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Variation at the M235T locus of the angiotensinogen gene and essential hypertension: a population-based case-control study from Rochester, Minnesota

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Abstract A variant of the angiotensinogen gene, M235T, has been associated with essential hypertension in selected subjects from Paris, France and Salt Lake City, Utah. In the present report, we studied a population-based sample consisting of 104 subjects diagnosed with hypertension before age 60 and 195 matched normotensive individuals from Rochester, Minnesota. We determined whether there was a relationship between the M235T polymorphism of the angiotensinogen gene and the occurrence of essential hypertension using two methods. First, a contingency chi-square analysis was carried out to test for an association between the M235T polymorphism and hypertension status. Second, multivariable conditional logistic regression was used to determine whether variation at the M235T polymorphism was a significant predictor of the probability of having essential hypertension. We detected no statistically significant association between the M235T polymorphism and the occurrence of essential hypertension. In particular, the association was not significant in either gender or in a subset of severely hypertensive subjects requiring two or more anti-hypertensive medications. Furthermore, variation in the number of M235T alleles did not make a significant contribution to predicting the probability of having essential hypertension, either alone or in conjunction with other predictor variables. These results suggest that the contribution of variation in the angiotensinogen gene to the occurrence of essential hypertension is less than initially suspected, or may not be constant across populations.

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Introduction

In westernized societies, essential hypertension affects more than 20% of the adult population and contributes to cardiovascular complications that are a major cause of morbidity and mortality in the general population. Biometric studies have provided strong evidence for a genetic component to the determination of interindividual blood pressure variation (Ward 1990). Although the genetic loci involved have not been identified, genes encoding the components of the renin-angiotensin system (RAS) are among the candidates, and polymorphic variations affecting the function or expression of these genes may constitute the basis for a genetic predisposition to essential hypertension. Stimulation of the RAS leads to the production of angiotensin II (Ang II), which raises blood pressure by causing vasoconstriction, aldosterone secretion, and increased sodium and water reabsorption by the kidneys. Ang II is produced by a series of chemical reactions, which includes the cleavage of angiotensinogen to angiotensin I by renin, followed by the cleavage of angiotensin I to angiotensin II by the angiotensin-converting enzyme (ACE). Epidemiologic and biochemical data support a direct relationship between plasma angiotensinogen and blood pressure levels (Fasola et al. 1968; Ménard et al. 1991; Watt et al. 1992; Kimura et al. 1992).

Jeunemaitre et al. (1992) have investigated the role of the gene coding for angiotensinogen in essential hypertension and have demonstrated linkage between a marker in this gene and an unidentified locus influencing the occurrence of essential hypertension. This result was recently confirmed by Caulfield et al. (1994) in 63 white European families. Jeunemaitre et al. (1992) have also shown that a $T^{704} \rightarrow C$ substitution in exon 2 of the gene, encoding a $Met^{235} \rightarrow Thr$ variant, referred to as M235T, is associated with elevated plasma angiotensinogen levels and essential hypertension in subjects from Paris, France and Salt Lake City, Utah. These results, directly implicating variation in the angiotensinogen gene in determining variation in plasma angiotensinogen and blood pressure levels, were

in agreement with previous epidemiological data demonstrating higher plasma angiotensinogen concentrations in hypertensive subjects and their offspring as compared to normotensive individuals (Fasola et al. 1968). However, the hypertensive patients studied by Jeunemaitre et al. (1992) were not representative of the general hypertensive population, and thus do not provide information about the impact of the M235T polymorphism on the occurrence of essential hypertension in the population-at-large. Therefore, we have examined the M235T variant of the angiotensinogen gene in a population-based sample of early-onset hypertensive subjects and their matched normotensive controls from Rochester, Minnesota.

Materials and methods

All of the individuals in this study were previous participants in the Rochester Family Heart study (RFHS), a population-based study initiated in 1984 to investigate the role of genetic factors in the occurrence of hypertension and cardiovascular disease in Rochester, Minn. Individuals participating in the RFHS are members of multigeneration pedigrees ascertained through households having two or more children in the schools of Rochester, Minn.; these pedigrees were selected without regard to health status of the family members. All individuals provided their informed consent to participate in the RFHS. Recruitment and examination protocols of participants in the RFHS have been described in detail by Turner et al. (1985, 1989) and Turner and Michels (1991).

Of the 3938 individuals who participated in the RFHS between 1984 and 1993, we selected 104 individuals who had been diagnosed with hypertension before age 60 and had a body mass index (BMI) of less than 32 kg/m^2. Subjects were defined as having essential hypertension if (1) they reported previous diagnosis of hypertension and were receiving anti-hypertensive treatment at the time of examination or (2) had a systolic blood pressure greater than 140 mm Hg or a diastolic blood pressure greater than 90 mm Hg at the time of examination and (3) had no evidence of causes of secondary hypertension. In this sample, 95% of hypertensive women and 87% of hypertensive men had been previously diagnosed and were taking blood-pressure-lowering medication at the time of examination.

To test for an association between the M235T polymorphism in the angiotensinogen gene and the occurrence of essential hypertension, we also selected 195 normotensive individuals. Up to two normotensive controls were matched to each hypertensive subject for gender, age (± 1 year), and BMI (± 2 kg/m^2). The normotensive subjects had a systolic blood pressure less than 140 mm Hg and a diastolic blood pressure less than 90 mm Hg at the time of participation in the RFHS, had no previous diagnosis of hypertension, and were never treated with anti-hypertensive medication. All hypertensive and normotensive subjects were unrelated Caucasians.

We have typed the M235T variant of angiotensinogen using single-strand conformation analysis (Orita et al. 1989) following the polymerase chain reaction (PCR) amplification of the appropriate region of the angiotensinogen gene. The M235T genotypes were readily distinguishable on the autoradiogram. Direct DNA sequencing of two amplification products (one of each homozygous genotype) was performed to verify that the variation detected by this method was at the expected nucleotide position (data not shown) as reported by Jeunemaitre et al. (1992).

To describe the distribution of quantitative variables in the groups of hypertensive and normotensive subjects, gender-specific means and standard deviations were computed. Within each gender, Student's *t*-test was used to evaluate differences in means between the hypertensive and normotensive groups. Satterthwaite's approximation to the *t*-test was used when the variances differed significantly between diagnostic groups.

Genotypes were scored for each individual, and allele frequencies were estimated by gene counting in hypertensive and normotensive subjects. Genotype frequencies did not differ from Hardy-Weinberg expectation in both groups (data not shown). Statistical comparison of allele frequencies of the M235T polymorphism between hypertensive and normotensive individuals was carried out using two methods. First, a contingency χ^2 analysis with one degree of freedom was used to test for an association between the relative frequencies of the M235T variant allele and hypertension status. Second, conditional logistic regression on the matched data set (Breslow and Day 1980; Hosmer and Lemeshow 1989) was used to determine whether allelic variation at the M235T locus was a significant predictor of the probability of having hypertension. Conditional logistic regression is a modification of the usual logistic regression method taking into account the matching of normotensive subjects to hypertensive subjects. The number of susceptibility alleles as defined by Jeunemaitre et al. (1992) was used as the predictor variable with three possible outcomes – 0, 1, and 2. Comparable results were obtained from a parallel analysis using two indicator variables to encode the three genotype categories (results not shown).

Results

Characteristics of the hypertensive subjects and their matched normotensive controls are described by gender in

Table 1 Characteristics of the samples matched for gender, age, and body mass index of hypertensive and normotensive subjects from Rochester, Minnesota by gender. Values given are averages ± one standard deviation (BP Blood pressure, Rx medication, Na/Li CNT sodium/lithium countertransport, RBC red blood cell, apo apolipoprotein

	Hypertensives	Normotensives
Women		
Sample size (*n*)	59	111
Age (years)	64 ± 10	63 ± 9
Age at diagnosis (years)	50 ± 6	–
Body Mass Index (kg/m^2)	25.6 ± 2.6	25.5 ± 2.5
Systolic BP (mm Hg)	144.5 ± 29.5	120.5 ± 11.6*, **
Diastolic BP (mm Hg)	77.8 ± 10.3	67.4 ± 7.4*, **
Antihypertensive Rx (Y/N)	56/3	–
Hormone use[a] (Y/N)	8/51	12/99
Total cholesterol (mg/dl)	224.3 ± 44.4	212.9 ± 36.9
Triglycerides (mg/dl)	137.9 ± 57.2	123.3 ± 49.9
Na/Li CNT (μmol/l RBC/h)	325.4 ± 87.8	258.1 ± 95.9*
apo CII (mg/dl)	2.9 ± 1.0	2.6 ± 0.8
Men		
Sample size (*n*)	45	84
Age (years)	60 ± 10	61 ± 10
Age at diagnosis (years)	48 ± 7	–
Body Mass Index (kg/m^2)	26.5 ± 2.5	26.6 ± 2.5
Systolic BP (mm Hg)	137.5 ± 19.4	116.5 ± 11.3*, **
Diastolic BP (mm Hg)	82.0 ± 9.0	70.9 ± 8.2*, **
Antihypertensive Rx (Y/N)	39/6	–
Total cholesterol (mg/dl)	200.8 ± 36.9	200.6 ± 38.9
Triglycerides (mg/dl)	158.2 ± 65.1	132.1 ± 64.1*
Na/Li CNT (μmol/l RBC/h)	367.2 ± 155.1	301.1 ± 120.8*, **
apo CII (mg/dl)	2.9 ± 1.0	2.4 ± 0.8*

*Statistically significant difference in means ($P < 0.05$) between hypertensive and normotensive subjects; **statistically significant difference in variances ($P < 0.05$) between hypertensive and normotensive subjects

[a]Estrogen or estrogen plus progesterone

Table 1. The group of 104 hypertensive subjects included 45 men and 59 women. The group of 195 normotensive subjects included 84 men and 111 women. Because of matching, the ratio of men to women did not differ significantly between diagnostic groups, and the distributions of age and BMI in normotensive subjects were not significantly different from those in hypertensive subjects of the same gender. The mean age at diagnosis of essential hypertension was 50 years for women and 48 years for men. Within each gender, average systolic and diastolic blood pressures were significantly higher in hypertensive subjects than in normotensive subjects (144 and 78 mm Hg, respectively, for hypertensive women vs 120 and 67 mm Hg, respectively, for normotensive women; 137 and 82 mm Hg, respectively, for hypertensive men vs 116 and 71 mm Hg, respectively, for normotensive men).

Since the prevalence of essential hypertension differs between men and women (Kaplan 1994), and angiotensinogen is known to be under the control of estrogens (Cain et al. 1971), we investigated the potential association between the M235T polymorphism and essential hypertension in each gender separately (Table 2). In women, there was no significant difference in allele frequencies between hypertensive and normotensive subjects (0.38 vs 0.36; $P = 0.70$). When women taking hormones were excluded from the analysis, the difference in allele frequencies between hypertensive and normotensive female subjects was larger (0.40 vs 0.34), but did not reach statistical significance ($P = 0.28$). Likewise, in men, there was no significant difference in allele frequencies between hypertensives and matched normotensive controls (0.44 vs 0.47; $P = 0.69$).

We next determined whether allelic variation at the M235T locus predicts the probability of having essential hypertension using conditional logistic regression on these matched data. The number of variant M235T alleles was introduced as the predictor variable in the conditional logistic regression model with three possible outcomes – 0, 1, or 2. Results from the conditional regression analysis on matched samples stratified by gender are summarized in Table 3. The number of variant alleles was not a significant predictor of the probability of having hypertension in either gender ($P = 0.78$ in women; $P = 0.59$ in men).

The probability of having hypertension is modified by a large number of factors. Therefore, we asked whether variation in the number of M235T alleles was a significant predictor of an individual's probability of having hypertension while simultaneously considering the effects of other risk factor variables. Two separate prediction equations were considered. In the first equation, the significant

Table 2 Allele frequencies of the angiotensinogen M235T variant in Rochester, Minnesota (n sample size; q_{M235T} allele frequency of the M235T variant, P probability of observing allele frequency differences by chance)

	n	q_{M235T}	P*
Women			
Hypertensive	59	0.38	
Normotensive	111	0.36	0.70
More severe hypertensive[a]	22	0.45	
Normotensive	41	0.35	0.27
Excluding women taking hormones			
Hypertensive	51	0.40	
Normotensive	99	0.34	0.28
More severe hypertensive	19	0.47	
Normotensive	38	0.35	0.22
Men			
Hypertensive	45	0.44	
Normotensive	84	0.47	0.69
More severe hypertensive	23	0.47	
Normotensive	44	0.49	0.90
Genders combined			
Hypertensives	104	0.41	
Normotensives	195	0.40	0.98
More severe hypertensives	45	0.47	
Normotensives	85	0.42	0.50

[a] Hypertensive individuals taking two or more anti-hypertensive medications

Table 3 Results of the conditional logistic regression analyses predicting the probability of having hypertension in Rochester, Minnesota (P probability of detecting a significant effect of the number of M235T alleles by chance, C.I. confidence interval)

Predictor variable	Parameter estimate[a]	P	Odds ratio[b] [95% C.I.]
M235T alleles only			
Women	0.065	0.78	1.068 [0.670–1.702]
Men	–0.143	0.59	0.866 [0.512–1.465]
M235T alleles with significant predictors of essential hypertension in this sample[c]			
Women	–0.004	0.99	0.996 [0.565–1.757]
Men	0.017	0.95	1.017 [0.562–1.841]
M235T alleles with significant predictors of essential hypertension identified in Turner et al. (1992)[d]			
Women	–0.033	0.91	1.034 [0.568–1.880]
Men	–0.017	0.96	0.983 [0.531–1.819]

[a] Change in the logarithm of the odds of having essential hypertension per unit increase in the number of M235T alleles

[b] Change in the odds of having essential hypertension per unit increase in the number of M235T alleles

[c] Significant predictors of essential hypertension identified by stepwise conditional logistic regression in this sample were sodium-lithium countertransport, sodium-lithium countertransport-squared (in women), and apolipoprotein CII levels (in men)

[d] Significant predictors of essential hypertension in Turner et al. (1992) were age, BMI, sodium-lithium countertransport levels, apolipoprotein CII levels, apolipoprotein CII levels squared, and apo AI levels (in women only)

predictor variables were identified in this sample by forward stepwise conditional logistic regression. both first- and second-order terms were allowed to enter the model. Information about the significant predictor variables was fixed in the model and then the information about the M235T allele was added to the model to test the null hypothesis that this allele did not significantly predict the probability of having essential hypertension. In the second equation, the significant predictor variables of essential hypertension in the RFHS identified by Turner et al. (1992) were first included, and then information about the number of M235T alleles a person carries was added to the model. The results of these analyses are summarized in Table 3. For women, the significant predictors of the probability of having hypertension were sodium/lithium countertranport level and sodium/lithium countertransport level-squared. For men, they were sodium/lithium countertransport and apolipoprotein CII levels. In neither gender did variation in the number of M235T alleles significantly predict variation in the probability of having hypertension after the effects of these predictors had been considered ($P = 0.99$, in women; $P = 0.95$, in men). Significant predictors of essential hypertension in women taking part in the RFHS as previously identified by Turner et al. (1992) were age, BMI, sodium/lithium countertransport level, plasma apolipoprotein CII level, apolipoprotein CII level-squared, and apolipoprotein AI level. For men, the predictors were the same as for women, except apolipoprotein AI level was not a significant predictor. Likewise, variation in the number of M235T alleles did not significantly predict variation in the probability of having hypertension in either gender after the effects of these risk factors had been considered ($P = 0.91$ in women; $P = 0.96$ in men).

The effect of the genetic variation at the M235T locus of the angiotensinogen gene may not be simply additive to the effects of variation in other predictors. Therefore, we asked whether the relationship between the probability of having hypertension and its predictors is influenced by the M235T polymorphism of the angiotensinogen gene. Within each gender, terms representing interactions between the number of M235T variant alleles and each predictor previously identified by stepwise conditional logistic regression (first-order terms only) were successively added to the model. Interactions between the number of M235T alleles and age or BMI were also considered. In neither gender was the relationship between the probability of having hypertension and its predictors modified by variation in the number of M235T alleles (result not shown).

A previous study reported an elevation of the frequency of the M235T allele in subsets of severely affected hypertensives as defined by the requirement for two or more blood pressure lowering medications (Jeunemaitre et al. 1992). In our sample, there were 45 individuals, 22 women and 23 men, with severe hypertension by this definition. There was no significant association in either gender between the M235T polymorphism and severe essential hypertension ($P = 0.27$ in women; $P = 0.90$ in men). However, because of the small number of severe hypertensive individuals in each gender, we next tested for an association of this polymorphism with severe essential hypertension for women and men combined. The estimated frequency of the M235T allele for each gender combined in this subset is shown in Table 2. The frequency of the variant allele was elevated in severe hypertensives relative to the overall sample of hypertensives (0.47 vs 0.41). Although the difference in M235T allele frequency between the severe hypertensives and their matched normotensive controls (0.47 vs 0.42) was greater than that between hypertensives and normotensives in the total sample (0.41 vs 0.40), it did not reach statistical significance ($P = 0.50$).

Table 4 summarizes the allele frequencies at the M235T locus obtained in three previous studies. The frequency of the M235T variant in the sample of normotensive controls from Rochester was not significantly different from those reported in other Caucasian normotensive populations (0.38 in Paris, France, 0.35 in Salt Lake City,

Table 4 Comparison of allele frequencies of the M235T polymorphism in several Caucasian populations (q_{M235T} allele frequency of the M235T variant of the angiotensinogen gene, W/M Women/Men, NR not reported, P probability of observing an allele frequency difference between hypertensive and normotensive subjects by chance)

Population	n	q_{M235T}	P
Rochester, Minn.			
Hypertensive (W/M)	104 (59/45)	0.41 (0.38/0.44)	0.98
Normotensive (W/M)	195 (111/99)	0.40 (0.36/0.47)	
Salt Lake City, Utah			
Hypertensive (W/M)[a]	264 (206/224)	0.44 (0.51/0.44)[a]	< 0.05
Normotensive (W/M)[a]	280 (NR)	0.35 (0.37/NR)[a]	
Paris, France[b]			
Hypertensive (W/M)[a]	166 (206/224)	0.52 (0.51/0.44)[a]	< 0.001
Normotensive (W/M)[a]	184 (NR)	0.38 (0.37/NR)[a]	
Paris, France[c]			
Hypertensive (mild)	136	0.44	0.25 < P < 0.50
Hypertensive (severe)	119	0.49	< 0.05
Normotensive	90	0.38	
Sydney, Australia[d]			
Hypertensive (W/M)	92 (44/48)	0.42 (0.43/0.42)	0.63
Normotensive (W/M)	95 (55/40)	0.39 (0.37/0.41)	

[a] For combined samples from Salt Lake City and Paris
[b] Data reported in Jeunemaitre et al. (1992)
[c] Data reported in Jeunemaitre et al. (1993)
[d] Data reported in Bennett et al. (1993)

Utah, 0.39 in Sydney, Australia, and 0.40 in Rochester). In contrast, the frequency of this allele was 0.71 in a sample of 80 Japanese normotensive females and 0.73 in a sample of 42 Hispanics from Utah (Ward et al. 1993). Therefore, it appears that the allele frequency variability at the M235T locus is small among Caucasian populations relative to that observed among other racial groups. In the hypertensive group, the M235T allele frequency in the sample of hypertensive males of Rochester was also comparable to that in other studies (0.44 in Rochester; 0.44 in Paris, and Salt Lake City, combined; 0.42 in Sydney), but greater variability in allele frequencies was observed among the hypertensive females in the three studies (0.51 in hypertensive females from Paris and Salt Lake City, combined; 0.43 in hypertensive females from Sydney; 0.38 in hypertensive females from Rochester).

Discussion

Several lines of evidence suggest that angiotensinogen gene variation may have a role in predisposing individuals to essential hypertension. First, angiotensinogen is a precursor of angiotensin II, a peptide with powerful vasoconstrictor and antinatriuretic effects. Second, there is a significant correlation between plasma angiotensinogen levels and diastolic blood pressure (Walker et al. 1979), and hypertensive subjects and their offspring have higher average plasma angiotensinogen levels than normotensives (Fasola et al. 1968). Third, blood pressure was shown to increase or decrease after injection of angiotensinogen or antibodies to angiotensinogen, respectively (Ménard et al. 1991; Gardes et al. 1989). Fourth, transgenic animal models overexpressing the angiotensinogen gene have higher blood pressure levels than controls (Kimura et al. 1992). Finally, two independent studies (Jeunemaitre et al. 1992; Caulfield et al. 1994) have reported linkage between the angiotensinogen gene and essential hypertension in selected hypertensive subjects from Paris, France, Salt Lake City, Utah, and southeast England.

We have studied a population-based sample of 104 subjects with onset of hypertension at less than 60 years and 195 matched normotensive individuals from Rochester, Minn. We used both a contingency χ^2 analysis and a multivariable conditional logistic regression analysis for matched data to determine whether there was a relationship between a polymorphism in the angiotensinogen gene, M235T, and the occurrence of essential hypertension in this cross-sectional study. We found no association between the M235T polymorphism of the angiotensinogen gene and hypertension, and we showed that variation in the number of M235T alleles did not significantly predict variation in the probability of having hypertension in either men or women. We also showed that the relationship between the probability of having hypertension and its predictors was not modified by the number of variant alleles at the M235T locus of the angiotensinogen gene. In addition, this polymorphism was not significantly associated with severe hypertension, as defined by the need for multiple-drug therapy.

To date, the association of the M235T variant with essential hypertension has been reported in two different studies (Jeunemaitre et al. 1992, 1993). In a study of 166 hypertensive subjects from Paris, France and 264 hypertensive subjects from Salt Lake City, Utah, Jeunemaitre et al. (1992) showed an elevated M235T allele frequency as compared to normotensive controls. Hypertensive subjects in that study belonged to sibships with a family history of hypertension ascertained either from a referral hypertension clinic (Paris sample) or from a self-reported health survey in the Utah population (Salt Lake City sample; Jeunemaitre et al. 1992; Williams et al. 1988). Association of the M235T variant allele of angiotensinogen with essential hypertension was also found in a second study of 119 hypertensives from Paris, France selected through a referral hypertension clinic and having a positive family history of hypertension (Jeunemaitre et al. 1993). Unlike these previous samples, our sample of hypertensive subjects was selected irrespective of family history of hypertension and not through a referral clinic. Absence of a strong genetic predisposition to hypertension, as implied by the absence of a familial history, may in part be responsible for the lack of association between the M235T polymorphism and hypertension observed in our sample. However, recent data reporting no association between the M235T variant and hypertension in a sample of 92 hypertensive subjects from Sydney, Australia, having both parents with hypertension (Bennett et al. 1993) and in a sample of hypertensive index cases from 63 multiplex pedigrees from England (Caulfield et al. 1994) indicate that absence of a family history of hypertension in our sample may not be sufficient to explain the discrepancy between the results reported here and those published in earlier reports (Jeunemaitre et al. 1992, 1993).

Jeunemaitre et al. (1992) had previously observed a stronger association of the M235T variant and severe hypertension defined by the requirement of two or more anti-hypertensive drugs or a diastolic blood pressure over 100 mm Hg. In addition, significant linkage of the angiotensinogen gene and severe hypertension, as defined above, was obtained in both the Paris and Salt Lake City samples. Bennett et al. (1993) reported no association of the M235T variant of angiotensinogen with hypertension in 92 severely hypertensive subjects, as defined by greatly elevated diastolic and systolic blood pressures (DBP = 112 ± 18 mm Hg; SBP = 172 ± 23 mm Hg). In this study, we found no association between genetic variation at the M235T locus of angiotensinogen and severe hypertension, as defined by the requirement for two or more antihypertensive medications.

Taken together, data from this and other studies suggest that the contribution of the angiotensinogen M235T polymorphism to essential hypertension in the general population may be less than originally suggested. Although, the effect of variation is this gene may be small in the general population, it may be greater in particular subsets of individuals. Therefore, the role of ascertainment

must be carefully considered if a clear picture of the influence of the M235T polymorphism is to be obtained. Because subjects selected through specialized clinics may have come to clinical attention because of occurrence of coronary heart disease, it is possible that the M235T allele may be associated with the cardiovascular complications of hypertension, rather than with elevated blood pressure itself. Interestingly, a polymorphism in the gene encoding another component of the RAS angiotensin-converting enzyme (ACE), has been associated with increased risk for myocardial infarction (Cambien et al. 1992).

The possibility must also be considered that the M235T polymorphism in the angiotensinogen gene does not lead to changes in function or regulation of the angiotensinogen protein. Data from Caulfield et al. (1994), confirming the linkage between a marker in the angiotensinogen gene and essential hypertension but not the association of the M235T polymorphism with essential hypertension, provide some support for this hypothesis. If the M235T polymorphism is not functional, its association with essential hypertension is likely due to the effects of allelic variation at an unknown functional site within or near the angiotensinogen gene and linkage disequilibrium between this unknown functional site and the M235T polymorphism. If one considers the possibility that the linkage disequilibrium between the two loci may be different among populations, then the hypothesis of the M235T polymorphism being a marker in linkage disequilibrium with a nearby functional site is compatible with the inconsistency in the results of previous association studies. Identification of functional sites following detection of significant linkage or association in studies of other complex traits has proven to be a formidable task. Several research strategies to identify these functional changes, such as cladistic analysis and DNA sequencing (Templeton et al. 1987; Deeb et al. 1992), have been proposed but have not yet yielded a comprehensive solution to this difficult problem.

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