Angiotensinogen Gene Polymorphisms M235T/T174M
No Excess Transmission to Hypertensive Chinese

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Abstract—The gene encoding angiotensinogen (AGT) has been widely studied as a candidate gene for hypertension. Most studies to date have relied on case-control analysis to test for an excess of AGT variants among hypertensive cases compared with normotensive controls. However, with this design, nothing guarantees that a positive finding is due to actual allelic association as opposed to an inappropriate control population. To avoid this difficulty in our study of essential hypertension in Anqing, China, we tested AGT variants using the transmission/disequilibrium test, a procedure that bypasses the need for a control sample by testing for excessive transmission of a genetic variant from parents heterozygous for that variant. We analyzed two AGT polymorphisms, M235T and T174M, which have been associated with essential hypertension in whites and Japanese, using data on 335 hypertensive subjects from 315 nuclear families and their parents. Except in the group of subjects younger than 25 years, M235 and T174 were the more frequently transmitted alleles. We found that 194 parents heterozygous for M235T transmitted M235 106 times (P = 0.22) and that 102 parents heterozygous for T174M transmitted T174 60 times (P = 0.09). Stratifying offspring by gender, M235 and T174 were transmitted 60 of 106 times (P = 0.21) and 44 of 75 times (P = 0.17), respectively, in men, and 46 of 88 times (P = 0.75) and 16 of 27 times (P = 0.44), respectively, in women. Our results were also negative in all age groups and for the affected offspring with blood pressure values ≥ 160/95 mm Hg. Thus, this study provides no evidence that either allele of M235T or T174M contributes to hypertension in this Chinese population. (Hypertension. 1999;33:698-702.)

Key Words: angiotensinogen ■ hypertension, essential ■ transmission/disequilibrium test

Hypertension, a leading cause of cardiovascular and renal disease in the United States, results from a combination of genetic and environmental factors. Because of the public health significance of this disorder, many studies on the genetic basis of hypertension have been performed.1–9 Several of these studies have tested genes with potential biological relevance to ascertain whether certain variants appear to influence the disease process.5–9 One frequently studied gene has been angiotensinogen (AGT), a logical candidate, given the strong correlation of plasma angiotensinogen levels and blood pressure.10 Located on 1q42 to 43, AGT comprises five exons and four introns spanning 13 kb.11 Although several polymorphisms in the AGT region have been identified,3 much interest has focused on two coding region polymorphisms, M235T and T174M, both in exon 2. More specifically, through case-control studies, many3–5 but not all6–9 investigators have concluded that the threonine allele (T235) of M235T and the methionine allele (M174) of T174M are associated with elevated risk for hypertension. The validity of these case-control studies, however, depends on the ability of identifying a suitable control group, since different genetic backgrounds with varying polymorphism frequencies can potentially lead to spurious results.

To eliminate the need for an external control group in our study of M235T and T174M in rural Chinese, we have collected nuclear families consisting of hypertensive individuals and their parents and have applied family-based association testing using the transmission/disequilibrium test (TDT).12 With TDT, parental alleles transmitted to the affected hypertensive offspring are compared with those not transmitted. As a result, each family provides its own controls, and false-positives due to poorly matched controls are avoided.

In addition to the sophistication of the TDT study design, the other defining feature of our investigation is the unique nature of the population considered. The rural community of Anqing, China, is more than simply a novel ethnic group for testing AGT. It is a population characterized by relatively low blood pressure (7 to 12 mm Hg below Western norms), infrequent use of blood pressure medication, and high popu...

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lation frequencies of T235 and T174. Consequently, we have the opportunity to investigate whether AGT plays an important role in hypertension in a population with these characteristics.

**Methods**

**Study Sample**

The study population was selected from the Anqing district in Anhui province, China. Because of limited public transportation, the population has a very stable base and is extremely homogeneous with respect to ethnicity, dietary habits, lifestyles, and environmental factors. The characteristics of the Anqing population have been described elsewhere. Initial recruitment (panel 1 families) yielded 488 individuals (156 nuclear families, with 176 hypertensive offspring and 312 parents) collected from 5 towns in Anqing: Huaining, Qiangjiang, Qianshan, Yuxi, and Susong. Because heterozygosity of M235T and T174M turned out to be low in this population, statistical power using only these 1 families would be somewhat limited, especially in the youngest age group. To achieve a greater number of heterozygous parents for each marker, we formed a second panel of trios from a random collection of families from 8 towns of Anqing: Tongcheng, Zongyang, Huaining, Wangjiang, Qianshan, Taihu, Yuxi, and Susong. Panel 2 families contained 477 individuals (159 nuclear families, with 159 hypertensive offspring and 318 parents). The index hypertensive offspring were selected on the basis of the following criteria: (1) systolic blood pressure ≥140 or diastolic blood pressure ≥90 mm Hg or current antihypertensive medication, (2) free of secondary hypertension due to renal insufficiency or diabetes mellitus, and (3) availability of both parents. If a hypertensive proband met these eligibility requirements in an initial screening survey, a field team, consisting of trained epidemiologists from Anhui MEIZHONG Institute for Biomedical Science and Environmental Health, was sent to the village to confirm the blood pressure measurements.

The study was approved by the Institutional Review Board of the Harvard School of Public Health, and all study subjects gave informed consent. All the procedures followed were in accordance with institutional guidelines. Blood samples were drawn from the hypertensive proband and his/her parents (as described below under Phlebotomy), and a previously validated questionnaire was administered by trained interviewers. Blood pressure, weight, and height were measured by standard methods. So that misclassification of hypertensive subjects was minimized, blood pressure measurements were taken on three separate dates. All protocol procedures were fully compatible with standard methods used in the United States, and anthropomorphic measurements were taken by trained and certified examiners as described previously.

**Blood Pressure Measurements**

Trained nurses measured blood pressure by plethysmography using a mercury-gravity manometer with an appropriately sized cuff. Measurements were taken with subjects in the seated position after they had rested for 10 minutes. This procedure was repeated on three separate dates. All protocol procedures were fully compatible with standard methods used in the United States, and anthropomorphic measurements were taken by trained and certified examiners as described previously.

**Phlebotomy**

Forearm venous blood samples, obtained by venipuncture, were collected in 10-mL Vacutainer tubes containing EDTA (2 tubes) and citrate (2 tubes). Tubes were kept on ice and subsequently for 10 minutes in a refrigerated centrifuge at 2000g. Plasma was removed from the cell pellet by pipetting. All samples were stored at −80°C.

**DNA Extraction**

DNA extraction was carried out at the Anhui MEIZHONG Institute for Biomedical Science and Environmental Health. Isolation of genomic DNA was performed with Puragene DNA isolation kits (Gentra Systems) by a modification of previously described DNA extraction methods. This DNA extraction protocol typically yields 300 μg DNA from 10 mL whole blood with and OD260/OD280 value of 1.8. After extraction, 50% of the DNA sample was prepared for shipment to the Harvard Program for Population Genetics for genetic analysis, and the other half was stored at −85°C as a backup sample.

**Genotype Analysis**

The M235T and T174M polymorphisms were investigated by polymerase chain reaction (PCR) amplification of genomic DNA followed by restriction-endonuclease digestion. The primers for PCR amplification of T174M polymorphism were 5′-TGGGCCCT-CTG GCCCTCTCTCTATCT-3′ (forward) and 5′-CACGCCGTAT-GAACCGTCAATCT-3′ (reverse). The primer sequences for M235T have been described previously. Genomic DNA (20 ng) was amplified in a reaction containing 200 mmol/L of each primer, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 200 mmol/L of each deoxynucleotide triphosphate, and 0.15 U Taq polymerase (Promega) in a volume of 15 μL. An initial denaturation (3 minutes at 94°C) was followed by 38 cycles of 15 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C. The specific mismatches incorporated into the antisense primer create a Tth111 I site if the T235 variant is present; subsequent digestion with this enzyme at 65°C thus results in diagnostic fragments that are visualized by ethidium bromide staining and UV transillumination after electrophoresis on a horizontal submarine 3.5% agarose gel. The T174M polymorphism was genotyped as an Ncol PCR restriction fragment length polymorphism using an analogous protocol, except for an annealing temperature of 64°C.

**Statistical Analysis**

To eliminate potential bias introduced by a poorly matched control population, we tested for association of the M235T and T174M polymorphisms with hypertension using TDT. Instead of using control subjects, TDT assesses whether one allele is transmitted more frequently from parents to affected offspring; thus, the relevant information is the number of times that an allele is transmitted from parents heterozygous for that allele. In this context, TDT involves identifying all parents heterozygous for either M235T or T174M and assessing whether any of the alleles is transmitted to hypertensive offspring more frequently than 50% of the time. The test assumes that there is no segregation distortion at the marker locus and that the contributions from male and female heterozygous parents to affected children are independent.

The computer program ANALYZE (ftp://linkage.cpmc.columbia.edu/software/analyze) was used to determine the allele transmission rates. Because of small sample sizes in some subgroups (eg, age <25 years), the probability of observing greater or equal transmission of an allele by chance (ie, the P value) was found using exact binomial calculation. Since effects may vary across subgroups, we also performed stratified analysis by gender, age group, and severity (with “severe” being defined as systolic blood pressure ≥160 or diastolic blood pressure ≥95 mm Hg). Although findings of previous investigations suggest that the T235 and M174 alleles are associated with an increased risk of hypertension, it is still controversial whether these alleles are functionally relevant variants or merely surrogates for the actual disease-causing mutation. In our population, we were interested in observing whether there was excess transmission of any allele; consequently, a two-sided test for statistical significance was performed. Our power calculations, which use exact binomial calculations for various transmission rates (f) and assume a significance level of 0.05, take into account the two-sided nature of our test. The program EH16 was used to assess linkage disequilibrium between M235T and T174M.

**Results**

Sociodemographic characteristics of the hypertensive offspring are shown in Table 1. Combining panel 1 and panel 2

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**Note:** The document contains a table that is not visible in the text. For a complete understanding, the table should be referred to in the original text. The table is likely to contain data related to sociodemographic characteristics of the hypertensive offspring, which are essential for the statistical analysis described in the Methods section.
TABLE 1. Demographic Characteristics of Hypertensive Offspring

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subcategory</th>
<th>Panel 1</th>
<th>Panel 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>131</td>
<td>74.4</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>45</td>
<td>25.6</td>
<td>62</td>
</tr>
<tr>
<td>Age, y</td>
<td>&lt;25</td>
<td>29</td>
<td>16.5</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>25–34</td>
<td>90</td>
<td>51.1</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>57</td>
<td>32.4</td>
<td>62</td>
</tr>
<tr>
<td>Severity</td>
<td>&lt;160/95</td>
<td>131</td>
<td>74.4</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>≥160/95</td>
<td>45</td>
<td>25.6</td>
<td>45</td>
</tr>
<tr>
<td>M235T*</td>
<td>M235/M235</td>
<td>6</td>
<td>3.5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>M235/T235</td>
<td>50</td>
<td>29.4</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>T235/T235</td>
<td>114</td>
<td>67.1</td>
<td>93</td>
</tr>
<tr>
<td>T174M*</td>
<td>T174/T174</td>
<td>148</td>
<td>86.0</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>T174/M174</td>
<td>23</td>
<td>13.4</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>M174/M174</td>
<td>1</td>
<td>0.6</td>
<td>1</td>
</tr>
</tbody>
</table>

*The number of subjects in the category is not equal to the number of offspring because of missing values in the genotyping. In panel 1, 6 parents and 4 offspring had missing values for T174M, and 6 parents and 6 offspring had missing values for M235T; in panel 2, 9 parents and 5 offspring had missing values for T174M, and 6 parents and 6 offspring had missing values for M235T, and 3 offspring had missing values for T174M.

Together, parental allele frequencies were 0.20 (M235) and 0.80 (T235) for M235T, and 0.92 (T174) and 0.08 (M174) for T174M. Both markers were in Hardy-Weinberg equilibrium. T174 and T235 were in strong linkage disequilibrium ($P<0.0001$), so information from these two loci is not independent.

From among our 315 nuclear families, we found 194 informative transmissions (ie, heterozygous parents) for M235T and 102 for T174M. Despite the power (Table 2) of this sample size (eg, ≥80% power for detecting actual transmission rates of M235 or T235 ≥0.61, or T174 or M174 ≥0.65), we did not detect statistically significant excess transmission of any allele. Interestingly, however, the trend in all subgroups except age <25 years was for reduced transmission of T235 and M174, the putative risk alleles proposed in other studies. Specifically, 106 of 194 heterozygous parents transmitted M235 ($P=0.22$) and 60 of 102 heterozygous parents transmitted T174 ($P=0.09$). M235 was transmitted 60 of 106 times to men ($P=0.21$) and 46 of 88 times to women ($P=0.75$), whereas T174 was transmitted 44 of 75 times to men ($P=0.17$) and 16 of 27 times to women ($P=0.44$). Moreover, M235 was transmitted more frequently to those older than age 35 (44 of 77 times, $P=0.25$) and to those aged 25 to 34 (44 of 77 times, $P=0.25$). T174 was also transmitted more frequently to those older than age 35 (25 of 39 times, $P=0.11$) and to those aged 25 to 34 (28 of 48, $P=0.31$). For those younger than age 25, T235 was transmitted 22 of 40 times ($P=0.64$) and M174 was transmitted 8 of 15 times ($P=0.99$). Similar results were also obtained when the data were stratified by severity of hypertension: M235 was transmitted 31 of 55 times to cases with blood pressure ≥160/95 mm Hg ($P=0.42$) and 75 of 139 times to cases with blood pressure <160/95 mm Hg ($P=0.40$); T174 was transmitted 17 of 26 times ($P=0.17$) and 43 of 76 times ($P=0.30$) to those in the same severity groups. Results were also negative when the two panels were considered separately (Tables 3 and 4).

**Discussion**

This investigation revealed no evidence for a significant association of essential hypertension with AGT polymorphisms M235T or T174M, even when we stratified offspring by age group, gender, or disease severity. These findings are in agreement with the results of our affected sib-pair linkage analysis of AGT in a separate collection of 310 hypertensive sibling pairs from the same geographic region. Thus, we conclude that neither M235T nor T174M plays a critical role in the pathogenesis of essential hypertension in this Chinese population.

TABLE 2. Power to Detect Excess Transmission of M235T or T174M Alleles

<table>
<thead>
<tr>
<th>Transmission Frequency (f)</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M235</td>
</tr>
<tr>
<td>0.60</td>
<td>0.76</td>
</tr>
<tr>
<td>0.61</td>
<td>0.84</td>
</tr>
<tr>
<td>0.62</td>
<td>0.90</td>
</tr>
<tr>
<td>0.63</td>
<td>0.94</td>
</tr>
<tr>
<td>0.64</td>
<td>0.97</td>
</tr>
<tr>
<td>0.65</td>
<td>0.99</td>
</tr>
<tr>
<td>0.66</td>
<td>0.99</td>
</tr>
<tr>
<td>0.67</td>
<td>0.99</td>
</tr>
<tr>
<td>0.68</td>
<td>0.99</td>
</tr>
<tr>
<td>0.69</td>
<td>0.99</td>
</tr>
<tr>
<td>0.70</td>
<td>0.99</td>
</tr>
<tr>
<td>0.71</td>
<td>0.99</td>
</tr>
</tbody>
</table>
In the present study, we have broadened the investigation of \textit{AGT} for a role in hypertension in two major ways. First, we have studied two novel (ie, not included in our previous linkage study\textsuperscript{17}) panels selected from the rural Chinese district of Anqing in Anhui province. These families are noteworthy not only because they are the first Chinese panels studied using TDT, but also because of several distinctive features. For example, as pointed out above, blood pressure in rural Chinese tends to run approximately 7 to 12 mm Hg lower than in Western populations,\textsuperscript{13} so a 140/90 mm Hg criterion captures only those at the very upper portion of the population blood pressure distribution. Moreover, because the use of blood pressure medication in rural China is uncommon, our hypertensive cases tend to reflect the natural progression of the disease. Ethnic, dietary, and lifestyle homogeneity also exists in this population.

Second, we have used TDT rather than a case-control study design, thereby eliminating the possibility of biased results due to poorly matched controls. Furthermore, TDT can be much more powerful than allele-sharing methods (eg, sib-pair analysis) for genes of modest effect.\textsuperscript{18} For example, despite an effective sample size of 194 for M235T or T174M, the statistical power to detect a modest excess of allelic transmission. Parental heterozygosity), we had more than 80% power to detect a 140/90 mm Hg criterion allele 4. But even when we stratified offspring by gender, we found no association of essential hypertension with either M235T or T174M. To isolate more severe cases, we also considered an alternative criterion for hypertension (systolic blood pressure $\geq 160$ or diastolic blood pressure $\geq 95$ mm Hg). Transmission of M235 and T174 exceeded 50% in each subgroup, although the excess did not reach statistical significance.

We gratefully acknowledge the assistance and cooperation of the faculty and staff of the Anhui Medical University, Anqing Public Health Bureau, Yijing Hospital, Huigong Hospital, Fushan Hospital, and Haikou Hospital. We are thankful to all the participants in our study.

### Acknowledgments

We gratefully acknowledge the assistance and cooperation of the faculty and staff of the Anhui Medical University, Anqing Public Health Bureau, Yijing Hospital, Huigong Hospital, Fushan Hospital, and Haikou Hospital. We are thankful to all the participants in our study.
Transmission of T235/M174 to Hypertensive Chinese

References


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