T+31C Polymorphism of Angiotensinogen Gene and Essential Hypertension

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Abstract—A common variant at codon 235 of the angiotensinogen gene with methionine to threonine amino acid substitution (AGT M235T) has been reported as a genetic risk for essential hypertension. However, the frequency of AGT T235 was heterogeneous among races, and a positive association between AGT M235T and hypertension was not settled. To examine the association in a general population of Japanese (n=4013), we introduced the TaqMan polymerase chain reaction method and examined the relation between hypertension and T+31C polymorphism, which was in absolute linkage disequilibrium with AGT M235T. The C+31 allele of AGT was significantly associated with the positive family history of hypertension (FH) but not with the presence of hypertension or blood pressure. The subjects with CC tended to have hypertensive relatives, especially a hypertensive father or siblings, and its statistical significance was stronger in men. Adjustment of confounding factor did not alter the results of simple association study, suggesting that this positive association with FH is independent and significant. Our findings revealed that the TaqMan polymerase chain reaction method is a powerful tool for genetic association study with a large number of subjects and that AGT T+31C is significantly associated with paternal FH. (Hypertension. 2001;37:281-285.)

Key Words: genetics ■ epidemiology ■ hypertension, essential ■ angiotensin ■ risk factors ■ cardiovascular diseases

Hypertension is a common disorder in which multiple genetic factors account for 40% of blood pressure variability. Because the renin-angiotensin system plays a central role in the control of blood pressure, genetic variants of the renin-angiotensin system have been examined as candidates for causing hypertension. Physiological studies revealed that angiotensinogen is genetically involved in the pathogenesis of essential hypertension.

In 1992, the angiotensinogen gene (AGT) polymorphism AGT M235T was reported to be associated with essential hypertension and increased concentration of plasma angiotensinogen in white subjects. A recent report suggested that a common variant of AGT G-6A in the proximal promoter, which is in almost complete linkage disequilibrium with M235T, leads to a higher basal transcription rate of the AGT gene. In regard to the association between T235 or the A-6 allele and hypertension, however, the results obtained from many case-control studies or affected sib-pair methods are still inconsistent. What is clear from the previous studies is only that the frequency of the T235 allele is significantly different among races. Even in a Japanese population, however, genetic studies concerning the effect of AGT/T235 could not reach a conclusion. Thus, we decided to examine the effect of the AGT allele for hypertension by studying a large general population. The advantage of this study design is that participants of this study consist of randomly selected urban residents and that it used the TaqMan chemistry method, which is a powerful tool for semiautomatic genotype determination in the large number of samples.

Methods

Study Population

Suita City is a satellite city located in the Osaka prefecture, the second largest urban area in Japan. The Suita Study was based on a random sample of 14 200 Japanese residents of Suita. Participants between the ages of 30 and 79 years were selected at random from the municipality population registry stratified by sex and age groups of 10 years. The basic sampling of the population started in 1989 with a cohort study base, and 51.7% (n=7347; mean age, 55.6 years at their first visit; percentage of men, 47.8%) of the subjects had paid their initial visit to the National Cardiovascular Center by February 1997. In addition to performing routine blood examinations, we extracted DNA from an extra 5 mL of blood withdrawn from those who visited the National Cardiovascular Center between May 1996 and February 1998. All participants were Japanese, and only those who gave informed consent for genetic analysis of angiotensinogen and storage of DNA samples were enrolled in the study.

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Blood Pressure Measurement and Family History of Hypertension

After >10 minutes of rest, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice by a single physician. According to the recent criteria of the sixth report of the Joint National Committee on Prevention, Detection, and Treatment of High Blood Pressure (JNC/VI),20 hypertension was defined as a mean SBP of ≥140 mm Hg, a mean DBP of ≥90 mm Hg, or the patient currently taking antihypertensive medication. The remaining population was simply defined as normotensive. Family history of hypertension was defined as having father, mother, or siblings with a history of hypertension (FH).

TaqMan Polymerase Chain Reaction Method

To deal with 4013 samples, we introduced the TaqMan chemistry method, which is able to determine the single nucleotide polymorphism (SNP) without gel electrophoresis. Because cleavage of allele-specific probes can be detected in a single polymerase chain reaction (PCR) by the use of a different reporter dye, typical PCR with the TaqMan probe can identify SNP. The TaqMan probe is a fluorogenic probe that consists of an oligonucleotide labeled with both a fluorescent reporter dye and a quencher dye. The fluorescent reporter dye, such as FAM and TAMRA, is covalently linked to the 5’ end of the nucleotide. Each of the reporters is quenched by TAMRA, typically located at the 3’ end. During the PCR cycle, 2 TaqMan probes hybridize competitively to a specific sequence of the target DNA, and the reporter dyes separate from the quencher dye, resulting in an increase in fluorescence of the reporter. The fluorescence level of PCR products was measured with the ABI PRISM 7200 Sequence Detector (Applied Biosystems, Inc), resulting in clear identification of 3 genotypes of SNP.

Determination of AGT/T+31C Polymorphism

We previously reported the T+31C polymorphism that was located in intron 1 of AGT and in complete linkage disequilibrium with G-6A and M235T31; the same result was obtained in Suita samples (n=500). Our previous investigation with 375 unrelated subjects revealed that there was no recombination among 3 polymorphisms: G-6A, T+31C, and M235T31 The following primers and probes were included in the reaction: a forward primer, 5’-ACA GCA GAA GG T AAG CCG G-3’; a reverse primer, 5’-CCT CCT AGC CCA CAG CTC A-3’; a T-allele-specific probe, 5’-Tet-CAT CCT GGC CGC GCG TCT GCT TCT GCT GGC CCT CAT-Tamra-3’; and a C-allele-specific probe, 5’-Tet-CAG CTC CTT CTC GGC CTT GCT TCT TCT-Tamra-3’. PCR was carried out with Gene Amp 9600 (Applied Biosystems, Inc) under the conditions as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 65.5°C for 60 seconds. To test the reliability of this method, we determined AGT/T+31C polymorphism of 500 subjects who were randomly selected from 4013 subjects by classic PCR–Hae III restriction fragment length polymorphism.

Statistical Analysis

All statistical analyses were conducted with the use of the Stat View 4.5J (Abacus Concepts) and JMP 3.1.5 (SAS). The difference in genotype or allele between normotensives and hypertensives was examined by χ² analysis. The association between angiotensigen T+31C polymorphism and clinical variables was examined by 1-way ANOVA. We assessed the quantitative effects of covariates by multiple logistic regression analysis with JMP.

Results

Study Population

There was no significant difference in age, gender, or blood pressure between those who participated in the genetic analysis and those who did not. From the 4013 subjects, 1520 hypertensive subjects and 2493 normotensive subjects were defined according to the criteria mentioned above. In the comparison of characteristics between hypertensives and normotensives, age, percentage of men, body mass index (BMI), FH, SBP, DBP, total cholesterol (T-chol), triglycerides (TG), fasting plasma glucose (FPG), and creatinine were significantly higher in hypertensives (Table 1). In contrast, smoking habits and HDL cholesterol (HDL-chol) level were significantly lower in hypertensives (Table 1).

Angiotensigen T31C Polymorphism and Hypertension

Genotype frequency of T+31C polymorphism between hypertensive and normotensive subjects was not significantly different (Table 2). In the subjects having the C+31I allele, the calculated odds ratio for hypertension is 0.95 (95% confidential interval [CI], 0.85 to 1.07). The lack of association with hypertension (Wald χ²=1.46, P=0.48) remained after the full adjustment for confounding factors (age, gender, BMI, smoking habit, T-chol, TG, HDL-chol, creatinine, and FPG).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hypertensives</th>
<th>Normotensives</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>1520</td>
<td>2493</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>65±0.3</td>
<td>57±0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender, % men</td>
<td>50</td>
<td>45</td>
<td>0.0002</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23±0.1</td>
<td>22±0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FH, %</td>
<td>40</td>
<td>32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Drinking habits, %</td>
<td>47</td>
<td>48</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol consumption, mL/d</td>
<td>15±0.6</td>
<td>14±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking habits, %</td>
<td>19</td>
<td>26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>149±0.4</td>
<td>117±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>89±0.2</td>
<td>75±0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T-chol, mmol/L</td>
<td>5.5±0.02</td>
<td>5.4±0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TG, g/L</td>
<td>1.4±0.2</td>
<td>1.2±0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-chol, mmol/L</td>
<td>1.6±0.01</td>
<td>1.5±0.01</td>
<td>0.003</td>
</tr>
<tr>
<td>FPG, mmol/L</td>
<td>5.5±0.02</td>
<td>5.3±0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>66±0.6</td>
<td>62±0.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Variables are mean±SEM.
Angiotensinogen T31C Polymorphism and FH

In contrast, the T+31C polymorphism was significantly associated with the FH (Table 3). The frequency of the C allele significantly increased in the subjects with a hypertensive father, mother, or sibling. The estimated odds ratio for the positive FH in individuals with the C allele was 1.20 (95% CI, 1.06 to 1.35). Because it has not been clarified whether the C allele has the dominant effect, the genotype-phenotype correlation was examined under the following situations: additive model (CC versus CT versus TT), dominant model (CC + CT versus TT), and recessive model (CC versus CT + TT). Although the significance of association among the 3 models is similar, the strongest association was obtained in the recessive model (Table 3), and the estimated odds ratio for the positive FH in individuals with CC genotype (versus CT + TT) was 1.20 (95% CI, 1.04 to 1.38).

Because the FH was associated with age, gender, BMI, and current existence of hypertension, we have taken them into consideration as confounding factors and examined the effect of T+31C polymorphism. Multiple logistic regression analysis revealed that the effect of T+31C polymorphism is significant (Wald $\chi^2 = 8.22, P = 0.016$). The estimated odds ratio for FH is 1.48 (1.12 to 1.97) in CC versus TT and 1.08 (0.81 to 1.47) in CT versus TT. To examine gender-specific association with hypertension, we calculated the adjusted odds ratio separately among men and women. The association with FH was significant in men but marginal in women. Especially in the father or siblings, the frequency of hypertensive relatives in CC subjects is twice that in TT subjects (Figure).

Discussion

A recent study by Corvol et al deduced 7 lessons from 2 candidate genes, AGT and epithelial sodium channel, from the previous genetic studies in hypertension. They pointed out the limitation of statistical power in previous studies and emphasized the importance of quality of studied population. The obtained results in the association between AGT M235T and essential hypertension were not consistent, especially in Asians and blacks compared with whites. An advantage of the present study is a very large number of participants (n = 4013) that equals to the grand total of subjects in meta-analysis of several studies, leads to make it possible to avoid the selection bias of examined population. We previously reported the positive association between homozygous deletion allele of the ACE gene and hypertensive men in the same cohort. The hypertensives were simply chosen after the recent criteria for hypertension (JNC/VI), and the remaining population was defined as normotensives. To succeed in the determination of AGT genotype in a large number of samples, we applied a new method, the TaqMan polymerase PCR method, and examined whether hyperten-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FH (+)</th>
<th>FH (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>CC</td>
<td>965</td>
<td>68.3</td>
</tr>
<tr>
<td>CT</td>
<td>407</td>
<td>28.8</td>
</tr>
<tr>
<td>TT</td>
<td>40</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Additive model: CC vs CT vs TT $\chi^2 = 9.1, P = 0.0104$
Dominant model: CC + CT vs TT $\chi^2 = 4.9, P = 0.027$
Recessive model: CC vs CT + TT $\chi^2 = 6.6, P = 0.0101$

Allele

| C       | 2,337 | 82.8  | 4,165  | 80.1  |
| T       | 487   | 17.2  | 1,037  | 19.9  |

$\chi^2 = 8.6, P = 0.0033$

Odds ratio = 1.20 (95% CI, 1.06–1.35)

Comparison in frequency of hypertensive relatives according to AGT T+31C genotypes among father, mother, and siblings. Length of bar indicates percentage of hypertensive relatives. Subjects with CC, CT, and TT were indicated by closed, hatched, and open bars, respectively. Probability values were calculated by 1-way ANOVA. Statistical significance ($P < 0.05$) was observed in father and siblings but not in mother.
sive risk associates with AGT T+31C polymorphism instead of M235T. The accuracy of the TaqMan PCR method having been confirmed, a unique association between AGT T+31C and hypertension was revealed.

AGT T+31C polymorphism was associated with FH but not with blood pressure or with the current presence of hypertension. This result was against our expectation, on the basis of our previous case-control study. A feasible explanation for the discrepancy between our investigations is due to the difference in the severity of hypertension or in the presence of FH. Whereas the definition of hypertension in the Suita Study is to simply satisfy the JNC/VI criteria, the previous case-control study recruited moderate to severe hypertensive cases with FH. The result of the latter appears to be similar to the results of the PEGASE study, which also recruited moderate to severe hypertensives. In the PEGASE study, the AGT M235T polymorphism was weakly but significantly associated with hypertension in men. When we reanalyzed the previous results of a case-control study in the association between hypertension and AGT M235T, significance was obtained in men (P=0.04) but not in women (P=0.07). In this general population, however, significant association with hypertension was not obtained either in men or in women. By putting these results together, it can be concluded that the variants (A-6, C+31, T235) may not strongly or directly increase the predisposition to hypertension.

On the other hand, a positive association was obtained in the association between FH and AGT T+31C polymorphism, suggesting that a possible relation between AGT and hypertension was not ruled out. Kunz and coworkers demonstrated a meta-analysis that examined the association between AGT M235T and hypertension on the basis of 5493 patients of 11 previous articles that studied a white population. Their subgroup analysis according to FH revealed that the pooled estimated odds ratio associated with the AGT T235 allele is 1.42 (1.25 to 1.61) in the cases with FH, whereas it is 1.08 (0.98 to 1.19) in cases with unknown FH. They implied the importance of study with a more rigorous design in the discussion, and our result is just one answer counter to their advice. Even in our study, however, the exact meaning of “family history of hypertension” in the genetics of hypertension is still unclear. The questionnaire to the participants could not check whether they had hypertensive children, with the result that the frequency of positive FH decreases in proportion to their age. On the other hand, the data of the subjects with hypertensive relatives revealed a unique difference in association with the AGT variant among father, mother, and siblings. As shown in Figure 1, the C+31 allele increases the frequency of FH by an additive or dominant manner, but its significance was obtained in the subjects with a hypertensive father and siblings but not mother. These gender-specific associations may reflect the effects of unknown genes on sex chromosomes or genomic imprinting. However, the questionnaire did not check the gender of siblings, either. Accordingly, it is not appropriate to overestimate from current results.

In this study, we regarded the T+31C polymorphism as a marker that is in an absolute linkage disequilibrium with M235T. However, this polymorphism of intron 1 might be involved in the direct regulation of AGT. When the effect of AGT T+31C was examined by MOTIF (Searching Protein and Nucleic Acid Sequence Motifs, http://www.motif.genome.ad.jp/), the homology score for several transcriptional factor binding sites is different between T+31 and C+31. The scores that corresponded to T+31 for ectopic viral integration site 1 encoded factor (Evi-1), Elf-1 (CP2, human, mouse), retroviral poly A downstream element (Poly), and heat shock factor (HSF, Drosophila) are 84%, 81%, 80%, and 80%, whereas those that corresponded to C+31 are only 74%, 69%, 70%, and 71%, respectively. A zinc finger protein that may be involved in leukemic transformation of hematopoietic cells, Evi-1, affects the signaling of transforming factor-β and interacts with Smad3. Elf-1 is a lymphoid-specific transcription factor that belongs to the ETS family and interacts with the promoter region of human endothelial nitric oxide gene. Although we did not demonstrate the functional analysis of T+31C polymorphism itself, these estimations based on the sequence difference suggested the possibility that this polymorphism is involved in transcription or mRNA stability of mRNA of AGT.

Conclusions
The TaqMan PCR method is proved to be a useful and reliable tool for analyzing large-sized samples of genetic study and revealed that a genetic variant of AGT T+31C is associated with FH but not with high blood pressure. Even if the final conclusion will be obtained from future results of this prospective study, this large cross-sectional analysis may be able to imply the real relation between AGT and hypertension.

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