Angiotensinogen Gene Haplotype and Hypertension
Interaction With ACE Gene I Allele

Chia-Ti Tsai, Daniele Fallin, Fu-Tien Chiang, Juey-Jen Hwang, Ling-Ping Lai, Kuan-Lih Hsu, Chuen-Den Tseng, Chiau-Suong Liau, Yung-Zu Tseng

Abstract—There are many reports demonstrating the association of renin-angiotensin system gene polymorphisms with hypertension in different populations. In the present study, we used haplotype analyses of the angiotensinogen gene with a new permutation-based hypothesis testing method to determine the association between multilocus angiotensinogen gene polymorphisms and hypertension in a relatively homogeneous Taiwanese population. We also genotyped angiotensin-converting enzyme gene insertion/deletion polymorphism and angiotensin II type 1–receptor gene A1166C polymorphism to detect epistatic gene-gene interactions. There were 408 patients with hypertension (hypertensives) and 286 controls. The angiotensinogen gene haplotype frequencies were significantly different between hypertensives and controls, and this finding was only present in subjects with angiotensin-converting enzyme gene II genotypes when the analysis was stratified by genotype of this polymorphism. In addition, the angiotensinogen gene haplotype structure of hypertensives was more heterogeneous than that of controls. Our results showed that angiotensinogen gene haplotypes were associated with hypertension and might act synergistically with I allele of the angiotensin-converting enzyme gene. **(*Hypertension*. 2003;41:9-15.)**

Key Words: haplotypes ■ hypertension, genetic ■ polymorphism ■ genetics ■ angiotensinogen ■ angiotensin-converting enzyme ■ receptors, angiotensin II

The genetic basis of essential hypertension is complex. Genetic-association study has been widely used to search for the susceptibility gene(s) of a complex trait disease. These studies look for a significantly increased or decreased frequency of a marker allele with a disease trait, which represents deviations from the random occurrence of the alleles with respect to disease phenotype. Allelic association can be explained either by direct biological action of the allele or by linkage disequilibrium (LD) with a nearby susceptibility gene. LD occurs when a particular marker allele lies so close to the disease-susceptibility allele that these alleles will be inherited together over many generations.

The renin-angiotensin system (RAS) genes have been most extensively studied as hypertension candidate genes. However, the results are different in different populations or studies. For example, the insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene showed an association with hypertension in Japanese,[1] but not in Chinese.[2] A coding polymorphism of the angiotensinogen (AGT) gene (M235T) showed an association with hypertension in Chinese,[3] but not in Japanese.[4] The A1166C transversion in the 3′ untranslated region of the angiotensin II type 1 receptor (AT_1R) gene has also shown an association with hypertension in a white population,[5] but not in another study with a different white population.[6]

The causes of these discrepancies are multiple. The most important cause is that hypertension is a polygenic disorder, but not a monogenic trait. One gene may be responsible for the disorder in one population, but not necessarily in another population. Other possible reasons include a spurious association caused by population stratification, epistatic gene-gene interactions when the effect of the studied gene is masked by the effect of other susceptible genes, or that a specific multilocus haplotype, rather than any of the single loci that define the haplotype, is more significant in determining the association.[7]

Genotyping of multiple diallelic sites, especially dense single nucleotide polymorphism (SNP) sites, is now available for genetic studies of human disease. It is more powerful to focus on the transmission of multilocus haplotypes, as opposed to alleles at individual loci. Therefore, haplotype analysis is mandatory in this regard and has become an increasingly popular tool for population-genetic studies and disease-gene discovery.

However, its application is limited by its complex statistical work and the requirement of either laboratory-based

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From the Division of Cardiology, Department of Internal Medicine (C.-T.T., F.-T.C., J.-J.H., L.-P.L., K.-L.H., C.-D.T., C.-S.L., Y.-Z.T.) and Department of Laboratory Medicine (F.-T.C.), National Taiwan University Hospital, Taipei, Taiwan; and Department of Epidemiology and Biostatistics, Case Western Reserve University (D.F.), Cleveland, Ohio.

Correspondence to Fu-Tien Chiang, MD, PhD, Department of Laboratory Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei 100, Taiwan. E-mail futienc@ha.mc.ntu.edu.tw

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chromosome isolation or recruitment of family members for phase information, which is laborious, and, furthermore, family members are usually not available. Therefore, the wealth of haplotype information has created a need for efficient statistical methods for estimating haplotype frequencies from a sample of genotyped but unphased diploid individuals. A number of methods have been described for estimating haplotype frequencies. Among them, the maximum-likelihood estimation method based on expectation maximization (EM) algorithm[8] can accommodate several loci with an arbitrary number of alleles, and its accuracy has been validated by several studies.[9,10] Detailed description of this method is beyond the scope of this report. In brief, the likelihood function for the unphased diploid genotypic data from a random preset of haplotype frequencies is constructed and is then maximized by iterative processes according to EM algorithm. The estimated haplotype frequencies are those that constitute the maximum of the likelihood function.

Recently, this method, used with permutation-based hypothesis testing, has been documented to be a powerful method for identifying disease-predisposing haplotypes in a case/control design with individuals in large, freely mixing populations.[11] Accordingly, we applied this powerful method to demonstrate the association of specific AGT gene haplotypes with hypertension in a relatively homogeneous Taiwanese population with a large sample size. In addition, we also genotyped ACE gene I/D polymorphism and AT_1R gene A1166C polymorphism simultaneously to detect any epistatic gene-gene interaction between these genes.

Methods

Study Subjects

This was a single-center, case-control study. Hypertensive subjects (hypertensives) were recruited consecutively from the cardiovascular clinic of the National Taiwan University Hospital from July 1995 through June 2000. The patients referred to this clinic were residents of metropolitan Taipei, Taiwan, although some of them might have come from other cities around Taiwan. They were the so-called "Taiwanese" or "Taiwan Chinese." Most of their ancestors moved to Taiwan from southeastern China about 500 years ago. They were not Taiwanese aborigines. The normotensive subjects (normotensives) were from the same areas as the hypertensives, were recruited during the same time period from the general clinic of this hospital, and had no history of hypertension, diabetes mellitus, renal insufficiency, significant hepatic disease, or apparent coronary artery disease.

Hypertensives were defined as those with a systolic blood pressure of ≥140 mm Hg or a diastolic blood pressure of ≥90 mm Hg, or who were being administered at least one antihypertensive agent. The possibility of secondary hypertension, such as that caused by primary aldosteronism, renal vascular hypertension, Cushing syndrome, acromegaly, or pheochromocytoma, was excluded by an extensive inpatient workup. Patients who had diabetes mellitus, renal failure, or significant hepatic disease or who were taking oral contraceptives, systemic corticosteroids, or excessive amounts of alcohol or herbal drugs were also excluded. Non–insulin-dependent diabetes mellitus was defined as a fasting blood glucose >126 mg/dL and/or being administered at least one oral hypoglycemic agent. No patient with insulin-dependent diabetes mellitus was found in our patient population. Normotensives were defined as those with a blood pressure <140/90 mm Hg. The blood pressure was determined using a conventional mercury sphygmomanometer on the right arm, with the patient in a seated position after he or she had rested for at least 30 minutes, and averaging 2 measurements taken on 2 occasions separated by an interval of 4 weeks.

TABLE 1. Demographic Data for Subjects

Demographics	Hypertensives (n=408)	Normotensives (n=286)	*P*
Gender, men/women	229/179	151/135	0.29
Age, y	61.4 (0.7)	53.1 (0.9)	<0.001
Body mass index, kg/m^2	26.1 (1.0)	23.6 (0.3)	0.03
SBP, mm Hg*	156 (14.3)	121 (10.2)	<0.001
DBP, mm Hg*	99 (7.0)	73 (6.7)	<0.001

Values are mean (SD). DBP indicates diastolic blood pressure; SBP, systolic blood pressure.

*Blood pressure values for hypertensives do not include individuals currently on medication.

Four hundred eight hypertensives (229 men and 179 women) and 286 normotensives (151 men and 135 women) were recruited for study. The study was approved by the local institution committee, and the subjects gave their informed consent. The demographic and laboratory data were collected from the medical chart records. The age, body mass index, systolic blood pressure, and diastolic blood pressure of the hypertensives were significantly higher than those of the normotensives (Table 1).

Identification of Diallelic Polymorphisms

Genomic DNA was extracted by a nonenzymatic method. DNA fragments were amplified by polymerase chain reaction (PCR). Genotyping of ACE gene I/D polymorphism was performed as previously reported.[2,12] Genotyping of AT_1R gene A1166C polymorphism was performed by the PCR-restriction fragment length polymorphism (RFLP) method.[13]

For genotyping of AGT gene polymorphisms, we used mini-PCR direct sequencing to identify the M235T and T174M amino acid polymorphisms (T4072C and C3889T dinucleotide polymorphisms, respectively) of the AGT gene, according to our previously reported methods.[3] By the same method, we also identified the G-6A and A-20C diallelic polymorphisms in angiotensinogen core-promoter element-1.[14] The functional studies of these 2 polymorphisms have been reported.[15,16] Based on the preliminary results of our transcriptional activity study, we found that upstream promoter regions other than core-promoter element 1 also played a critical role in transcriptional control.[17] Therefore, we also identified 2 polymorphisms with functional significance at positions −152 and −217 relative to the transcriptional start site (G-152A and G-217A, respectively).

Statistical Methods

The between-group demographic and blood pressure data were compared by the Student unpaired *t* test for continuous data and by the χ^2 test for categorical data. Allele frequencies were calculated from the genotypes of the subjects. Differences in allele frequencies and genotype distributions between the hypertensives and normotensives were compared using the χ^2 test or the Fisher exact test. Hardy-Weinberg equilibrium (HWE) was assessed by the χ^2 test. After applying the Bonferroni correction for multiple tests, the significance level was $P<0.006$ (0.05/8 for 8 loci) for allele, genotype, and HWE tests. The measure of LD known as D′,[18] which is corrected for allele frequencies at each of the loci, was computed for alleles at pairs of SNP loci in the AGT gene. Tests of departures from LD were performed using the composite test described by Weir[19] for the normotensive group. The EM-based haplotype frequency estimations and permutation-based hypothesis testing procedures were performed based on our previous work.[11] The significance level was $P<0.05$ for the omnibus test, and 8×10^{-4} (0.05/64) for individual haplotype analyses (64 haplotypes for 6 loci).

We used 2 methods to evaluate the gene-gene interaction. First, we stratified the patients according to the genotype of one gene and performed the analysis of the other gene in different strata defined by the genotype of the former gene. This included tests of homogeneity of odds ratios across strata by Mantel-Haenszel test in single-locus

TABLE 2. Single-Locus Allele Frequencies and Genotype Distributions

Locus	Genotype			Allele Frequency
ACE I/D*†	II	ID	DD	Frequency I
Hypertensives	130 (32)	197 (48)	81 (20)	0.56
Normotensives	51 (18)	155 (54)	80 (28)	0.45
AT_1R A1166C	AA	AC	CC	Frequency C
Hypertensives	378 (92.8)	29 (7)	1 (0.2)	0.04
Normotensives	270 (94)	16 (6)	0 (0)	0.03
AGT G-217A	GG	GA	AA	Frequency A
Hypertensives†	297 (73)	86 (21)	25 (6)	0.17
Normotensives	196 (68)	82 (29)	8 (3)	0.17
AGT G-152A	GG	GA	AA	Frequency A
Hypertensives	374 (92)	30 (7)	4 (1)	0.05
Normotensives	257 (90)	29 (10)	0 (0)	0.05
AGT A-20C*†	AA	AC	CC	Frequency C
Hypertensives†	379 (93)	23 (6)	6 (1)	0.04
Normotensives†	284 (99.2)	1 (0.4)	1 (0.4)	0.01
AGT G-6A	GG	GA	AA	Frequency A
Hypertensives	18 (4)	102 (25)	288 (71)	0.83
Normotensives	9 (3)	69 (24)	208 (73)	0.85
AGT T174 mol/L	TT	TM	MM	Frequency M
Hypertensives†	326 (80)	70 (17)	12 (3)	0.12
Normotensives	231 (81)	53 (18)	2 (1)	0.10
AGT M235T	MM	MT	TT	Frequency T
Hypertensives	16 (4)	97 (24)	295 (72)	0.84
Normotensives	8 (3)	68 (24)	210 (73)	0.85

Values are mean (%). ACE indicates angiotensin-converting enzyme; AGT, angiotensinogen; and AT_1R, angiotensin II type 1 receptor.

*Allele frequency significantly different between hypertensives and normotensives as determined by χ^2 or Fisher exact test; $P<0.006$ after Bonferroni correction.

†Genotype distribution significantly different between hypertensives and normotensives by χ^2 or Fisher exact test; $P<0.006$ after Bonferroni correction.

‡Genotypes significantly different from Hardy-Weinberg expectation by χ^2 test; $P<0.006$ after Bonferroni correction.

analysis and haplotype analysis of the AGT gene as mentioned above in different strata of different ACE gene I/D or AT_1R gene A1166C genotypes. The significance levels of probability values were corrected according to the number of tests performed after stratification. Second, we also analyzed the pairwise LD in unlinked loci, which could possibly be generated by unlinked gene-gene interactions.[20] The significance levels were also corrected according to the number of tests performed.

Results

Single-Locus Analyses

The results of the single-locus analyses are shown in Table 2. ACE gene I allele and AGT gene C-20 variant were significantly associated with hypertension by both genotype and allele frequency analyses.

HWE Tests for All Eight Diallelic Polymorphisms and LD Analyses for the Six SNPs in the AGT Gene

Tests of HWE were performed for all loci among the hypertensives and normotensives separately. Significant departures from HWE are labeled in Table 2. Many loci were not in accordance with HWE in the hypertensives.

The results of pairwise LD between 6 diallelic polymorphisms of the AGT gene in the normotensives are shown in Table 3. There was nearly absolute LD at the pair of G-6A and M235T. The 2 polymorphisms were seen almost together and occurred with the same frequency (G-6/M235 and A-6/T235) (Table 4). The D′ value was approximately 1.0 when any locus was paired with M235T. Therefore, all the other 5 polymorphisms in the AGT gene were in quasi-complete LD with M235T, each variant being only a subset of the T235.

The pairwise LD was also evaluated in pairs of unlinked loci for detection of unlinked gene-gene interactions. Of the 8 diallelic polymorphisms in 3 chromosomes, there were 13 pairs of unlinked loci (6 between AGT and ACE genes, 6 between AGT and AT_1R genes, and 1 between ACE and AT_1R genes). Six of these 13 pairs (A-20C/ACE I/D, A-20C/AT_1R A1166C, G-152A/ACE I/D, G-152A/AT_1R A1166C, G-217A/ACE ID, and G-217A/AT_1R A1166C) exhibited significant LD ($P<0.0001$; the significance level was $P<0.004$ [0.05/13] after applying the Bonferroni correction) in the hypertensives. However, no LD was detected in any of these 13 pairs in the normotensives.

Haplotypes of the AGT Gene and Their Association With Hypertension

Table 4 displays the results of 6-locus haplotype frequency analyses for the AGT gene in the hypertensives and normotensives. The omnibus haplotype profile test[11] was significant ($\chi^2=53.96$, $P=0.001$), which indicated the overall haplotype frequency profiles were different between the hypertensives and normotensives, and thus there might be some disease-predisposing or -protecting haplotypes in the hypertensives.

Accordingly, in the individual haplotype analyses, we identified 5 haplotypes (GGAGCC, GGCACC, GGCATC,

TABLE 3. Pairwise Linkage Disequilibrium and Statistical Significance

Locus	G-217A	G-152A	A-20C	G-6A	T174M	M235T
G-217A	—	0.945	0.010	0.047	0.892	0.017
G-152A	−0.17	—	0.002	0.033	0.776	0.024
A-20C	−0.21	0.30	—	0.002	0.0003	0.002
G-6A	1.00	0.85	−0.24	—	0.001	<0.0001
T174M	−0.12	−0.11	0.63	1.00	—	0.001
M235T	1.00	0.99	1.00	0.99	1.00	—

Linkage disequilibrium is D' below the diagonal, and statistical significance is *P* value above the diagonal for the 6 diallelic polymorphisms in the angiotensinogen gene in the normotensives.

TABLE 4. Haplotype Frequency Estimates of Angiotensinogen Gene in Hypertensives and Normotensives and Significance Levels of Comparison from Permutation Tests

Haplotype*						Overall	Hypertensives	Normotensives		
−217	−152	−20	−6	3889†	4072†	(n=694)	(n=408)	(n=286)	χ^2	P‡
G	G	A	A	C	C	0.519	0.508	0.533	0.89	0.193
G	G	A	A	T	C	0.085	0.082	0.090	0.32	0.450
G	G	A	A	C	T	0.005	0.007	0.002	1.81	0.206
G	G	A	A	T	T	0.002	0.004	0.000	2.18	0.190
G	G	A	G	C	C	0.015	0.023	0.005	7.15	0.014
G	G	A	G	T	C	0.002	0.004	0.001	0.96	0.489
G	G	A	G	C	T	0.135	0.131	0.143	0.43	0.488
G	G	A	G	T	T	0.002	0.003	0.000	1.60	0.814
G	G	C	A	C	C	0.004	0.007	0.000	3.84	0.040
G	G	C	A	T	C	0.012	0.019	0.002	8.65	0.008
G	G	C	A	C	T	0.001	0.003	0.000	1.46	0.517
G	G	C	G	T	T	0.001	0.000	0.000	1.42	0.262
G	A	A	A	C	C	0.035	0.027	0.048	4.09	0.046
G	A	A	G	C	C	0.001	0.001	0.001	0.00	0.941
G	A	A	G	C	T	0.003	0.003	0.000	1.96	0.717
G	A	A	G	T	T	0.000	0.001	0.000	0.30	0.720
G	A	C	A	C	C	0.008	0.012	0.002	4.62	0.041
A	G	A	A	C	C	0.159	0.155	0.167	0.36	0.516
A	G	A	A	T	C	0.003	0.002	0.005	0.55	0.614
A	G	A	A	C	T	0.003	0.005	0.000	2.76	0.131
A	G	A	G	C	T	0.001	0.002	0.000	1.03	0.978
A	G	C	A	T	C	0.001	0.001	0.000	0.70	0.415
A	A	A	A	C	T	0.001	0.001	0.000	0.56	0.848
A	A	C	A	C	C	0.001	0.001	0.000	0.70	0.423
Log (ln) likelihoods						−1847.6	−1161.4	−659.3	53.96§	0.001

*Haplotypes are not listed if their estimated frequencies are zero in hypertensives and normotensives.

†C3889T dinucleotide polymorphism corresponds to T174M amino acid polymorphism, and T4072C corresponds to M235T.

‡*P* values based on 10 000 permutations.

§Likelihood ratio test statistic values for omnibus test.

GACACC, and GAAACC) with significantly higher or lower haplotype frequency in the hypertensives than in the normotensives at the significance level $P<0.05$, but the differences were not significant after the use of stringent Bonferroni correction for multiple tests ($P<8\times10^{-4}$). There were also several haplotypes that were only found in the hypertensives, such as GGAATT and GGAGTT, although their frequencies were not to a significant level by permutation test. In other words, the haplotype structure of the hypertensives was more heterogeneous than that of the normotensives.

For a pair of diallelic loci (such as A/a and B/b), there should be 4 haplotypes (AB, Ab, aB, and ab). In the normotensives, there were only 3 haplotypes for any locus paired with M235T, generating a D′ value of 1.0,[21] whereas there were 4 haplotypes for each locus paired with M235T in the hypertensives. This was compatible with the finding that the haplotype structure of the hypertensives was more heterogeneous than that of the normotensives.

Gene-Gene Interactions

In the single-locus analyses, ACE gene I allele and AGT gene C-20 allele were associated with hypertension. No significant difference of odds ratio for hypertension with at least one copy of I allele was noted when stratified on AGT gene A-20C polymorphism (AA and AC+CC) ($\chi^2=0.47$, $P=0.49$ for test of homogeneity of odds ratio; combined Mantel-Haenszel odds ratio=1.5, $P=0.025$). There was also no significant difference of the analysis of A-20C polymorphism with hypertension when stratified on ACE gene I/D polymorphism (II and ID+DD) ($\chi^2=0.06$, $P=0.813$ for test of homogeneity for odds ratio; combined Mantel-Haenszel odds ratio for hypertension with at least one copy of C-20 allele=8.8, $P=0.0004$). The significance level was $P<0.025$ (0.05/2) because 2 stratifications were performed.

The results of gene-gene interactions using the stratification method and haplotype analysis are shown in Table 5. The significance level was $P<0.025$ (0.05/2) because 2 strata

TABLE 5. Haplotype Analyses of Angiotensinogen Gene According to ACE Gene Insertion/Deletion Polymorphism Genotype

	II (n=181)		ID and DD (n=513)	
Haplotype	Hypertensives (n=130)	Normotensives (n=51)	Hypertensives (n=278)	Normotensives (n=235)
GGAACC	0.487*	0.637	0.519	0.525
GGAATC	0.065	0.078	0.089	0.091
GGAACT	0.000	0.000	0.009	0.002
GGAATT	0.008	0.000	0.002	0.000
GGAGCC	0.039*	0.000	0.015	0.006
GGAGTC	0.000	0.000	0.006	0.001
GGAGCT	0.135	0.127	0.131	0.145
GGAGTT	0.006	0.000	0.000	0.000
GGCACC	0.011	0.000	0.004	0.002
GGCATC	0.034*	0.000	0.012	0.000
GGCACT	0.008	0.000	0.000	0.000
GGCGCT	0.001	0.000	0.000	0.000
GGCGTT	0.000	0.010	0.000	0.000
GAAACC	0.021*	0.000	0.030	0.044
GAAGCC	0.000	0.000	0.003	0.004
GAAGCT	0.006	0.010	0.000	0.000
GAAGTT	0.000	0.000	0.002	0.000
GACACC	0.026*	0.000	0.005	0.000
GACATC	0.000	0.000	0.000	0.002
GACGCT	0.000	0.000	0.000	0.000
AGAACC	0.131	0.127	0.165	0.175
AGAATC	0.006	0.000	0.000	0.006
AGAACT	0.012	0.000	0.002	0.000
AGAGCT	0.004	0.000	0.000	0.000
AGCATC	0.000	0.000	0.002	0.000
AAAACT	0.000	0.000	0.002	0.000
AACACC	0.000	0.000	0.002	0.000
Log (ln) likelihoods	χ^2=37.49†	P=0.04†	χ^2=25.04†	P=0.186†

I indicates insertion polymorphism and D, the deletion polymorphism, of the ACE gene.
*$P<0.05$ based on 10 000 permutations.
†Omnibus test statistic values and P values based on 10 000 permutations.

were created and 2 omnibus tests were performed. When the patients were stratified according to ACE gene I/D genotype, the number of patients with II genotype was greatly decreased and was lower than that with ID+DD genotypes. However, the association of the AGT haplotypes with hypertension was only found in patients with II genotype at the significance level $P<0.05$, but not at $P<0.025$. No similar finding was noted when stratified by AT_1R gene A1166C genotype.

Discussion

Haplotype analysis is a powerful tool for identifying candidate genes for complex trait disease. Our results show that hypertensives have a AGT haplotype profile that is significantly different from that of the normotensives, which implies that the AGT gene may be a susceptible locus for essential hypertension in the Taiwanese population. Furthermore, the association was more evident in patients with ACE gene II genotype than in patients with ID or DD genotype, which indicates the possibility of a gene-gene interaction. Our study also showed that more information could be obtained from haplotype analyses than from single-locus analyses.

Haplotypes of the AGT Gene and Association With Hypertension

Haplotype analyses of the AGT gene and its association with hypertension have also been reported in whites[22] and Japanese.[23] Jeunemaitre et al[22] have shown that T235/A-6 haplotype may be the ancestral form of the human AGT gene and was associated with hypertension. They also identified another 8 SNPs in the AGT gene, all of which were in complete LD with M235T/G-6A. Sato et al[23] also identified 8 SNPs and demonstrated that only M235/G-6 haplotype was significantly associated with a hypotensive effect.

However, it was surprising to note that haplotype GGAGCC (G-6/T235) was associated with hypertension in

our study. The frequency of this haplotype was the highest in the hypertensives with ACE gene II genotype. This means that T235 allele was not always associated with A-6 allele in our hypertensive subjects, especially in the hypertensives with ACE gene II genotype. In fact, the D′ value was 0.86, but not 1.0, in our hypertensive subjects. This finding was unlikely to result from misgenotyping, because we used a direct sequencing method to determine genotypes, and was contradictory to the concept that T235 allele and A-6 allele appear absolutely together.[15] The AGT haplotype structure was also more heterogeneous in the hypertensives than in the normotensives. These phenomena could possibly be explained by more point mutations or recombinations in the AGT gene during the phylogenetic history of our hypertensive population.

Our result also suggests the possibility that the mechanism of the association of T235 allele with hypertension is not through its association with A-6 allele, which was found to be associated with higher basal AGT gene transcriptional activity.[15] In fact, we found an opposite result: that G-6 variant was associated with a higher transcriptional activity than was A-6 variant in our laboratory.[17] However, despite the higher transcriptional activity, single G-6 variant was not sufficient to cause hypertension because it was not significantly associated with hypertension in single-locus analyses. It is possible that nonfunctioning T235 allele is linked to other susceptible loci in the AGT gene or to another susceptible gene other than the AGT gene, which is as yet unidentified, and exerts its hypertensive effect in conjunction with G-6 allele to cause hypertension.

Gene-Gene Interactions Between the RAS Genes

Significant LD was also noted in many unlinked loci between the AGT and ACE genes in the hypertensives, but not in the normotensives. This amount of LD is high compared with that in human populations for unlinked loci[24] and may come from epistatic gene-gene interactions.[20] In addition, an association of the AGT gene haplotypes with hypertension is more apparent in the presence of II homozygotes, which implies the possible synergistic effect of the AGT haplotypes and ACE I allele.

The association of ACE gene I allele with hypertension may be counterintuitive, because individuals with D allele also have higher serum ACE levels in the Taiwanese.[2] However, I allele has also been shown to be associated with hypertension,[25] insulin resistance[26] and metabolic syndrome.[27] The mechanism of the association of I allele with hypertension in our population and the relationship between I allele, hypertension, and insulin resistance must be further clarified.

Advantages and Limitations

There were several advantages in our study. First, we selected the polymorphisms in the promoter region of the AGT gene based on functional transcription studies, although many of the other polymorphisms may serve only as markers without functional significance. Second, we developed and used the rapid minisequencing method to detect the diallelic polymorphisms of the AGT gene in every subject.[3] This PCR direct-sequencing method decreases inaccuracy of genotyping by PCR-RFLP owing to incomplete cutting. Furthermore, we used stepdown PCR, which increased the detection accuracy of ACE gene I/D heterozygotes.[12]

Third, the accuracy of haplotype frequencies estimation by the EM-based maximum-likelihood method has been validated in many studies.[9,10] The accuracy of this estimation method depends on several conditions, such as sample size (>100 chromosomes), the number of loci (>5 loci), and dispersion of haplotype frequency values (with some very common haplotypes and many rare haplotypes).[9] Many of these conditions were fulfilled in our study. Therefore, the accuracy of haplotype frequencies estimation was good in our study.

Nevertheless, there are also limitations in our studies. First, the normotensive subjects were relatively young compared with the hypertensives. This age difference might reduce the power of our study because some younger normotensives might develop hypertension later in life. Second, many LDs between unlinked loci were found in the hypertensives. Although they most likely resulted from epistatic gene-gene interactions,[20] the problem of selective population stratification in the hypertensives could not be definitely excluded. The use of microsatellite markers unlinked to hypertension candidate genes is necessary to exclude this possibility.[28] However, our study was conducted in a relatively homogeneous Taiwanese population, and we did not find any LD in pairs of unlinked loci in the normotensives. The possibility of selective population stratification solely in the hypertensives was low in a relatively homogenous population. Third, we only demonstrated the epistasis of the RAS genes statistically and obtained a probability value that was only borderline significant after multiple-test correction.

Perspectives

The present study highlights the potential benefits of haplotype analysis and evaluation of gene-gene interactions in the genetic studies of hypertension. These benefits are consistent with the concept of multilocus haplotype effect within the same gene and multiple-gene interaction effect for a complex trait disease. A transcriptional activity study of a specific haplotype may be performed in the future to determine the biological mechanism of the haplotype effect. In addition, the use of animal models to prove the true existence of an interaction between the AGT and ACE genes and subsequently to determine the mechanism of this interaction is warranted in future studies.

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