Evaluation of the Angiotensinogen Locus in Human Essential Hypertension
A European Study

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Abstract—Different family and case-control studies support genetic linkage and association at the human angiotensinogen (AGT) locus with essential hypertension. To extend these previous observations, a European collaborative study of nine centers was set up to create a large resource of affected sibling pairs. The AGT locus was studied using a highly polymorphic dinucleotide repeat in the 3’-flanking region of the gene in 350 European families, comprising 630 affected sibling pairs. Statistical analyses using two different methods did not show any evidence for linkage either in the whole panel or in family subsets selected for severity or early onset of disease. Although several arguments from association studies suggest a role of the AGT gene in essential hypertension, this large family study did not replicate the initial linkage reported in smaller studies. Our results highlight the difficulty of identifying susceptibility genes by linkage analysis in complex diseases. (Hypertension. 1998;31:725-729.)

Key Words: angiotensinogen ■ hypertension, essential ■ genetics ■ microsatellite repeats

Several genetic studies have been performed to evaluate the role of the AGT gene in essential hypertension after initial positive results were obtained by linkage and association in French and Utah families.1 This first study showed an excess of allele sharing at the microsatellite marker at the 3’-flanking region of the AGT gene in hypertensive sibling pairs and a significant allele frequency difference between hypertensive subjects and unrelated controls for two substitutive polymorphisms of the gene, located at codon 174 (T174M) and 235 (M235T). The T235 allele of the M235T polymorphism, the frequency of which was increased in hypertensives, was also associated with an increased level of plasma AGT in hypertensive patients. Subsequently, Caulfield et al12 confirmed a linkage of the AGT locus to hypertension in affected sibling pairs, although results were at variance in some respects from the study of Jeunemaıˆtre et al.1 In particular, a marked frequency difference was observed between case and control subjects for the A6 and A7 allele of the AGT microsatellite, and no difference was observed for the M235T polymorphism between case and control subjects. Several case-control studies have also been performed with the M235T polymorphism of the AGT gene as the genetic marker with conflicting results: some of these studies were negative,2–8 whereas others were positive.9–14 In addition to data obtained in humans, linkage of the agt locus in the rat hereditary model of hypertension has been sought. The data obtained in different strains of rats are conflicting, since Hübner et al15 found no linkage in a cross between stroke-prone spontaneously hypertensive rats and Wistar-Kyoto rats, whereas Lodwick et al16 found linkage in a cross between spontaneously hypertensive and Wistar-Kyoto rats.

To investigate the reported linkage in a large number of human hypertensive families, we established a collaboration among nine European centers collecting families with multiple cases of essential hypertension. In these families, linkage to AGT was studied using the AGT microsatellite and different affected sib-pair statistical methods of analysis.

Methods

Hypertensive Sibships

The study was approved by local review committees, and all subjects gave informed consent. A total of 350 hypertensive sibships were selected in collaborating centers in France (Paris, n=155), Germany (Berlin North, n=36; Berlin South, n=18; Regensburg, n=16), Italy (Milan, n=34), the Netherlands (Rotterdam, n=11), and the UK (Glasgow, n=10; London, n=26; Oxford, n=44) according to the following criteria: (1) onset of hypertension at <60 years of age; (2)
Selected Abbreviations and Acronyms

AGT = angiotensinogen
BMI = body mass index
DBP = diastolic blood pressure
SBP = systolic blood pressure

established hypertension as defined either by a DBP >90 mm Hg in treated patients or by a DBP >95 mm Hg on two consecutive visits for those untreated; (3) absence of secondary forms of hypertension, as determined by appropriate clinical investigation in the collaborating center; and (4) families with at least two siblings affected by hypertension. Subjects with a history of alcohol intake greater than three drinks per day, oral contraceptive therapy, diabetes mellitus, or renal impairment were excluded. Blood pressure was measured with subjects in the supine position with a sphygmomanometer.

Normotensive subjects (n=331) from hypertensive families had SBP <145 mm Hg, DBP <90 mm Hg, and no history of antihypertensive treatment or chronic disease.

Application of these criteria led to the identification of 350 sibships in which two or more offspring were hypertensive (255 pairs, 72 trios, 19 quartets, 3 quintets, and 1 sextet). A total of 630 affected sibling pairs were thus evaluated. All individuals in the study were European Caucasians. Their clinical characteristics are listed in Table 1.

Genotype Analysis of the Dinucleotide Repeat Polymorphism at the Human AGT Locus

The AGT dinucleotide repeat genotypes were determined in included subjects by polymerase chain reaction and acrylamide gel electrophoresis as described in Kotelevtsev et al.7 Genotypes were read either on standard sequencing gels using [γ-32P]ATP end-labeled primers or by an ABI 373 automated sequencer using fluorescent labeled primers. Allele identity was checked in all gels using CEPH individuals No. 1413.1 and 1413.2 who bear the 7/9 and 5/6 alleles, respectively. Estimated allele frequencies of the AGT microsatellite are shown in Table 2.

Statistical Analysis

Databases from the different centers were electronically transferred to the analyzing center. The allele frequencies were estimated from the pedigree data with the ILINK program of the LINKAGE package (Table 2). Genetic linkage was tested using two different nonparametric methods for sib-pair analysis, no significant excess of hypertensive sibships are shown in Table 3. Using two different programs for sib-pair analysis, no significant excess of shared alleles was observed in the whole panel. Marker information on other siblings and one or both parents was incorporated when available. A one-sided \( t \) test with \((n-1) df\) (where \( n \) is the total number of affected sib pairs) is performed to determine whether this mean proportion is greater than .5 (the expected value under the null hypothesis of no linkage). The result of this test is obtained by using the program SIBPAL of the SAGE package.20

The second method is based on the number of shared and nonshared alleles among affected sib pairs computed from informative meioses. When there are multiple siblings in a sibship, this method weights for the pair numbers. The number of shared and nonshared alleles is calculated. Under the null hypothesis of no linkage, the number of shared and nonshared alleles is equal to half of the estimated sum of shared and nonshared alleles. A \( \chi^2 \) test with 1 df is computed to compare the observed values and the expected ones. This method is computed in the program SIBPAIR of the ANALYZE package.21 The MAPMAKER/SIBS package was used to calculate the relative risk ratio deduced from the allele sharing for the AGT microsatellite alleles.22

Results

Because parental genotypes were not available, allele frequencies were estimated by ILINK (LINKAGE) on the total group of families. No significant difference was found between the allele frequency observed in each group and the overall estimated allele frequency.

Results of linkage analysis with the AGT microsatellite in hypertensive sibships are shown in Table 3. Using two different programs for sib-pair analysis, no significant excess of shared alleles was observed in the whole panel.

The statistical analysis was also performed on subgroups of families defined according to criteria used in the initial AGT

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### Table 1. Clinical Parameters of Populations Studied

<table>
<thead>
<tr>
<th>Cities</th>
<th>Sibships (Pairs)</th>
<th>Subjects (M/F)</th>
<th>Age, y</th>
<th>Onset, y</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>Rx, %</th>
<th>BMI, kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berlin North</td>
<td>36 (58)</td>
<td>82 (39/43)</td>
<td>54±9.6</td>
<td>39±12.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>27.3±3.7</td>
</tr>
<tr>
<td>Berlin South</td>
<td>18 (34)</td>
<td>44 (16/27)</td>
<td>60±9.2</td>
<td>NA</td>
<td>176±23.1</td>
<td>104±12.5</td>
<td>32 (74)</td>
<td>27.7±4.4</td>
</tr>
<tr>
<td>Regensburg</td>
<td>16 (39)</td>
<td>42 (22/20)</td>
<td>59±6.1</td>
<td>NA</td>
<td>165±18.1</td>
<td>100±5.5</td>
<td>24 (57)</td>
<td>27.0±3.5</td>
</tr>
<tr>
<td>London</td>
<td>26 (28)</td>
<td>53 (25/28)</td>
<td>61±12.5</td>
<td>NA</td>
<td>168±11.0</td>
<td>103±6.1</td>
<td>NA</td>
<td>25.7±3.8</td>
</tr>
<tr>
<td>Oxford</td>
<td>10 (14)</td>
<td>22 (6/16)</td>
<td>NA</td>
<td>50±14.0</td>
<td>141±10.3</td>
<td>92±7.7</td>
<td>19 (86)</td>
<td>28.0±3.7</td>
</tr>
<tr>
<td>Rotterdam</td>
<td>11 (32)</td>
<td>31 (16/15)</td>
<td>64±9.3</td>
<td>NA</td>
<td>156±21.4</td>
<td>86±13.2</td>
<td>22 (71)</td>
<td>25.2±2.7</td>
</tr>
<tr>
<td>Milan</td>
<td>34 (54)</td>
<td>76 (39/37)</td>
<td>54±11.0</td>
<td>45±9.9</td>
<td>165±23.3</td>
<td>106±9.6</td>
<td>50 (66)</td>
<td>26.4±3.4</td>
</tr>
<tr>
<td>Paris</td>
<td>155 (266)</td>
<td>361 (185/176)</td>
<td>52±9.7</td>
<td>40±10.5</td>
<td>166±19.3</td>
<td>106±9.7</td>
<td>304 (84)</td>
<td>25.5±3.6</td>
</tr>
</tbody>
</table>

Rx indicates number of subjects taking antihypertensive medication; NA, not available. Values are mean±SD.
TABLE 3. Linkage Analysis With AGT Microsatellite in the Hypertensive Sibships Using Two Different Methods

<table>
<thead>
<tr>
<th>Sib Pair</th>
<th>Sample</th>
<th>(\pi \pm SE)</th>
<th>(P)</th>
<th>(P) (SAGE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All affected</td>
<td>630</td>
<td>0.49±0.01</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>176</td>
<td>0.51±0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>180</td>
<td>0.48±0.02</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sib Pair Shared/Not Shared</th>
<th>P (ANALYZE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All affected</td>
<td>370.9/382.9</td>
</tr>
<tr>
<td>Males</td>
<td>124.1/117.5</td>
</tr>
<tr>
<td>Females</td>
<td>114.9/121.7</td>
</tr>
</tbody>
</table>

\(n\) indicates number of sib pairs; \(\pi\), mean proportion of alleles identical by descent.

study and in other subsequent studies. These criteria included a BMI <27 kg/m², severe hypertension (DBP >100 mm Hg or at least two antihypertensive treatments), or an early age of onset (<45 years of age). Application of these criteria, isolated or in combination, did not result in any significant evidence for linkage in the subjects defined. Only a marginal excess of shared versus nonshared alleles was observed when all these criteria were used together, but the number of analyzed pairs was small in this case (Table 4).

Because results are influenced by allele frequency estimation, two different analyses were also performed using control or patient AGT microsatellite allele frequencies. Both analyses gave similar results.

To calculate the relative risk ratio for a sib attributable to the AGT locus, we used the MAPMAKER/SIBS package. The estimated relative risk ratio associated with AGT microsatellite allele sharing is equal to .83. From these family data it is possible to exclude the hypothesis that the AGT locus is associated with a \(\lambda \geq 1.25\) at the exclusion threshold of logarithm of the odds \(< -2\).

### Discussion

This study of a large number of Caucasian hypertensive sibships (\(n=350\)), using the AGT microsatellite located in the 3'-flanking region as a genetic marker, is the largest panel of affected hypertensive pairs (\(n=630\)) yet studied for linkage at the AGT locus. The AGT microsatellite is a highly informative marker, and statistical analysis was performed using different programs developed for analyzing sib-pair studies. Therefore, this study should have the power to replicate initial positive results found in smaller panels of families, if the effect of the AGT locus is as large as the previous studies suggest. However, neither the result in the whole panel of sibships nor the result in subgroups of families selected according to previously defined criteria, which have increased the evidence for linkage at AGT in other studies, provided any evidence for linkage.

In view of the negativity of the results, we reanalyzed the initial panel of French families included in the first report of Jeunemaître et al, which is included as part of this larger panel. We confirmed the presence of a linkage by the Lange method of sib-pair analysis used in the first report, which is based on identity by state. The probability value as indicated in the original report \((P<.05)\) can only be considered as suggestive of a linkage of hypertension to the AGT locus. Moreover, use of an allele frequency estimated by maximum likelihood (ILINK) gave a nonsignificant probability value. In view of the low genetic determination of essential hypertension and the likely high number of genes involved, it remains unlikely that a highly significant probability value can be found, even with a biological relevant gene, unless a huge number of families is included in the study.

The absence of replication of the initial study could have several possible causes. The first possibility is that the initial series of subjects included patients with a more severe genetic predisposition to hypertension and possibly a stronger family history of hypertension. Another possibility is that the initial linkage result was a false-positive, which would not be expected to be confirmed in an independently ascertained panel.

Sib-pair methods of linkage analysis are highly sensitive to allele frequency estimation when parental genotypes are not known. Thus, difficulties in allele frequency estimation might have influenced previously published positive results.

However, several lines of genetical, biological, and experimental data suggest that AGT is involved in hypertension. Several association studies have replicated results found in the initial study, the 235T allele of the M235T polymorphism.

### TABLE 4. Linkage Analysis With AGT Microsatellite in Families According to Severity of Hypertension, Age of Onset, and BMI Using Two Different Methods

<table>
<thead>
<tr>
<th>Sib Pair</th>
<th>Sample</th>
<th>(\pi \pm SE)</th>
<th>(P)</th>
<th>SAGE</th>
<th>ANALYZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Status 1 (onset &lt;45 y)</td>
<td>All affected</td>
<td>181</td>
<td>0.48±0.02</td>
<td>NS</td>
<td>120.2/123.6</td>
</tr>
<tr>
<td>Status 2 (DBP ≥100 mm Hg or AHD ≥2)</td>
<td>All affected</td>
<td>295</td>
<td>0.49±0.02</td>
<td>NS</td>
<td>176.9/176.4</td>
</tr>
<tr>
<td>Status 3 (DBP ≥95 mm Hg or AHD ≥1) and (BMI ≤27 kg/m²)</td>
<td>All affected</td>
<td>242</td>
<td>0.49±0.02</td>
<td>NS</td>
<td>157.1/153.5</td>
</tr>
<tr>
<td>Status 4 (DBP ≥100 mm Hg or AHD ≥2) and (onset &lt;45 y) and (BMI ≤27 kg/m²)</td>
<td>All affected</td>
<td>30</td>
<td>0.56±0.06</td>
<td>NS</td>
<td>24.8/20.6</td>
</tr>
</tbody>
</table>

AHD indicates antihypertensive drugs; \(n\), number of sib pairs; and \(\pi\), mean proportion of alleles identical by descent.
being more frequent in hypertensive than in normotensive subjects. A recent study using AGT haplotypes reproduced the initial data in a large case-control population involving 477 hypertensive and 364 normotensive Caucasian subjects. However, this is not the case for all studies published, and the discrepancy between positive and negative results might come from variations in ethnic groups studied, from differences in the severity of hypertension in affected patients studied, or from spurious association due to unrecognized population stratification.

The 235T allele is associated with an increased level of plasma AGT and this relationship between the AGT genotype and plasma AGT has been confirmed in independent studies. An increased plasma AGT level is potentially a cause of elevated angiotensin II generation. This possibility is further supported by the establishment of transgenic mice expressing high levels of rat agt and having elevated blood pressure and the development of strains of mice with an in situ duplication of the agt gene. Plasma agt levels increase progressively with the number of agt copies, reaching 145% of the normal level in the four-copy animals, with correlated blood pressure increase and pressure filtration.

A critical point, therefore, is the interpretation of the discrepancy between negative results from this large sib-pair study and the positivity of several association studies. Sib-pair studies are considered to be more robust but to have a smaller power than association studies to detect the implication of a locus in complex diseases. However, the negativity of this large linkage study does not argue for a strong effect of AGT on hypertension. Any selection criteria that would decrease hypertension heterogeneity, or even help to distinguish phenotypically hypertensive patients in whom AGT is at play, could facilitate the detection of a linkage. However, no such criteria are available at the present time.

To identify a particular biological or paraclinical phenotype associated with functional genetic variation at the AGT locus, genotype-phenotype relationships should be studied using the functional variant(s) of AGT or marker(s). Because the M235T polymorphism is associated with plasma AGT level variations but does not seem to be responsible by itself for the change in the AGT gene regulation, the functional variant has been searched for with both genetic and molecular biology approaches. Recent in vitro data from Inoue et al suggest that the A(-6)G polymorphism of the AGT promoter is functional. That study found that a reporter gene was expressed at a higher level when the A(-6) allele was present on the AGT promoter driving the reporter gene transcription. Combined segregation-linkage analyses of plasma AGT level and genetic marker data, such as those performed in the case of the human angiotensin I–converting enzyme gene polymorphism, will help in understanding whether the A(-6)G polymorphism, or others as yet undiscovered, can explain the genetic effect observed on the plasma AGT level.

Finally, these results have important consequences for the future design of family studies in genetic research on hypertension. They suggest that even for a gene for which evidence has accumulated supporting its involvement in hypertension, the study of a large panel of families can be negative. This emphasizes the need for collection of even larger panels of families, whose structure would allow the use of various types of genetic analysis including not only linkage studies but also linkage disequilibrium studies, such as the haplotype relative risk method or the transmission disequilibrium test. In addition, careful characterization for intermediate phenotypes may be used to identify families with a genetic predisposition resulting from variation in a particular physiological system.

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