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Three single-nucleotide polymorphisms of the angiotensinogen gene and susceptibility to hypertension: single locus genotype vs. haplotype analysis

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Wu, Shyh-Jong, Fu-Tien Chiang, Wei J. Chen, Pi-Hua Liu, Kwan-Lih Hsu, Juey-Jen Hwang, Ling-Ping Lai, Jiunn-Lee Lin, Chuen-Den Tseng, and Yung-Zu Tseng. Three single-nucleotide polymorphisms of the angiotensinogen gene and susceptibility to hypertension: single locus genotype vs. haplotype analysis. *Physiol Genomics* 17: 79–86, 2004. First published February 17, 2004; 10.1152/physiolgenomics.00133.2003.—Although some single polymorphism analyses of the angiotensinogen (AGT) gene have been found to be associated with hypertension, the results are still inconsistent. The objectives of this study are to evaluate the association of the genotype and haplotype distributions of three single-nucleotide polymorphisms (SNPs) (G−217A, A−6G, and M235T) in the AGT gene with hypertension. In a sample of 461 hypertensive and 327 normotensive patients in Taiwan, we found that −217AA and −6GG homozygotes conferred independently an increased risk to hypertension ($P = 0.008$ and $P = 0.037$, respectively), as illustrated by their significant associations with hypertension in both single SNP and pair-wise SNPs analyses. Meanwhile, a very weak linkage disequilibrium was found between the G−217A and the A−6G polymorphisms in terms of r^2 (<0.05). On the basis of likelihood ratio test, only the set of haplotypes that constituted the A−6G and the M235T polymorphisms was associated with hypertension ($\chi^2 = 20.91$, $P = 0.0008$), which was mainly due to the increased frequency of the recombinant haplotypes (−6A ≡ 235M and −6G ≡ 235T), and a pathophysiological role in the predisposition to hypertension was hence indicated. In functional assays, the promoter activities of the haplotypes −217A ≡ −6A and −217G ≡ −6G were significantly higher than the most common haplotype −217G ≡ −6A. These results highlight the necessity of a thorough analysis of all reported variants of a candidate gene in the elucidation of genetic susceptibility to a complex disease like hypertension, even when the variants are in the same haplotype block.

renin-angiotensin system; genetic polymorphism; linkage disequilibrium; recombination; transcriptional activity

HYPERTENSION is a complex disorder that is thought to result from an interplay between an individual's genetic background and various environmental factors (24). Genetic linkage and candidate gene association studies have implicated various loci and genes in predisposition to hypertension (16, 20, 25, 32, 37, 44, 46). However, an analysis of human genetic markers revealed that 8–10% of the genetic variance is found among continental population groups (34). Because of the ethnic divergence of gene polymorphisms, consistent associations have been difficult to demonstrate, at least up to the present.

The primary genes that comprise the renin-angiotensin system (RAS) have been the focus of an enormous number of association studies over the last decade, especially those associated with hypertension. Angiotensinogen is the precursor of angiotensin II, which acts as a physiologically important regulator of blood pressure and electrolyte homeostasis, as well as a growth factor of cardiac myocytes. An angiotensinogen M235T polymorphism has been found to be associated with high plasma AGT levels in subjects that are homozygous for the T variant and hence associated with hypertension in some European and Asian populations (7, 16), but not all populations (3, 18, 35, 38). The inconsistencies of gene association studies by a single-nucleotide polymorphism (SNP) analysis observed in different populations demonstrate the challenges of dissecting complex multifactorial traits like essential hypertension.

One possible way to surpass the limitation of previous association studies is to look for susceptible haplotypes of the AGT gene for hypertension rather than focusing on the M235T polymorphism solely. The promoter polymorphisms A−6G and G−217A, which may play regulatory roles in the AGT gene expression (12, 13, 43, 45), were suggested to be located in the same haplotype block with the M235T polymorphism (46, 47). Whether these SNPs could be treated as a haplotype tag to investigate the association with a complex disease remain unresolved issues. Recent studies have revealed that nucleotides form haplotype blocks that are interspersed with presumed recombination hotspots (14, 40) in the genome of humans and other species. Additionally, there is great interest in the identification of susceptible genes underlying the complex disease through whole genome linkage disequilibrium (LD) mapping using comparisons of allele frequencies in affected and unaffected individuals with SNPs (1, 17, 21, 33). However, the feasibility of such research is currently under debate, and patterns of LD are well known for being noisy and unpredictable (17, 19, 33, 42). The haplotype blocks can span an interval ranging from <1 kb to 173 kb in length (6, 10). Recently, the diversity of haplotype structures of the human AGT gene between populations has come to light (27, 47). To date few studies have investigated how the actual combinations of these vital SNPs (G−217A, A−6G, and M235T) affect gene expression and whether the various haplotypes and genotypes

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of these polymorphisms conceal some pathophysiological or evolutionary roles in the human population. In this study, we aimed to elucidate the biological meaning of the positional relationship among the G−217A, A−6G, and M235T polymorphisms of the AGT gene by comparing the magnitude of LD, the haplotype structure, and the genotype distribution of the three polymorphisms between the hypertensive and normotensive subjects in a Taiwanese population. We also examined the transcriptional activity of different haplotype structures of the AGT promoter in an in vitro system.

MATERIALS AND METHODS

Subjects. A detailed description of the patient collection has been published previously (4). In brief, hypertensive and normotensive patients were recruited from an outpatient clinic of the National Taiwan University Hospital from July 1995 through June 2002. They were so-called "Taiwanese" or "Taiwan Chinese." They mainly resided in the metropolitan area of Taipei city, which is located at the northern end of Taiwan. The diagnosis of essential hypertension was considered on the basis that no known cause of high blood pressure in the subjects could be detected after a complete clinical and biochemical examination. All the hypertensive subjects included in the present analyses met at least one of the following two criteria for the diagnosis of hypertension: prior diagnosis of hypertension by a physician and use of prescription antihypertensive medication, and/or having a systolic blood pressure (SBP) ≥140 mmHg and/or mean diastolic blood pressure (DBP) ≥90 mmHg. The blood pressure was measured by conventional mercury sphygmomanometer and determined by averaging two separate measurements taken 4 wk apart. Patients with diabetes mellitus and secondary etiologies of hypertension, such as renal vascular hypertension, or endocrine diseases, were excluded. Normotensive subjects who were apparently healthy on the basis of their clinical history and laboratory tests (with SBP/DBP less than 140/90 mmHg) were recruited. In total, 461 hypertension patients and 327 normotensive subjects were enrolled in this study. All subjects gave informed consent as approved by the Institutional Review Board at the National Taiwan University Hospital. The demographic characteristics of all participants are shown in Table 1.

Genomic DNA preparation and genotyping. Genomic DNA extraction and genotyping was performed as described previously (4). Briefly, genotyping for the molecular variants M235T, A−6G, and G−217A was conducted by direct sequencing using a dye-terminator cycle sequencing method (Perkin-Elmer, Foster City, CA) and performed on an automatic sequencing apparatus (ABI Prism 373A sequencer, Perkin-Elmer). The primers used for polymerase chain reaction (PCR) and DNA sequencing of M235T were as follows: forward primer, 5′-GAT GCG CAC AAG GTC CTG TC-3′; reverse primer, 5′-GCC AGC AGA GAG GTT TGC CT-3′. Another primer pair for A−6G and G−217A was as follows: forward primer, 5′-CTG TGC TAT TGT TGG TGT T-3′; reverse primer, 5′-GCT TAC CTT CTG CTG TAG T-3′. The reverse primers were used as PCR sequencing primers. The results were analyzed using incorporated sequencing analysis software (Perkin-Elmer).

Construction of expression variants. The reporter plasmid p−217G−6A and p−1222SEAP were constructed by ligating −614 to +41 and −1222 to +41 DNA fragment of the promoter region of the human AGT gene, respectively, to the front of the secreted human placental alkaline phosphatase (SEAP) gene in the expression vector pSEAP2-Basic (BD Biosciences Clontech, Palo Alto, CA). Serial deletion mutants in the 5′-flanking region of p−1222SEAP were constructed by using the Exonuclease III digestion method (Erase-a-Base System, Promega), as shown in Fig. 1. All inserts and mutants were verified by DNA sequencing. Site-directed mutagenesis at the −6 and −217 sites of the −614 to +41 AGT promoter was carried out by a Quick Change Site-Directed Mutagenesis method (Stratagene, Amsterdam, The Netherlands). The primers for A−6G were as follows: forward primer, 5′-TGA CCC GGC CAG GGG AAG AAG-3′; reverse primer, 5′-TTC TTC CCC CGG CCG CCT CAC-3′. The primers for G−217A were as follows: forward primer, 5′-CCC TGC ACC AGC TCA CTC TGT TC-3′; reverse primer, 5′-AGA GTG AGC TGG TGC AGG GTC-3′.

Cell culture and transient transfection. Human hepatoma cell lines HuH-7 (26) and HepG2 were maintained in Dulbecco's modified Eagle's media (DMEM) with 10% fetal calf serum (GIBCO-BRL; Invitrogen, Grand Island, NY) and cultured at 37°C under an atmosphere containing 5% CO_2. Plasmid DNA for transfection was prepared using Qiagen columns (Qiagen, Hilden, Germany). DNA concentrations were determined by spectrophotometry and checked by gel electrophoresis and ethidium bromide staining. The cells were freshly subcultured at a density of 5×10^5 cells per dish on 60-mm plastic dishes. They were transfected 24 h later using a transient liposome cotransfection protocol (TransFast Transfection Reagent; Promega, Charbonniere-les-bains, France) with 4 μg of reporter plasmids and 1 μg of pCAT control vector for chloramphenicol acetyltransferase (CAT) expression as an internal control to normalize efficiency of transfection. For each construct, the experiments were performed in quadruplicate. Twenty-four hours after transfection, the medium was changed to a fresh complete medium with the same volume per dish. After another 48 h, the conditioned cell culture medium was collected for measurement of SEAP activity of each construct by Great EscAPe SEAP Chemiluminescence Detection (BD Biosciences Clontech) and Luminometer (Labsystems Luminoskan). The CAT activities of the cell extracts were measured using a CAT ELISA (Roche Diagnostics, Meylan, France).

Statistics. The relationships between various genotypes and hypertension were examined by using logistic regression analyses, in which hypertension status was treated as the dependent variable, individual genotypes as the independent variables, and sex and age as covariates. Hardy-Weinberg equilibrium (HWE) was assessed using the χ^2 test. Estimation of haplotypes frequency and testing whether there was a significant LD were performed using the Stata command "hapipf" with the Stata package (**http://www.stata.com**). Potential associations between the set of haplotypes and hypertension status were examined by employing an omnibus likelihood ratio test with permutation-based hypothesis testing procedures as described by Fallin et al. (8). Following the suggestion in a recent review (42), two important pairwise measures of LD, $|D'|$ (22, 23) and r^2 (11), were then calculated using the Stata command "pwld" (**http://www-gene.cimr.cam.ac.uk/clayton/software/**). Except where otherwise noted, high values of LD were defined as $r^2 > 1/3$ as suggested by Ardlie et al. (2) or as $|D'| > 0.7$ as suggested by Gabriel et al. (10).

Two-way analysis of variance (ANOVA) and Student's *t*-test were performed to compare the expression activity of reporter constructs in at least four independent transfection experiments. Data are expressed as means ± SE, and a P value of <0.05 was considered statistically significant.

Table 1. *Baseline characteristics of the study participants*

Characteristic	Hypertensive Subjects (n = 461)	Normotensive Subjects (n = 327)	P
Sex, men/women	292/169	199/128	0.48
Age, yr	59.1±10.1	54.3±14.7	<0.001
BMI, kg/m^2	25.2±3.3	23.6±3.4	<0.001
SBP, mmHg	155.8±15.4	114.7±14.5	<0.001
DBP, mmHg	94.2±10.3	72.4±7.7	<0.001

Values are means ± SD, where indicated. BMI, body mass index. SBP, systolic blood pressure. DBP, diastolic blood pressure.

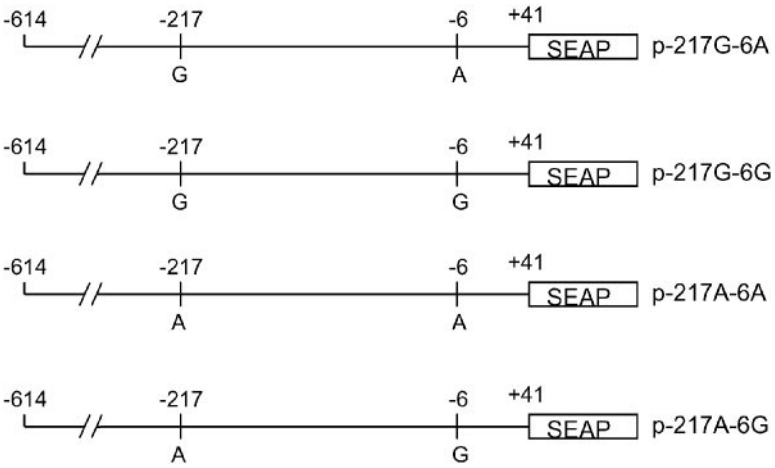

Fig. 1. Construction of expression vector containing angiotensinogen (AGT) promoter-SEAP fusion genes. The −614 to +41 DNA fragment of the AGT gene was cloned and inserted into the pSEAP-Basic reporter plasmid in front of the SEAP gene, p−217G−6A. Site-directed mutagenesis was performed at the −6 and −217 positions, and both positions of the constructed vectors formed p−217G−6G, p−217A−6A, and p−217A−6G, respectively. SEAP, secreted human placental alkaline phosphatase.

RESULTS

Genotypes associated with hypertension. The relations between the genotypes of each of the three AGT gene SNPs and hypertension status and the test of HWE are shown in Table 2. All the three SNPs were not deviant from HWE, with the exception of the G−217A and A−6G in the hypertensive group. The logistic regression analyses found that the risk of hypertension was significantly higher for the least common homozygotes −217AA and −6GG, but only borderline for the 235MM, compared with the most common genotype in each polymorphism. Meanwhile, the risks for the heterozygotes were not different from that of the most common homozygotes (odds ratio around 1). When the three genotypes were reduced to two by treating the least common genotype as the recessive one, similar results were obtained for each polymorphism. Thus a recessive model was adopted for the subsequent genotype analyses.

To determine whether the three recessive homozygotes of the AGT gene are independently associated with hypertension, logistic regression analyses of two-SNP genotypes for the risk of hypertension were further conducted by reducing the genotypes of each SNP into those with the risk recessive genotype or not (Table 3). For the simplification of notation, the risk genotype in each SNP is denoted as $-217AA^{+}$, $-6GG^{+}$, and $235MM^{+}$, respectively, while the rest of nonrisk genotypes are pooled as $-217AA^{-}$, $-6GG^{-}$, and $235MM^{-}$, correspondingly. Compared with the doubly nonrisk genotype in each pair of SNPs, three of the two-SNP genotypes were found to have significantly higher risk for hypertension: $-217AA^{-}/-6GG^{+}$, $-217AA^{+}/-6GG^{-}$, and $-217AA^{+}/235MM^{-}$. That is, two

Table 2. *One-polymorphism genotype distribution of the AGT gene in hypertensive and normotensive subjects*

Genotype	Hypertensive Subjects, *n*	Normotensive Subjects, *n*	OR (95% CI)	
G−217A				
GG	317 (69.5%)	237 (72.9%)	1.0	
GA	112 (24.6%)	82 (25.2%)	1.04 (0.74–1.45)	0.901
AA	27 (5.9%)	6 (1.9%)	3.71 (1.56–8.82)	0.008
HWE	$\chi^2 = 13.99$†	$\chi^2 = 0.13$		
Recessive model				
GG and GA	429 (94.1%)	319 (98.2%)	1.0	
AA	27 (5.9%)	6 (1.9%)	3.38 (1.37–8.38)	0.008
A−6G				
AA	316 (68.8%)	229 (70.5%)	1.0	
AG	121 (26.4%)	90 (27.7%)	0.94 (0.71–1.34)	0.874
GG	22 (4.8%)	6 (1.8%)	2.67 (1.06–6.66)	0.037
HWE	$\chi^2 = 5.15$*	$\chi^2 = 0.71$		
Recessive model				
AA and AG	437 (95.2%)	319 (98.2%)	1.0	
GG	22 (4.8%)	6 (1.8%)	2.64 (1.04–6.74)	0.042
M235T				
TT	320 (70.6%)	231 (71.1%)	1.0	
TM	115 (25.4%)	89 (27.4%)	0.93 (0.67–1.30)	0.674
MM	18 (3.8%)	5 (1.5%)	2.60 (0.95–7.10)	0.063
HWE	$\chi^2 = 3.36$	$\chi^2 = 1.19$		
Recessive model				
TT and TM	435 (96.0)	320 (98.5)	1.0	
MM	18 (4.0)	5 (1.5)	2.49 (0.89–6.94)	0.082

Odds ratio (OR) is estimated from logistic regression adjusted for sex and age (95% CI is in parentheses). HWE, Hardy-Weinberg equilibrium. OR, odds ratio; CI, confidence interval. *$P < 0.05$. †$P < 0.01$.

Table 3. *Odds ratio of double-recessive genotypes of the two AGT SNPs for the risk of hypertension*

G−217A/A−6G				A−6G/M235T				G−217A/M235T			
Genotype	Hyper, *n*	Normo, *n*	OR (95% CI)	Genotype	Hyper, *n*	Normo, *n*	OR (95% CI)	Genotype	Hyper, *n*	Normo, *n*	OR (95% CI)
AA^-GG^-	407 (89.3%)	313 (96.3%)	1.0	GG^-MM^-	426 (94.5%)	317 (98.1%)	1.0	AA^-MM^-	405 (90.2%)	312 (96.6%)	1.0
AA^-GG^+	22 (4.8%)	6 (1.9%)	2.8 (1.1–7.1)*	GG^-MM^+	4 (0.9%)	0		AA^-MM^+	18 (4.0%)	5 (1.6%)	2.6 (0.9–7.3)
AA^+GG^-	27 (5.9%)	6 (1.9%)	3.5 (1.4–8.7)†	GG^+MM^-	7 (1.6%)	1 (0.3%)	6.8 (0.8–52.5)	AA^+MM^-	26 (5.8%)	6 (1.9%)	3.4 (1.4–8.4)†
AA^+GG^+	0	0		GG^+MM^+	14 (3.1%)	5 (1.6%)	1.8 (0.6–5.4)	AA^+MM^+	0	0	

Genotype in each single-nucleotide polymorphism (SNP) with superscript "+" indicates those persons who have the designated risk genotype, whereas genotype with superscript "−" indicates those without the risk genotype. For example, for the two-SNP genotype of G−217A/A−6G, AA^+GG^- includes those with genotypes of −217AA/−6AA or −217AA/−6AG; AA^-GG^+ includes those with −217GG/−6GG or −217GA/−6GG; whereas AA^-GG^- includes those with −217GG/−6AA, −217GG/−6AG, −217GA/−6AA, or −217GG/−6AG. Odds ratio (OR) is estimated from logistic regression with adjustment for sex and age. *$P < 0.05$. †$P < 0.01$. Hyper, hypertensive subjects. Normo, normotensive subjects.

promoter recessive genotypes, $-217AA^+$ and $-6GG^+$, seemed to have an independent association with hypertension, whereas the $235MM^+$ did not. Although this assertion was compromised by the nonsignificantly elevated risk of hypertension for $-6GG^+/235MM^-$, its odds ratio was very high, and the failure in reaching statistical significance was likely to be accounted for by its rare frequency.

Haplotypes associated with hypertension. Because the three SNPs examined in this study have been assigned to the same haplotype block, we first compared the three-SNP haplotypes distribution between the two groups. Judged from the likelihood ratio test, there was a significant LD among the three SNPs of the AGT gene in both groups as well as the total sample, and the set of haplotypes was found to be associated with hypertension (Table 4). However, to take a closer examination on whether there was unequal LD between SNPs, we also estimated the pairwise LD between the three SNPs (Table 5). In terms of $|D'|$, the LD in each pair was very strong in both the hypertensive and the normotensive subjects (all >0.8). However, in terms of r^2, only the LD between the A−6G and the M235T could be described as high; the r^2 values between the G−217A and the A−6G as well as between the G−217A and the M235T were very weak (both <0.05). Similarly, only the set of haplotypes composed of A−6G and M235T was associated with hypertension, whereas the other two pairs of two-SNP haplotypes did not exhibit such an association with hypertension.

Comparing the distribution of the haplotype of A−6G/M235T between the two groups of subjects, we found that haplotypes −6A ≡ 235T (denoted as AT) and −6G ≡ 235M (denoted as GM) were almost 99% in the normotensive subjects and hence can be treated as the wild-type haplotypes. In contrast, the frequencies of the recombinant haplotypes, −6A ≡ 235M (denoted as AM) and −6G ≡ 235T (denoted as GT), were much increased in the hypertensive patients. To illustrate the effect of these recombinant haplotypes, we divided the genotypes into those with wild-type haplotypes only and those with at least one recombinant haplotype and then conducted a simple logistic regression analysis (Table 6). Indeed, the risk of hypertension was increased four times more for those with at least one recombinant haplotype.

Transcriptional activity of different promoter haplotypes. A set of SEAP reporter plasmid was constructed by subcloning the 5′ franking promoter region of the AGT gene (−614 bp ~ +41 bp). Then three comparative promoters with one-point substitution were established by site-directed mutagenesis (Fig. 1), which resulted in four reporter constructs with a base difference at two positions, −217 and −6 of the AGT promoter. This promoter length was near the one that showed maximal expression activity, of which serial 5′ deletions of −1222/+41 AGT promoter region were constructed and assayed (Fig. 2). First, we performed a parallel transient transfection to assay the basal transcriptional activity of the different constructs in HuH-7 cell lines (Fig. 3*A*). The results showed

Table 4. *Frequency of three-polymorphism haplotypes of the AGT gene in hypertensive and normotensive subjects*

Haplotype			Overall	Hypertensive Subjects	Normotensive Subjects		
−217	−6	235	(*n* = 773)	(*n* = 450)	(*n* = 323)	χ^2	*P*
G	A	T	0.6551	0.6245	0.6966	8.65	0.001
G	A	M	0.0101	0.0163	0.0016	8.16	0.013
G	G	T	0.0209	0.0294	0.0093	7.40	0.004
G	G	M	0.1477	0.1476	0.1486	0.00	0.951
A	A	T	0.1629	0.1772	0.1440	3.04	0.054
A	A	M	0.0018	0.0031	0.0000	2.00	0.279
A	G	T	0.0000	0.0000	0.0000	0.00	0.816
A	G	M	0.0015	0.0019	0.0000	1.23	0.982
Test for LD							
LR[a] χ^2, df = 4			748.04	424.25	378.62		
P			$<10^{-5}$	$<10^{-5}$	$<10^{-5}$		
Case control comparison							
LR[b] χ^2, df = 7						26.88	0.0006

[a]Likelihood ratio (LR) test by comparing a model allowing for linkage disequilibrium (LD) vs. an independent model. [b]Omnibus likelihood ratio test for an association between the set of the haplotypes and hypertension status. *P* values are based on 10,000 permutations.

Table 5. *Frequency of two-locus haplotypes of the AGT gene and pairwise LD measures in hypertensive and normotensive subjects*

G−217A/A−6G				A−6G/M235T				G−217A/M235T			
Haplotypes				Haplotypes				Haplotypes			
−217	−6	Hyper	Normo	−6	235	Hyper	Normo	−217	235	Hyper	Normo
G	A	0.6399	0.6986	A	T	0.8033	0.8405	G	T	0.6558	0.7060
G	G	0.1781	0.1567	A	M	0.0171	0.0016	G	M	0.1616	0.1500
A	A	0.1803	0.1448	G	T	0.0304	0.0093	A	T	0.1772	0.1438
A	G	0.0017	0.0001	G	M	0.1491	0.1485	A	M	0.0054	0.0001
$\lvert D' \rvert$		0.9477	1			0.8746	0.9876			0.8218	1
R^2		0.0438	0.0315			0.6970	0.9192			0.0303	0.0297
Test for LD[a]		41.2 ($<10^{-5}$)	10.0 (0.016)			381.3 ($<10^{-5}$)	368.4 ($<10^{-5}$)			24.7 ($<10^{-5}$)	9.3 (0.002)
Case control comparison[b]		$\chi^2 = 6.27$, $P = 0.100$				$\chi^2 = 20.91$, $P = 0.0008$				$\chi^2 = 6.37$, $P = 0.126$	

[a]Likelihood ratio test by comparing a model allowing for LD vs. an independent model; χ^2 statistics and its corresponding P value are presented; all df = 1. [b]Omnibus likelihood ratio test for an association between the set of the haplotypes and hypertension status; P values are based on 10,000 permutations.

that the promoter activity of the p−217G−6A (the most common haplotype of this population) was significantly lower than those of p−217G−6G and p−217A−6A ($P < 0.001$ for both) in HuH-7 cells. It is worth noting that the haplotype containing two risk alleles in the reporter construct (p−217A−6G), which is extremely rare in the human population, also expressed higher basal transcriptional activity than the p−217G−6A one ($P < 0.001$), but no additional increase in the expression activity was found when compared with those haplotypes with single risk allele, i.e., p−217G−6G and p−217A−6A. Next, we further conducted the same experiments in HepG2 cells by only comparing the basal transcriptional activity of the three common haplotypes. Similar results were also found in HepG2 cells (Fig. 3*B*).

DISCUSSION

With the three polymorphisms of the AGT gene examined in this study, we found that the risk of hypertension increased significantly only for those with −6GG or −217AA homozygotes genotypes. In contrast, many previous studies examined the relationship between these polymorphisms and hypertension solely on allele frequencies. Among them, the results on the relations between hypertension and A−6G (or and M235T) have been conflicting (3, 16, 18, 28, 35, 38), whereas the relation between hypertension and G−217A in a previous study (13) was similar to our finding. Furthermore, genetic diversity may exist in different populations (27, 31, 39, 47) because of evolutionary factors, including natural selection, gene flowing, population subdivision, and gene recombination. For the human AGT gene, allele frequencies of the A−6G and M235T polymorphisms vary substantially in different populations, with the −6G and 235M being the minor alleles in African, Japanese, and Taiwanese populations (12, 13, 18) but not the minor ones in Caucasians (12, 13, 18, 27, 36).

Table 6. *Odds ratio of the two-locus A−6G/M235T genotypes for the risk of hypertension*

A−6G/M235T Genotypes	Hypertensive Subjects, *n*	Normotensive Subjects, *n*	OR (95% CI)
Group 1: Of wild-type haplotypes only (AT, GM)	408 (90.5%)	315 (97.5%)	1.0
Group 2: Containing at least one recombinant haplotype (AM, GT)	43 (9.5%)	8 (2.5%)	4.15 (1.92–8.95)

Group 1 includes AT/AT, AT/GM, and GM/GM. *Group 2* includes AT/AM, AM/AM, GT/AM, GM/AM, AT/GT, GT/GT, and GM/GT. Odds ratio (OR) was obtained from simple logistic regression without other covariates; 95% CI is in parentheses.

Compared with previous in vitro experiments, the association of −6GG with hypertension is different from the association with the −6A allele first reported by Inoue et al. (12), whereas the association of −217AA with hypertension is consistent with the association with the −217A allele first reported by Jain et al. (13). Nevertheless, our results are compatible with the recent findings that transgenic mice carrying the haplotype −6G ≡ 235M of the AGT gene had a small but significant increase in blood pressure and compensatory downregulation of endogenous renin expression in vivo (5). Another recent report also indicated that sitting SBP significantly increased in the 235MM homozygotes, which is in strong LD with −6G allele in various populations, in the control group after 6 yr (30).

Even though the molecular distance between A−6G and G−217A (211 bp) is very short, an unexpected result in this study is that examining the two polymorphisms individually revealed more information than by treating them as belonging to the same haplotype block. Furthermore, in assessing the magnitude of LD between two sites, we found that the pattern of $\lvert D' \rvert$ is different from that of r^2. Although the D' is widely used for the identification of haplotype blocks (29, 42), its value reaches boundary (1 or −1) if one haplotype is missing (2) and hence is less sensitive in distinguishing the different magnitudes of LD than is r^2, which reaches boundary only when two haplotypes are missing. In addition, for small to moderate sample sizes, estimates of D' can exhibit a considerable upward bias (41). This may explain the result that when the estimated $\lvert D' \rvert$ was 1 between the G−217A and the A−6G in the normotensive group, r^2 remained extremely low (<0.05). The reason of $\lvert D' \rvert$ equaling one might be mainly due to the almost nonexistence of the haplotype −217A ≡ −6G in the normotensive group. Similar upward bias of $\lvert D' \rvert$ was found

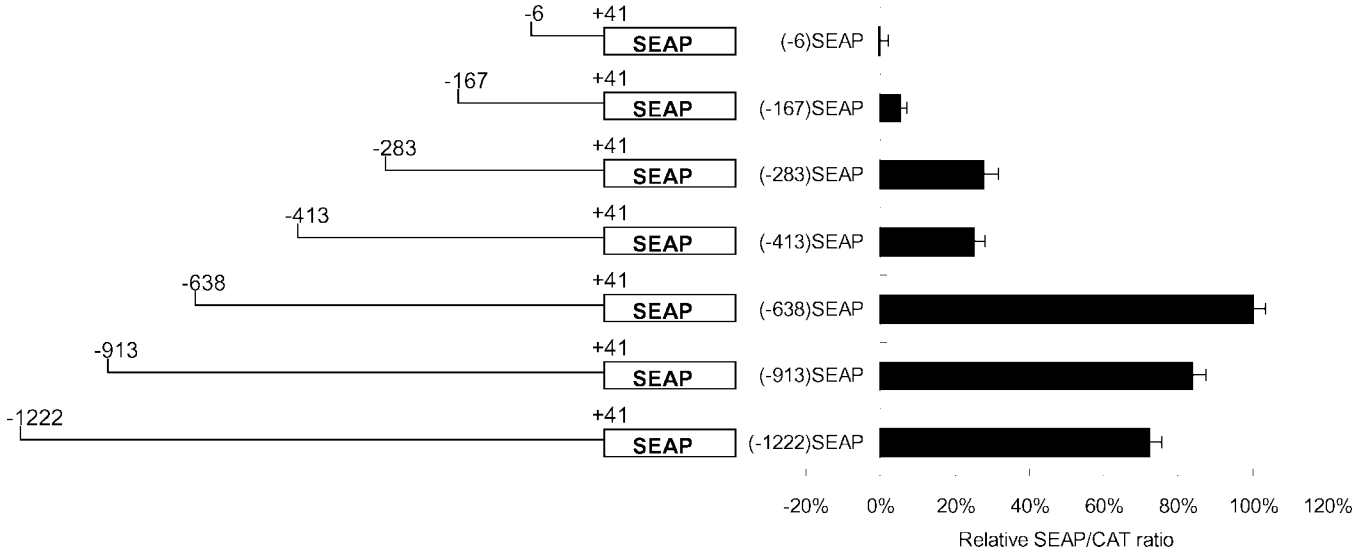

Fig. 2. Transient transfection of HuH-7 cells with deletion constructs of the 5′-flanking region of the human AGT promoter driving SEAP as a reporter gene. *Left*: schematic diagram of human AGT 5′-deletional constructs. *Right*: the means of relative SEAP/CAT ratios of each construct in at least three independent experiments are represented. For each construct, the experiments were performed in quadruplicate. CAT, chloramphenicol acetyltransferase.

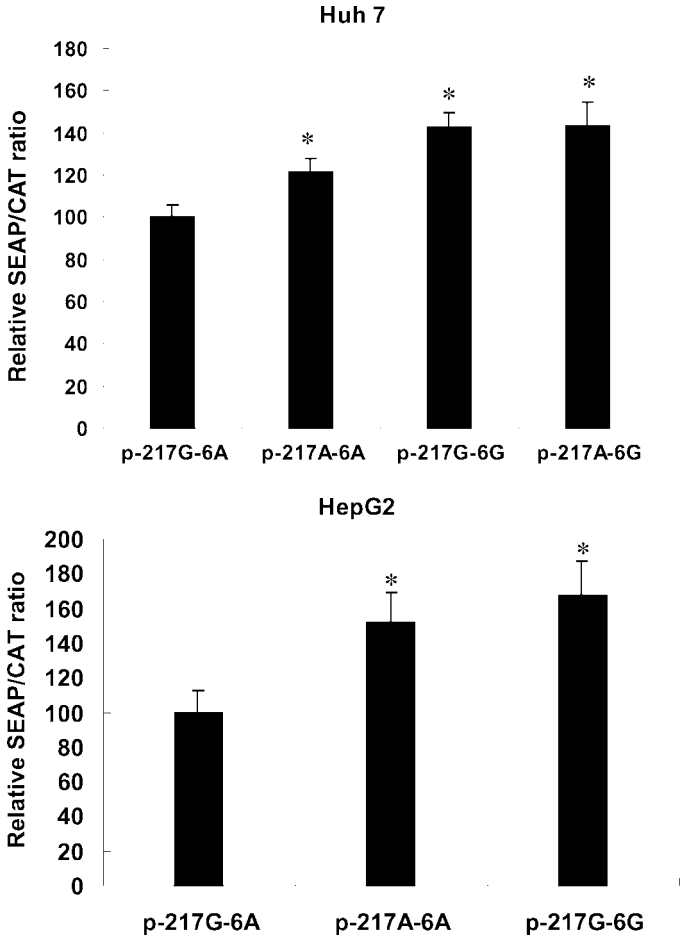

Fig. 3. Relative transcriptional activities of the different haplotype reporter constructs in HuH-7 and HepG2 cells. *A*: the means of relative SEAP/CAT ratios of four independent experiments are represented in HuH-7 cells. The reporter construct p−217G−6A is designated as 100. *$P < 0.001$ relative to p−217G−6A construct, ANOVA. *B*: The means of relative SEAP/CAT ratios of four independent experiments are represented in HepG2 cells. The reporter construct p−217G−6A is designated as 100. *$P < 0.001$ relative to p−217G−6A construct, ANOVA.

in a study examining the LD between the G−217A and M235T in African Americans, in which the $|D'|$ estimate was equal to 1 but was not statistically significant after adjusting for multiple tests (47). The same phenomenon was also observed in another report (27), although not discussed.

Under these circumstances, r^2 is a better indicator of LD. Pairwise r^2 values indicated that a strong LD existed only between the A−6G and the M235T polymorphisms. This strong LD was similar to those of previous reports in Caucasian and Japanese populations (15, 27, 47). However, a very weak LD existed between the G−217A variant and the other two SNPs, M235T and G−6A. There are two possible explanations for these observations. From the point view of reflecting recombination fractions, a low r^2 indicates that historical recombinations or a hotspot have occurred within such a near molecular distance in this promoter region. This would contradict the claim that the three SNPs were located in the same haplotype block (46, 47).

However, the other possibility is that both promoter variants were related to different functional expression pathways such that there was little association between the two in the evolution process. The A−6G variant is located in the region of AGCE1 element (AGT core promoter element 1; positions −25 to −1) that controls the basal transcriptional activity of the gene (45), whereas the G−217A variant is located in the potential hormonal responsive element (HRE), which could be further regulated by the stimulation of extraneous factors (13). Our results of in vitro assay did show that, compared with the wild haplotype, the increase in basal transcriptional activity of the p−217A−6A construct tended to be slightly lower than that of the p−217G−6G construct in both HuH-7 and HepG2 cells, although not reaching statistical significance. Because our assay did not employ extraneous stimulants, the construct of double risk alleles, p−217A−6G, did not exhibit an additional increase in the transcriptional activity. The possibility of different expression pathways is further implicated by our finding that the recessive genotypes −217AA and −6GG had

an independent association with hypertension. The latter finding further highlights the necessity of conducting both two-locus genotype analysis (Table. 3) and two-locus haplotype analysis (Table 5). Taken together, the two promoter polymorphisms are likely to play independent roles in regulating the whole AGT gene expression and should be examined separately rather than using one as representing the haplotype tag.

The departures from HWE of the G−217A and the A−6G in the hypertensive subjects were due to an excess of homozygosity (−217AA and −6GG). According to a recent simulation study that examined the impact of Hardy-Weinberg disequilibrium on the estimation of haplotype frequency via the expectation-maximization algorithm (9), the accuracy was little influenced by this kind of departure from HWE. Thus our results of haplotype analysis are unlikely to be affected by these violations of HWE.

Consistent with the proposition that only between the A−6G and the M235T existed a strong LD, only the set of haplotypes composed of these two SNPs was associated with hypertension. The major differences in the haplotype distributions of the two groups were mainly due to the increased frequency of the recombinant haplotypes (−6A ≡ 235M and −6G ≡ 235T) in the group of hypertensive patients, which led to its slightly lower value of $|D'|$ and r^2 compared with that of the normotensive subjects. Whether an increase in the recombination between the A−6G and M235T plays a pathophysiological role in the predisposition to hypertension warrants further investigation.

In summary, our analyses of population association indicate that the two promoter genotypes, −217AA and −6GG, have independent contribution to hypertension, which are supported by our functional assay and consistent with recent studies. Despite the short physical distance between the G−217A and the A−6G, in which the latter is in strong LD with the M235T as expected, the LD between them is very weak and hence implies that the two promoter polymorphisms are related to different functional expression pathways. Revealed by the increased frequency of the recombinant haplotypes between the A−6G and the M235T in the hypertensive subjects, the role of disruption in the conserved interval in the predisposition to hypertension is indicated. These results also highlight the necessity of a thorough analysis of all reported variants of a candidate gene in the elucidation of genetic susceptibility to a complex disease like hypertension, even when the variants are in the same haplotype block.

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