the risk for essential hypertension in a genetically homogeneous Tibetan population by using the classic EH/EH+ program, which is a likelihood method based on haplotype frequencies for depicting allelic associations, and a promising new data–mining approach, multifactor dimensionality reduction or MDR (*20*, *21*), which seeks to reduce the dimensionality of multilocus genotype space to facilitate the identification of gene-gene interactions.

Firstly, we found that except for a marginal association of A−20C allele distribution and hypertension, no significant association in relation to genotype or allele distributions of the studied polymorphisms was found between hypertensives and normotensives. Although no significant association was found between the M235T polymorphism and essential hypertension, this polymorphism could be associated with essential hypertension through moderate linkage with the A−20C or A−6G polymorphism. Therefore, we cannot rule out the possibility that M235T is linked to other loci with functional significance. Moreover, our results differed from those in previous studies that reported a positive association (*8*, *29*), but agreed with those in other studies that reported no association (*30*, *31*). Ethnic differences might be partially responsible for the observed discrepancies in the hypertension risk profile. As exemplified by the M235T polymorphism, the 235T allele varies widely in frequency across different entire populations, occurring in 35%–50% of whites (*8*, *23*), 75%–85% of African Americans (*32*), 75%–90% of Asians (*24*, *30*, *31*, *33*), and ≥90% of Africans (*11*, *34*). Hypothetically, the role played by *AGT* in hypertension might be different among populations with different genetic contexts of *AGT*. In view of this, it is important to construct a database of the polymorphisms potentially related to essential hypertension in each ethnic population. In addition, another possible explanation for the above-described discrepancies is that the underlying genetics of hypertension may not be based on single genes that exert major effects, but on interactions among genetic and environmental factors (*35*). Each of these factors could hinder the detection of a modest contribution of an individual locus to a trait such as hypertension.

Secondly, although our single-locus analysis did not provide substantial evidence of an association between the studied polymorphisms and essential hypertension, the more powerful haplotype analysis suggested an association between *AGT* and essential hypertension. Haplotype analysis is thought to be useful for the identification of not only rare disease genes but also common disease genes, and is considered more powerful than the analysis of a single polymorphism (*36*). Our haplotype analysis indicated that the H_1 haplotype may be the risk-conferring factor for hypertension predisposition, even after the rigorous Bonferroni correction. A previous study examining the haplotypes of A−20C and A−6G polymorphisms suggested that haplotype frequencies in white males differed significantly between hypertensives and normotensives, with the (−20)A-(−6)A haplotype being significantly more frequent in hypertensives (*18*), which was

consistent with our results. Further, another study performed by Gu *et al*. indicated that genetic variants in the regulatory regions of *AGT* showed a strong association with blood pressure reactivity (*37*). In addition, these authors revealed the collective interaction of promoter and genic polymorphisms in *AGT* on the blood pressure reactivity and BMI both in blacks and whites. Moreover, we further noted that the linkage patterns were heterogeneous among different ethnic populations. For example, the A−6G and M235T polymorphisms have been shown to be in complete linkage disequilibrium in white subjects (*8*), while they were in moderate linkage disequilibrium in the present Tibetan population. Given the importance of natural selection in human evolution, haplotype structures or haplotype frequencies in *AGT* might be dissimilar among populations of different origin (*38*), even among populations with similar distributions of linkage (*39*). Although haplotype analysis preponderates over single-locus analysis, it may represent a first step in detecting and characterizing the complexity of common diseases such as hypertension. The interpretation of single-approach results cannot be extrapolated without further validation among different populations or using different analytical approaches.

Thirdly, as the cost of genotyping continues to plummet, one of the greatest challenges facing human geneticists is the identification and characterization of susceptibility genes for common multifactorial human diseases. Due to the large number of potential genotypes along with the increasing number of possible combinations, it may become impossible to recruit enough subjects into epidemiological studies to represent every possible genotype combination. This problem has been referred to as the "curse of dimensionality" (*40*). To overcome this limitation, the MDR method was developed and has been successfully applied to detecting gene-gene and gene-environment interactions for a variety of clinical endpoints (*41–44*). Accordingly, we adopted the MDR method to explore the synergistic effects of the studied polymorphisms on susceptibility to essential hypertension. Using MDR, we preliminarily selected the final, best model, which included the A−20C and A−6G polymorphisms, by evaluating the magnitude of cross-validation consistency and prediction error. Since the two selected polymorphisms were not associated with essential hypertension, there is evidence for the existence of epistasis between the two polymorphisms. Taken together, under the epistasis model, we found a strong synergistic effect among apparently dissociated polymorphisms, which was beyond the power of single-locus analysis. Thus, we here preliminarily hypothesize that interactions between the A−20C and A−6G polymorphisms in *AGT* promoter might affect the transcription of the gene and/or the stability of the resulting mRNA, and thus play an important role in the pathogenesis of essential hypertension. This issue is beyond the scope of our present study, and will require further investigations from the biological and functional points of view, which are still ongoing by our group.

Several strengths and limitations of the study should be acknowledged. The strengths of the present study are as follows. 1) Our study had a door-to-door cross-sectional design, as previously described (*16*, *17*), and thus differed from a study with a hospital-based design which runs the risk of population admixture and stratification. 2) The study population was genetically homogeneous due to a relatively low rate of migration and lack of intermarriage with other ethnic groups over the course of hundreds of years. Moreover, the enrolled Tibetans were all permanent residents of the city of Lhasa, which has an altitude of 3,700 m above sea level (see Methods). 3) In light of the potential for mistyping of *AGT* genotypes, as described by Lizanecz *et al*. (*45*), we checked the accuracy of our RFLP screening method by randomly sequencing 10% of the selected samples. The 100% correspondence in results between the two methods indicated that the possibility of mistyping was unlikely. 4) In this study, we adopted the haplotype analysis for the multi-allelic association and a promising data–mining method, MDR, for overcoming the limitations of traditional parametric statistical approaches for the detection, characterization and interpretation of epistasis.

However, the present study also had some notable limitations. Because the study had a cross-sectional design, it inevitably suffered from the limitations of this type of study, *i.e.*, the inability to prove the existence of a cause-effect relationship. In addition, we only genotyped three polymorphisms of *AGT* and did not examine other candidate genes that might be associated with hypertension. Moreover, the polymorphisms selected in our study do not cover the gene fully and extensively. Since hypertension is multifactorial in nature, the use of association studies in large epidemiological cohorts with a large number of polymorphisms throughout the entire genome or throughout a single gene is a new strategy for identifying genes that contribute to hypertension susceptibility, and such a study will be needed to confirm any results from the present analysis. In addition, given the sample size of the study population, our results should be considered preliminary and cannot be directly extrapolated to define the contributions of these polymorphisms to hypertensive patients. Confirmation in a larger study is thus critical.

To sum up, the present study provided evidence of a strong synergistic effect between the A−20C and A−6G polymorphisms in *AGT*. Also, our observations leave open the question of whether the synergistic effect of the selected polymorphisms affects the transcription of *AGT* and/or the stability of the resulting mRNA. Further studies are warranted to examine this issue. Moreover, we have proposed a viable data–mining method to improve the identification of polymorphism combinations associated with hypertension risk.

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