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Exclusion of Angiotensinogen Gene in Molecular Basis of Human Hypertension: Sibpair Linkage and Association Analyses in Australian Anglo-Caucasians

William Y.S. Wang,[1] Cheryl L. Glenn,[1] Weiyi Zhang,[1] Adam V. Benjafield,[1] Dale R. Nyholt,[2] and Brian J. Morris[1]*

[1]Hypertension Gene Laboratory, Department of Physiology and Institute for Biomedical Research, The University of Sydney, Sydney, New South Wales, Australia
[2]Genomics Research Centre, Griffith University, Gold Coast, Queensland, Australia

Linkage with essential hypertension has been claimed for a microsatellite marker near the angiotensinogen gene (*AGT*; chromosome 1q42), as has association for the *AGT* variants M235T, G(–6)A and A(–20)C. To more rigorously evaluate *AGT* as a candidate gene for hypertension we performed sibpair analysis with multiple microsatellite markers surrounding this locus and using more sophisticated analysis programs. We also performed an association study of the *AGT* variants in unrelated subjects with a strong family history (two affected parents). For the linkage study, single and multiplex polymerase chain reaction (PCRs) and automated genescan analysis were conducted on DNA from 175 Australian Anglo-Celtic Caucasian hypertensives for the following markers: *D1S2880*–(2.1 cM)–*D1S213*–(2.8 cM)–*D1S251*–(6.5 cM)–*AGT*–(2.0 cM)–*D1S235*. Statistical evaluation of genotype data by nonparametric methods resulted in the following scores: Single-point analysis – SPLINK, $P > 0.18$; APM method, $P > 0.25$; ASPEX, MLOD < 0.28; SIB-PAIR, $P > 0.24$; Multipoint analysis – MAPMAKER/SIBS, MLOD < 0.24; GENEHUNTER, $P > 0.35$. Exclusion scores of Lod –4.1 to –5.1 were obtained for these markers using MAPMAKER/SIBS for a λ_s of 1.6. The association study of G(–6)A, A(–20)C and M235T variants in 111 hypertensives with strong family history and 190 normotensives with no family history showed significant linkage disequilibrium between particular haplotypes, but we could find no association with hypertension. The present study therefore excludes *AGT* in the etiology of hypertension, at least in the population of Australian Anglo-Celtic Caucasians studied. Am. J. Med. Genet. 87:53–60, 1999.

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INTRODUCTION

Essential hypertension is a common, complex, polygenic disease that affects one in five adults in Western society. The renin-angiotensin system is a key regulator of salt and fluid homeostasis and blood pressure. Such control could involve not just increased secretion of renin, which is rate limiting in the reaction, but also angiotensinogen, whose concentration in plasma is close to the K_m [Gould and Green, 1971; Reid et al., 1978]. Since angiotensinogen is ~20% higher in patients with essential hypertension and shows a positive correlation with blood pressure [Walker et al., 1979; Bennett et al., 1993; Schrader et al., 1996] the angiotensinogen gene (*AGT*; chromosome 1q42 [Isa et al., 1990]) has attracted attention as a candidate gene for this condition. In support of such a possibility, a Utah/Paris study of affected sibpairs using a microsatellite marker located < 2.5 kilobase-pairs (kb) [Kotelevtsev et al., 1991] 3′ of *AGT* has demonstrated linkage with essential hypertension [Jeunemaitre et al., 1992]. Apparent confirmation was provided subsequently in a pedigree study by others [Caulfield et al., 1994]. However, a recent sibpair study of the same *AGT* marker failed to demonstrate linkage [Brand et al., 1998].

Case-control studies have also produced conflicting results. The initial report showed association of a

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Dale R. Nyholt's present address is the Laboratory of Statistical Genetics, Rockefeller University, New York, New York.

*Correspondence to: Brian J. Morris, DSc, Hypertension Gene Laboratory, Department of Physiology and Institute for Biomedical Research, Building F13, The University of Sydney, NSW 2006, Australia. E-mail: brianm@physiol.usyd.edu.au

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Met235Thr (M235T) variant with hypertension [Jeunemaitre et al., 1992] and this has been confirmed in some [Jeunemaitre et al., 1993; Schmidt et al., 1995; Johnson et al., 1996; Krizanova et al., 1997; Schunkert et al., 1997], but not in other [Bennett et al., 1993; Caulfield et al., 1994; Fornage et al., 1995; Hingorani et al., 1995; Tiret et al., 1995; Hegele et al., 1996; Hingorani et al., 1996; Kiema et al., 1996; Beige et al., 1997] studies of Caucasian populations. A further study found no relationship with ambulatory blood pressure [Beige et al., 1997]. Another saw an association with nonmodulation in hypertensive men and postmenopausal women [Hopkins et al., 1996], which they claimed might explain the original linkage finding for male–male, but not female–female hypertensive sibpairs [Jeunemaitre et al., 1992].

To date, linkage studies have tested only the *AGT* marker. In view of the controversy surrounding *AGT* in hypertension, we decided to test additional microsatellite polymorphisms near the *AGT* locus and to use more sophisticated programs for data analysis. We also performed a case-control study of several *AGT* polymorphisms that have shown association with hypertension, viz. the G(−6)A, A(−20)C and M235T bi-allelic variants [Jeunemaitre et al., 1992, 1997]. Such an evaluation by both linkage and association provides timely information on the important question of whether or not *AGT* is involved in the onset of essential hypertension.

MATERIALS AND METHODS

Subjects

Linkage study. We recruited 239 individuals from 104 sibships (= 2 affected sibs) by public advertising. The study had ethical approval and the subjects gave informed consent. All were Anglo-Caucasians, mainly from eastern Australia, principally Sydney. The criteria for inclusion were a systolic/diastolic blood pressure of >140/90 mmHg on three separate occasions prior to treatment having been initiated, and lack of diabetes, renal disease or secondary causes of hypertension. Each subject provided a 50 ml blood sample, collected while the patient was in the sitting position. After adjustment [Hodge, 1984] for 16 trios, 6 quartets and 1 quintet, the weighted sibpair number was 175. The characteristics of the sibs are shown in Table I, where it should be noted that, as might be anticipated, blood pressure data does not follow a normal distribution (skewness = +0.59 and +0.55 for systolic and diastolic, respectively).

TABLE I. Characteristics of Patients and Controls*

	Linkage study HT sibs	Association study	
		HT	NT
n	239	111	190
Male/female	85/154	54:57	109:81
Age (y)	61 ± 10	53 ± 12	48 ± 10
BMI (kg/m^2)	27.2 ± 5.1	26.0 ± 4.6	26.0 ± 4.3
Systolic pressure pretreatment (mmHg)	171 ± 24 (n = 104)	174 ± 25 (n = 92)	120 ± 11
Diastolic pressure pretreatment (mmHg)	103 ± 10 (n = 96)	112 ± 17 (n = 92)	73 ± 8

*Values are mean ± S.D.

Association study. This study compared hypertensives who had two affected parents with normotensives whose parents both had normal blood pressure. Ascertainment details were as described previously [Bennett et al., 1993]. Characteristics of the subjects are shown in Table I. Plasma parameters (mean ± SE), determined by methods detailed before [Bennett et al., 1993], were as follows (for the hypertensive vs normotensive groups, respectively): angiotensinogen (pmol/ml), 1402 ± 40 vs 1172 ± 182 (P = 0.0001); renin (pmol angiotensin I.ml^{-1}.h^{-1}), 10.3 ± 1.4 vs 8.3 ± 0.6 (P = 0.1); angiotensin converting enzyme (nmol Gly–Gly.min^{-1}.ml^{-1}), 82 ± 4 vs 85 ± 3 (P = 0.6); total cholesterol (mmol/l), 5.8 ± 0.1 vs 5.2 ± 0.1 (P = 0.0003); triglycerides (mmol/l), 2.6 ± 0.2 vs 1.5 ± 0.1 (P = 0.0001); high density lipoprotein cholesterol (mmol/l), 1.1 ± 0.05 vs 1.4 ± 0.04 (P = 0.0001); low density lipoprotein cholesterol (mmol/l), 3.6 ± 0.1 vs 3.2 ± 0.1 (P = 0.006).

Genotyping

Microsatellite markers. DNA was isolated from whole blood by a modified salting out method [Miller et al., 1988] or using a kit (Qiagen, cat no. 29304, Germany). Five markers were tested. Fluorescently labeled polymerase chain reaction (PCR) primers for *D1S213* and *D1S235* were from panel 1 of the Applied Biosystems Inc (ABI; Foster City, CA) PRISM™ Linkage Mapping Set, for another two (*D1S251* and *D1S2880*) synthesis was performed by Bresatec (Adelaide, South Australia) and primers for the *AGT* 3′ microsatellite were made by Life Technologies (Melbourne, Australia). *D1S213* was labeled with TET, *D1S235* was labeled with FAM, and *AGT*, *D1S251*, and *D1S2880* were labeled with HEX. The sequences of the ABI primers were as described by the supplier. In the case of the others, primer sequences were: *AGT* – (forward) 5′–TAG ATC TCT CAG CTA TTA CAA GG–3′ (HEX-labeled), (reverse) 5′–GTT TCA GAG AAA CTG ACC TGT GG–3′ ; *D1S251* – (forward) 5′–GTC TCC AGC CTG CCA–3′ (HEX-labeled), (reverse) 5′–GAC CAA GCA ACT TCA CTC–3′; *D1S2880* – (forward) 5′–CGT GGT TCT AAT CGG C–3′ (HEX-labeled), (reverse) 5′–CAT CAT TTG CTT GCT GC–3′. Genotypes were determined by either PCR for an individual marker or by multiplex PCR in which markers from the same ABI panel and labeled with the same dye were used in a common PCR mixture. Since PCR products for these differed in size they could be distinguished subsequently by electrophoresis. Each 15 μl PCR mix consisted of 50 ng of genomic DNA, each primer (83 nmol/l for the ABI primers, 40 nmol/l for *AGT* primers, and 450 nmol/l for *D1S251* and *D1S2880* primers), 200 μmol/l each dNTP, 2 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, and 0.75 Units of AmpliTaq Gold™ (Perkin-Elmer, Norwalk, CT). Composition was the same for single and multiplex mixtures, with the exception of primers in the

multiplex mix, which were as specified for each marker previously [Veenstra-WanderWeele, 1997]. Amplification was carried out on a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). For single marker PCRs, this involved an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94, 55 and 72°C for 1 min each, and then a final extension step at 72°C for 10 min. For the multiplex reactions, a 'hot-start' protocol was employed, involving 94°C for 5 min, followed by 10 cycles of 94, 60 and 72°C for 30 s each, 15 cycles of 94, 58 and 72°C for 30 s each, and then 20 cycles of 94, 55 and 72°C for 30 s each, finishing with a step at 72°C for 30 min. PCR products were electrophoresed on an ABI 377 automated sequencer and genotypes were assigned using ABI Genotyper™ software. To confirm accuracy of genotype assignment, for the *AGT* marker, genotyping was performed twice and was also determined by a different Laboratory (Griffith University, Gold Coast), using an ABI 310 capillary electrophoresis system.

Bi-allelic* AGT *polymorphisms. In the association study, genotypes for the *AGT* A(−6)G and A(−20)C polymorphisms were determined by PCR RFLP analysis. Primers for detection of each were: (upstream) 5′–AGA GGT CCC AGC GTG AGT GTC–3′ (−166 to −144 with respect to the *AGT* transcription start site, +1) and (downstream) 5′–AGC CCA CAG CTC AGT TAC ATC 3′ (nt +81 to +101) [Ishigami et al., 1997]. Each 40 μl reaction mixture contained 120 ng genomic DNA, 0.2 μmol/l each primer, 0.2 mmol/l each dNTP, 10 mmol/l Tris-HCl, pH 9.0, 50 mmol/l KCl, 2 mmol/l $MgCl_2$, and 1 U AmpiTaq™ DNA polymerase (Perkin-Elmer). PCR involved 95°C for 3 min, followed by 36 cycles of 94, 60 and 72°C for 1 min each, and finally 72°C for 15 min. For determination of A(−6)G genotype, an aliquot of 4 μl of PCR product was then digested for 10 h at 50°C with 5 U *Bst*ZI (Promega, Madison, WI) made up to 12 μl with buffer and bovine serum albumin, according to the directions of the supplier. After electrophoresis on a 4% agarose gel, a 267 bp ethidium bromide stained band was indicative of the A(−6) allele (designated *A*) and bands of 154 and 113 bp showed the G(−6) allele (designated *G*) was present. The procedure for determination of the A(−20)C genotype was the same, except that PCR products were incubated at 37°C with 5 U *Eco*O109I (New England BioLabs, Beverly, MA), which yielded bands of 205 and 62 bp (A(−20) allele; designated *a*) or 141, 64 and 62 bp (C(−20) allele; designated *C*). In the case of the M235T polymorphism (alleles *M* and *T*), genotypes were determined as described previously [Bennett et al., 1993]. All genotype determinations were performed on two separate occasions to confirm genotype assignment.

Statistical analysis

Allele sharing by descent (IBD) or by state (IBS) was determined using 7 linkage programs suitable for complex traits in order to test whether there was concordant sibpair sharing of alleles more often than expected under random Mendelian segregation. Since each program has relative advantages and disadvantages, the use of several is deemed desirable [Davis and Weeks, 1997]. These included SPLINK [Holmans and Clayton, 1995], which uses allele shared IBD estimates for all possible pairs in a sibship and computes probabilities for each marker genotype when parents are not available, ASPEX [Hauster et al., 1996], which uses an alternate restriction to SPLINK when performing maximum likelihood calculations, MAPMAKER/SIBS [Kruglyak and Lander, 1995], the Affected Pedigree Member (APM) method [Weeks and Lange, 1988; Brown et al., 1994; Schroeder et al., 1994], which uses a nonrandom cosegregation statistic to test distortions in alleles shared IBS at a marker locus, SIB-PAIR [Duffy, 1997], and GENEHUNTER [Kruglyak et al., 1996], which can be used for multipoint analysis of pedigree data and can be applied to sibpair data. The significance thresholds we set for acceptance of linkage were as recommended [Lander and Kruglyak, 1995]. In the case–control study, differences were tested by χ^2 analysis (df = 1 for alleles; df = 2 for genotypes). Determination of linkage disequilibrium between *AGT* polymorphisms involved analysis of haplotype frequencies in the largest group as described by Hill [1974].

RESULTS

Sibpair Linkage Study

The markers used spanned a recombination fraction (θ) of 0.335 of the q arm of chromosome 1 and were highly informative, having an average heterozygosity of 0.79 ± 0.05 SD. Allele frequencies for the marker closest to *AGT* were also determined in the normotensive group and resembled the frequencies seen in the hypertensive sibs (Fig. 1). Table II shows the outcome of single point analyses of genotype data by SPLINK and APM, as well as multipoint analysis by MAPMAKER/SIBS. Values obtained using ASPEX were MLOD = 0.00, SIBPAIR were P = 0.24–0.50, and GENEHUNTER were nonparametric lod (NPL) = −2.2–0.39 and P = 0.35–0.99. For each marker, all of the statistical parameters generated consistently indicated an absence of excess allele sharing in the hypertensive sibpairs. Results for pairs with age of onset < 45 y (n = 87), those with pre-treatment diastolic pressure > 100 mmHg (n = 86), or male–male pairs (n = 100) were also not significant (e.g., P = 0.54, 0.55 and 0.51, respectively, by SPLINK). In addition we calculated exclusion values using MAPMAKER/SIBS under the

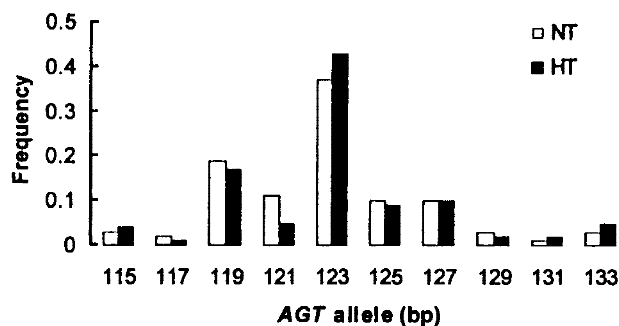

Fig. 1. Allele frequencies for *AGT* marker in the hypertensive sibs and the unrelated normotensive control group.

TABLE II. Results of Statistical Analyses of Genotype Data for Microsatellite Markers Near Angiotensinogen Locus*

	Locus statistics				SPLINK		APM		MAPMAKER/Sibs	
Locus	Distance (θ)	Locus het.	No. of alleles	No. of genotypes	*P*-value	Associated Lod	T-statistic	Theoretical *P*-value	MLS	Exclusion Lod
D1S2880	0.021	0.77	11	227	0.57	0.00	−4.10	1.00	0.00	−4.80
D1S213	0.028	0.83	11	234	0.41	0.04	−0.87	0.81	0.04	−4.40
D1S251	0.077	0.86	14	213	0.50	0.01	−2.27	0.99	0.01	−4.60
AGT	0.020	0.76	11	222	0.58	0.00	−1.14	0.87	0.00	−5.07
D1S235		0.73	13	206	0.58	0.00	−1.70	0.96	0.00	−4.05

*θ, recombination fraction; Het., heterozygosity; MLS, multipoint maximum Lod score.

assumption of no dominance variance for a hypothetical locus with $\lambda_s = 1.6$ and using a conventional exclusion threshold of Lod = −2. The Lod scores obtained (Table II) indicated an absence of linkage of the *AGT* region with hypertension.

Association Study

Genotype and allele frequencies for the M235T, G(−6)A and A(−20)C variants are shown in Table III. Hardy-Weinberg equilibrium was observed. No significant differences in frequencies were seen between the hypertensive and normotensive groups, with the exception of a weak ($P = 0.04$) association of the A(−20)C variant with hypertension. This can, however, be discounted on the basis of the multiple comparisons being undertaken. Haplotype analysis involving these variants also did not show association with hypertension (data not shown). Comparison of blood pressure and other parameters in Table I across genotypes did not demonstrate any significant tracking (data not shown). Furthermore, χ^2 analysis after stratification of genotype data according to mean plasma angiotensinogen, blood pressure, BMI, age of onset, sex, and other parameters still did not reveal any significant association with hypertension. We noted strong linkage disequilibrium between the G(−6)A, A(−20)C and M235T variants of *AGT*, the A(−6), C(−20) and T235 alleles being co-inherited (Table IV).

TABLE III. Association Analysis of *AGT* Variants in Hypertension*

		Genotype frequencies			Allele frequencies	
			M235T variant			
Group	n	*MM*	*MT*	*TT*	*M*	*T*
HT	111	34	61	16	129	93
		(0.31)	(0.55)	(0.14)	(0.58)	(0.42)
NT	190	55	96	39	206	174
		(0.29)	(0.51)	(0.20)	(0.54)	(0.46)
χ^2			1.8		0.9	
P			0.4		0.4	
			G(−6)A variant			
Group	n	*GG*	*GA*	*AA*	*G*	*A*
HT	111	33	63	15	129	93
		(0.30)	(0.57)	(0.13)	(0.58)	(0.42)
NT	190	30	104	56	216	164
		(0.16)	(0.55)	(0.29)	(0.57)	(0.43)
χ^2			0.3		0.1	
P			0.9		0.8	
			A(−20)C variant			
Group	n	*aa*	*aC*	*CC*	*a*	*C*
HT	111	84	25	2	193	29
		(0.76)	(0.23)	(0.02)	(0.87)	(0.13)
NT	190	120	65	5	305	75
		(0.63)	(0.34)	(0.03)	(0.80)	(0.20)
χ^2			5.0		4.4	
P			0.081		0.037	

*Values in brackets are fraction.

DISCUSSION

The present study found no evidence for linkage of markers in the vicinity of *AGT* with essential hypertension. A negative sibpair linkage result in a complex polygenic trait may indicate either that the loci tested are not linked to the condition or the size of the study group was insufficient to provide sufficient power to demonstrate a small genetic contribution of loci in this region to the trait. It has been estimated that in hypertension with strong family history and disease onset before age 55 years, relative risk to a sibling (λ_s) would be approximately 3.8 [Williams et al., 1991]. Analyses by others have suggested that for a complex disease ~100 sibpairs would be sufficient to provide 90% power to show significant linkage at the Lod = 3 ($P = 0.0001$ [Lander and Kruglyak, 1995]) level if its λ_s exceeds ~3.5 [Weeks and Lathrop, 1995]. For a disease with multiple weak contributing loci (e.g., λ_s values of ~1.6), the 175 sibs we tested should have 90% power to provide evidence at the Lod = 2 ($P = 0.001$) level [Weeks and Lathrop, 1995]. That our sibs do indeed have sufficient power to reveal a locus for hypertension is evident from recent findings in the same cohort [Rutherford et al., 1997] and in a study by others [Julier et al., 1997] of linkage on chromosome 17 with hypertension. Our sib number was, moreover, comparable to that used in the original work that claimed hypertension linkage after testing the same *AGT* microsatellite marker, viz. 135 sibpairs after weighting [Hodge, 1984] for the Paris sibs and 244 for the Utah sibs, significance being obtained at the $P < 0.05$ level for the former (8% excess of shared alleles), but not for the latter (4% excess) [Jeunemaitre et al., 1992]. For early-onset (< 45 y), the excess was 12% and 7% in Paris and Utah, respectively ($P = 0.02$ and 0.07) and for severe hypertension (diastolic = 100 mmHg or = 2 drugs), excess was 15% and 18% ($P = 0.02$ and 0.01). For our sibs, age of onset of hypertension was 43 ± 10 SD years and when we confined our analyses to those with onset < 45 y we still did not find significance. Similarly, significance was not achieved for our severely affected sibs using the same criteria as the Utah/Paris study. In the Utah/

TABLE IV. Estimate of Pairwise Haplotype Frequency and Disequilibrium Statistic for G(−6)A, A(−20)C and M235T Variants of *AGT* (n = 190)

Variants	Estimated haplotype frequency				*D*	*P**
	M/G	M/A	T/G	T/A		
M235T/G(−6)A	0.534	0.035	0.008	0.423	96.5%	<0.001
	M/a	M/C	T/a	T/C		
M235T/A(−20)C	0.538	0.004	0.264	0.194	96.4%	<0.001
	G/a	G/C	A/a	A/C		
G(−6)A/A(−20)C	0.568	0.234	0.000	0.197	100%	<0.001

*Significance of the standardized disequilibrium whose square (under the null hypothesis, $D = 0$) is approximately distributed as $\chi^2_{1\ d.f.}$, as described in Hill [1974].

Paris work significance was confined to male–male pairs (13% excess overall, $P < 0.01$; and 33%, $P < 0.0001$, for those with severe hypertension), but our male–male pairs did not show significant linkage.

A noteworthy result of our analyses was the finding of exclusion scores of Lod −4.1 to −5.1 for a λ_s of 1.6. This is well below the generally accepted threshold for non-linkage of −2. Therefore, utilizing a population of Australian Anglo-Celtic Caucasians, these data provide convincing evidence that the *AGT* region does not play an important role, if any, in essential hypertension etiology.

Consistent with the present findings, a multicenter analysis in Europe of the *AGT* marker using the programs SIBPAL and SIB-PAIR has also failed to demonstrate linkage of the *AGT* marker with hypertension [Brand et al., 1998]. Moreover, their reanalysis of *AGT* marker data from the original Utah/Paris study using the ILINK program (which generates maximum likelihood estimates) did not show linkage, leading them to suggest that the initial study could have been a false-positive. This group concluded that if there is a contribution it could be so small that only an enormous number of sibs would have sufficient power to detect it.

Significant linkage for the *AGT* microsatellite marker (but no association for the M235T variant) has also been reported for 63 Caucasian pedigrees (= 2 affected) in London [Caulfield et al., 1994]. There are, however, a number of problems with this data and its analysis. One is the fact that although the frequency of the two major alleles of the (GT)- repeat polymorphism were markedly different between patients and controls, there was no difference between alleles of the M235T variant, even though these two polymorphisms are in linkage disequilibrium with each other, suggesting genotype sizing differences. Consistent with the latter, the most common allele was much lower in their hypertensives (0.07 vs. 0.31 in controls) than was seen by [Jeunemaitre et al., 1992, 1997] (0.34 vs. 0.41 in controls). In our study the frequency of the most common allele was 0.43 and in the report that first described this marker it was 0.40 [Kotelevtsev et al., 1991]. Furthermore, the second most common allele was elevated (0.36 vs. 0.17 in controls) in the London hypertensives, but did not differ in the Utah or Paris subjects [Jeunemaitre et al., 1992, 1997]. The Caulfield group also reported linkage for Afro-Carribean blacks, although quite different alleles were shared in excess [Caulfield et al., 1995]. Furthermore, they used only the APM method for analysis. Because APM uses just IBS information and relies on user-specified allele frequency to test the null hypothesis it is very sensitive to misspecification of allele frequencies [Weeks and Lange, 1992]. Lastly, their findings differ from Jeunemaitre et al. [1992] in that 28 families with female–female pairs showed a greater allelic excess (29%; $P < 0.001$) than 18 with male–male pairs (17%; $P = 0.02$) and the excess was similar (25%; $P < 0.001$) for 31 families with 70 severely affected (diastolic > 100 mmHg) patients as for the whole group (26%; $P < 0.001$).

Thus closer examination of the various data to date, especially in the light of more recent findings, such as described herein, provide little reassurance that *AGT* is linked to hypertension.

Our case-control study of the G(−6)A and A(−20)C variants, like M235T [Bennett et al., 1993], also failed to support association of these *AGT* variants with hypertension. The G(−6)A and M235T variants [Jeunemaitre et al., 1997] and the A(−20)C and M235T variants [Sato et al., 1997] have been reported by others to be in linkage disequilibrium and our data confirm this for both variants in a Caucasian population. The number of subjects we used should have provided sufficient power, as judged from previous estimates [Cox and Bell, 1989] and our positive results for variants of other genes in the same subjects, where our use of only hypertensives with two hypertensive parents, and normotensives with two normotensive parents, would have increased the genetic input and power of our study, so increasing the likelihood of revealing an association [Chambers and Morris, 1996; Morris et al., 1997; Benjafield et al., 1998]. The present findings thus add to the disparity between reports for *AGT* variants in various Caucasian populations, as well as in other racial groups [reviewed in Morris, 1999]. Interestingly, one study of French Caucasians found association with hypertension only in patients who were not overweight [Tiret et al., 1995]. However, a study of a UK Caucasian group in East Anglia failed to find association even after subdivision according to BMI, sex or age [Hingorani et al., 1996] and we too could not see differences by subgroup analyses. Curiously, the initial M235T association was much stronger in females, whereas the linkage data were only significant for males [Jeunemaitre et al., 1992]. T235 allele frequency is quite variable, ranging from 0.35 [Jeunemaitre et al., 1992] to 0.49 [Caulfield et al., 1994] in different Caucasian groups, suggesting that cryptic ethnic or other differences between various populations of the same race may be contributing to the disparate findings

[Morgan et al., 1996]. Thus the M235T variant may not be an ideal marker for a causative variant, if there is one. Other polymorphisms in disequilibrium with T235, such as allele $(GT)_{16}$ of the dinucleotide repeat and the A(–6) or C(–20) alleles of the promoter polymorphisms, may have similar problems. Potential problems may be exacerbated when using APM, due to its inherent sensitivity to variations in allele frequency [Weeks and Lange, 1988; Morgan et al., 1996]. Thus at least some of the positive findings to date for *AGT* have the potential to be spurious.

In considering the findings from linkage and association studies it should be noted that what is tested differs for each approach and that each has different strengths and weaknesses. Linkage studies prefer multi-allelic markers to better track inheritance, whereas in association studies bi-allelic markers are preferred. Linkage studies test whether a disease and an allele show a correlated transmission (concordant inheritance) within a pedigree, whereas association studies test for correlated occurrence in a population [Lander and Schork, 1994]. Linkage may be seen without association, as can happen when there are many independent trait-causing loci in a population, so that association with any particular allele is weak, and association may be detected without linkage, such as when an allele explains only a minor proportion of the variance for a trait, meaning that even though the allele is more frequent in affected individuals, it does not predict disease status within a pedigree very well [Lander and Schork, 1994]. This situation could apply to hypertension considering its relatively small relative risk value and complexity and the expectation that many hypertension susceptibility loci may each contribute to a relatively minor proportion of the variance. A potential confounding factor in association studies is selection bias (all of our subjects in every group were respondents to requests for volunteers). However, the possibility of population stratification artefact does not apply to linkage analysis, since this is insensitive to population structures. Thus a negative result for *AGT* from both association and linkage analysis helps provide some assurance about the validity of the conclusion reached, but does not completely rule out a minor contribution to the disease tested.

There may be an effect of genotype on plasma angiotensinogen concentration [Jeunemaitre et al., 1992; Yanai et al., 1996; Inoue et al., 1997; Zhao et al., 1999]. The elevated plasma angiotensinogen in hypertension could be at least partly a result of stimulation of hepatic synthesis by hypertension-associated hyperinsulinemia [Schrader et al., 1996] or of adipose synthesis in the insulin resistant state of obese hypertensives [Aubert et al., 1998]. Moreover, *AGT* genotype is associated with insulin resistance [Sheu et al., 1998]. Any rise in plasma angiotensin that ensues would, by normal negative feedback mechanism, be quickly offset by a corresponding suppression in renin secretion. Indeed, as long as renin secretion can feed back normally, blood pressure is not angiotensinogen dependent [Gahnem et al., 1994; Sealey and Laragh, 1995; Catanzaro et al., 1999]. The net effect would be the absence of any change in the biologically active effector, angiotensin II. Thus there is no logical reason why a genetically determined elevation in plasma angiotensinogen in hypertension might play a role in the etiology of this condition. Finally, recent congenic experiments involving a sponaneously hypertensive rat chromosomal segment containing the angiotensinogen gene have shown that even though there is a quantitative locus for blood pressure in this region, this is not explained by differences in angiotensinogen expression or plasma concentrations [St Lezin et al., 1999].

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