Polymorphism of CYP11B2 Determines Salt Sensitivity in Japanese

Naoharu Iwai, Kazuaki Kajimoto, Hitonobu Tomoike, Naoyuki Takashima

Abstract—Aldosterone plays essential roles in body fluid and electrolyte homeostasis and blood pressure. However, the association between polymorphisms in the CYP11B2 gene and hypertension is controversial. We resequenced CYP11B1 and CYP11B2 and identified 35 polymorphisms in this region. We performed association studies between the plasma aldosterone concentration and 13 polymorphisms in this region in 1443 subjects. The subjects were all obtained from the Suita Cohort Study. Multiple regression analysis indicated that aldosterone levels were determined by renin activity, age, total cholesterol, and hematocrit. Residuals of the aldosterone levels after adjusting for these confounding factors were nominally associated with the T(-344)C (P=0.0026), C(595)T (P=0.0180), -(4837)C (P=0.0310), and G(4936)A (P=0.0498) polymorphisms. Only the T(-344)C polymorphism was significantly associated with the aldosterone level after a correction for multiple testing (Bonferroni). A significant interaction was observed between the T(-344)C polymorphism and renin activity in determining aldosterone levels. Moreover, a significant interaction was observed in 2063 subjects between urinary sodium excretion, which reflects sodium intake, and the T(-344)C polymorphism in determining systolic blood pressure. Only subjects with the TT genotype showed a positive correlation between urinary sodium excretion and systolic blood pressure. In vitro experiments confirmed the functional significance of this T(-344)C polymorphism in terms of angiotensin II reactivity. Thus, the T(-344)C polymorphism in CYP11B2 appears to affect salt sensitivity in Japanese and to have clinical significance. (Hypertension. 2007; 49:825-831.)

Key Words: aldosterone ■ polymorphism ■ hypertension ■ salt ■ genetics

Aldosterone synthase (CYP11B2), a cytochrome P450 enzyme that is mainly expressed in the zona glomerulosa of the adrenal cortex, is a key enzyme in aldosterone synthesis.1,2 Aldosterone controls the sodium balance and, thus, influences blood pressure regulation. Rare mutations of this gene are associated with either markedly elevated aldosterone levels and hypertension or insufficient aldosterone synthesis and sodium wasting.3–6 Thus, CYP11B2 is a candidate gene that may contribute to salt-sensitive hypertension.

The T(-344)C polymorphism, which is located at the putative binding site for the steroidogenic transcripational factor, has been reported to be associated with hypertension7–12 and/or hypertension-related phenotypes, such as the plasma aldosterone level,12,13 urinary aldosterone excretion,8,14 aldosterone:renin ratio,7,11,12,15–17 and left ventricular hypertrophy.18

We reported previously that the T(-344)C polymorphism was unlikely to influence the blood pressure status in the Japanese population based on an analysis of 4049 subjects recruited from the Suita Study.19 However, in the previous study, we had not determined sodium and potassium intakes, aldosterone levels, and plasma renin activity levels, which all seem to be important factors in determining the significance of CYP11B2 polymorphisms. Moreover, other polymorphisms than the T(-344)C in the CYP11B2 region may be associated with aldosterone production and/or hypertension. Thus, in the present study, we resequenced the CYP11B2 gene regions and performed extensive association studies between the polymorphisms in this region and phenotypes related to possible functions of CYP11B2.

Methods

Study Population

The selection criteria and study design of the Suita Study have been described previously.20–22 The sample consisted of 14200 men and women (30 to 79 years of age at enrollment), stratified by gender and 10-year age groups (10 groups and 1420 subjects in each group), who had been randomly selected from the municipal population registry. They were all invited, by letter, to attend regular cycles of follow-up examination (every 2 years). We routinely check 10 to 15 participants per day. DNA from leukocytes was collected from participants who visited the National Cardiovascular Center between April 2002 and March 2005. The present study included 2 study populations. Study population I consisted of 2779 subjects whose DNA was collected between April 2002 and October 2003. In this population, we collected spot urine specimens for the assessment of urinary sodium and potassium excretion. Study population II consisted of 1995 subjects whose DNA was collected between November 2003 and March 2005. In this study population, we measured...
plasma renin activity (PRA) and plasma aldosterone concentrations (PACs). All of the participants were Japanese, and only those who gave their written informed consent for genetic analyses of cardiovascular diseases were included. The study protocol was approved by the institutional ethics committee.

Blood pressure was measured after 10 minutes of rest in a sitting position. Systolic (SBP) and diastolic blood pressure (DBP) values were the means of 2 physician-obtained measurements. Physicians obtained detailed personal medical information directly from the participants. The diagnosis of hypertension was based on blood pressure measurement (SBP ≥ 140 mm Hg, or DBP ≥ 90 mm Hg) or the current use of antihypertensive medication.

Twenty-four–hour urinary sodium and potassium excretion was estimated from spot urine specimens as reported previously. Spot urine specimens were collected between 9:30 AM and 10:30 AM after an overnight fast. Twenty-four–hour urinary sodium and potassium excretion was calculated by adjusting for age and BMI.

### TABLE 2. Characteristics of Study Population II

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>699</td>
<td>568</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>Male, %</td>
<td>42.2</td>
<td>42.3</td>
<td>42.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age, y</td>
<td>63.5±11.3</td>
<td>63.4±10.9</td>
<td>64.5±11.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>BMI</td>
<td>22.50±2.05</td>
<td>22.46±3.06</td>
<td>22.39±3.06</td>
<td>n.s.</td>
</tr>
<tr>
<td>Log(PAC)</td>
<td>4.48±0.37</td>
<td>4.45±0.38</td>
<td>4.40±0.40</td>
<td>0.0471</td>
</tr>
<tr>
<td>Log(PRA)</td>
<td>−0.25±0.83</td>
<td>−0.16±0.78</td>
<td>−0.12±0.81</td>
<td>0.0525</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>121.5±18.0</td>
<td>119.4±17.2</td>
<td>120.0±18.5</td>
<td>0.0994</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>74.7±10.0</td>
<td>73.8±8.9</td>
<td>74.5±9.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>211.7±32.3</td>
<td>210.3±32.3</td>
<td>210.9±36.0</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Characteristics of the study population II are shown according to the genotypes of the T(−344)C polymorphism of CYP11B2. Residual of log(PAC) was calculated by adjusting for age, total cholesterol, hematocrit, and log(PRA). BMI indicates body mass index.

al. Thus, the final number of subjects was 1443 (Table 2). Of these 1443 subjects, 696 subjects had visited us between April 2002 and October 2003, and their clinical data at that time were included in study population I.

### Sequence Analysis of CYP11B2 and CYP11B1

The promoter and exon regions of CYP11B2 and CYP11B1 were resequenced in 96 subjects with a high or low aldosterone/renin ratio (top 48 + bottom 48 in 1995 subjects in study population II). Because the homology between these 2 genes is very high, sequence amplification primers were designed to correspond with gene-specific regions (Table S1, available online at http://hyper.ahajournals.org). The linkage disequilibrium structure among the polymorphisms in this region was calculated (R2 and D’ using the SNPAlalyze statistical package (Dynacom). Based on this linkage disequilibrium structure (Figure 1), 13 polymorphisms were selected for genotyping by the TaqMan method (Table S2).

### Genotype Determination and Statistical Analysis

All of the statistical analyses were performed using the JMP statistical package (SAS Institute Inc). PRA and PAC levels were logarithmically transformed to obtain a normal distribution. Multiple logistic and regression analyses were performed with other covariates. Residuals were the observed values minus predicted values based on confounding factors. Residuals of SBP and DBP were calculated by adjusting for age and body mass index. Because the relationship between age and DBP was not linear, a polynomial equation (n=2) was applied to accommodate a decline in DBP in elderly subjects. Haplotype-based association analyses were performed using the SNPAlalyze statistical package (Dynacom).

### Functional Analysis of the T(−344)C Polymorphism

To examine whether the T(−344)C polymorphism modulates the expression level of the CYP11B2 gene, the region from −435 (promoter) to +812 (exon2) of CYP11B2 (nucleotide number indicates the relative position from the translation initiation codon) was amplified and subcloned into the HindIII and XhoI sites of pG3L3-Basic Vector (Promega). This promoter region has been reported to be enough to maintain promoter activity and angiotensin II responsiveness. We included intron 1 in the construct, because our preliminary experiments indicated that this construct enhanced the expression level of the reporter transcript. Primers used for amplification were as follows: HindIII-forward, 5’-aagcttcatggctgatcagc-3’, and XhoI-reverse, 5’-ctcgag-attgatcag-3’. We constructed 4 types of expression vectors corresponding with the 4 haplotypes defined by the T(−344)C and C(595)T polymorphisms. We used 2 cell lines, namely, Y1 and...
H295R adrenocortical cells, for promoter reporter analyses. No significant modulation of promoter activity by angiotensin II was observed in Y1 cells (data not shown). H295R adrenocortical cells were cultured in a medium containing a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 15 mmol/L of HEPES, 0.5 mmol/L of sodium pyruvate, 1.25 g/L of sodium bicarbonate, 6.25 μg/mL of insulin, 6.25 μg/mL of transferrin, 6.25 ng/mL of selenium, 1.25 mg/mL of bovine serum albumin, 5.35 g/mL of linoleic acid, and 2.5% of 1-Serum (BD Bioscience). Transient transfection was performed using Lipofectamine2000 reagent (Invitrogen). Cells were seeded onto 6-well plates to 70% to 80% confluence and, 24-hour later, transiently cotransfected with 4 μg of a reporter plasmid and 1 μg of pAcGFP1-C1 vector, which expresses green fluorescence protein (GFP; Clontech), per well. After incubation for 6 hours, cells were washed with 1× PBS and further incubated in fresh medium for 24 hour in the presence or absence of angiotensin II (0.5 μmol/L). This incubation period of 6 hours was based on the previous study.25 Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol and was briefly treated with RNase-free DNaseI. RT-PCR was carried out with the following amplimers: Ex1-S (5′-gcaaaggcagaggtgtgcgt) and Ex2-AS (5′-cgccacatttgtgcccacga) for CYP11B2 and AcGFP-S (5′-cctgatcgagctgaatggcg) and AcGFP-A (5′-tgctggtagtggtcggccag) for GFP. PCR products were subjected to 1.5% agarose gel

Figure 1. Linkage disequilibrium among the single nucleotide polymorphisms in the CYP11B1 and CYP11B2 genes. In this schematic of the CYP11B2 and CYP11B1 genes, □ indicates 5′- and 3′-untranslated regions, and ■ indicate coding regions. Two measures of linkage disequilibrium are shown: D′-values in the upper right triangle and R2 values in the lower left triangle. Color-coded scales for D′-values or R2 values (measures of linkage disequilibrium (LD strength) are on the right.
Results

Sequence Analysis of CYP11B2 and CYP11B1

Sequence analysis in this region identified 35 polymorphisms (Table S3). Based on the linkage disequilibrium structure in this region, 13 polymorphisms were selected for genotyping (Table S2). These 13 polymorphisms were sufficient to cover all of the major haplotypes in this region (Figure 1).

Association Analysis Between Polymorphisms and Renin/Aldosterone Profiles

Multiple regression analysis indicated that log(PAC) was determined \(R^2=0.219\) by log(PRA) \((t=11.40; P<0.0001)\), age \((r=-0.822; P<0.0001)\), total cholesterol level \((r=2.70; P=0.0070)\), and hematocrit \((r=0.792; P<0.0001)\). Cholesterol is a substrate for aldosterone synthesis. Higher hematocrit may reflect dehydration and, thus, may be positively associated with higher aldosterone levels. Residuals of log(PAC) were calculated by adjusting for the above confounding factors. Of the 13 polymorphisms genotyped, the T(−344)C polymorphism was significantly associated with the residuals of log(PAC) after the Bonferroni correction \((P=0.0026 \times 13 = 0.0338)\). Haplotype analysis did not increase the power of the association with aldosterone levels (data not shown).

Intriguingly, a significant interaction \((P=0.0277)\) was observed between the T(−344)C (TT/TC+CC) polymorphism and log(PRA) in determining log(PAC) levels (Figure 2). Aldosterone levels were linearly correlated with renin activity. However, the slope was different between the TT and TC+CC genotypes. Subjects with the TT genotype seem to have inappropriately higher aldosterone levels under high salt intake (low renin activity).

Association Analysis Between Blood Pressure and the T(−344)C Polymorphism

Next, we investigated whether the T(−344)C polymorphism might influence blood pressure levels in Japanese. Although the TT genotype tended to be associated with higher SBP in study population II, we may not be able to assess the significance of this polymorphism without knowing the sodium balance (see Discussion section). Thus, this assessment was performed in study population I, which included 2063 subjects whose sodium and potassium excretion was estimated from a spot urine specimen.

Multiple regression analysis indicated that SBP levels were determined \(R^2=0.175; P<0.0001\) by age \((t=18.26; P<0.0001)\), body mass index \((t=9.76; P<0.0001)\), and urinary sodium \((t=3.78; P=0.0002)\) and potassium \((t=-2.81; P=0.0050)\) excretion. Intriguingly, whereas the T(−344)C genotype had no significant influence on SBP \((P=0.7179)\), a significant interaction was observed between urinary sodium excretion and the T(−344)C polymorphism \((TT/TC+CC; P=0.0137)\). This means that a significant positive correlation between SBP and sodium excretion was observed in subjects with the TT genotype but not in subjects with the TC or CC genotype (Figure 3). However, a similar interaction was not observed for DBP.

Functional Analysis of the T(−344)C Polymorphism

To investigate whether the T(−344)C polymorphism might modulate the expression of CYP11B2, we constructed reporter plasmids that expressed a fusion transcript under the CYP11B2 promoter, which contained the T(−344)C polymorphism \((P=0.0310)\), and G(4936)A \((P=0.0498)\) showed a \(P<0.05\) association with aldosterone levels. Only the T(−344)C polymorphism was significantly associated with the residuals of log(PAC) levels after the Bonferroni correction \((P=0.0277)\). Haplotype analysis did not increase the power of the association with aldosterone levels (data not shown).

Association Analysis Between Blood Pressure and the T(−344)C Polymorphism
CYP11B2

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Aldosterone plays essential roles in body fluid and electrolyte

expression vector. The promoter activities were assessed under the
presence or absence of angiotensin II (0.5 μM/L). The relative intensity of the PCR product from the CYP11B2 reporter plasmid to the PCR product from AcGFP1 vector was assessed by
densitometer. Data are mean±SD (N=8 for each group). Representa-
tive photograph is shown above (N=4 for each group).

**Figure 4.** Promoter reporter assay on the T(−344)C polymor-
phism. H295R cells were transiently cotransfected with a reporter construct (see Methods section) and AcGFP1 expres-
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Discussion

Aldosterone plays essential roles in body fluid and electrolyte
homeostasis and blood pressure. However, the association
between polymorphisms in the CYP11B2 gene and hypertension is controversial.7–19

In the present study, we performed extensive association
studies between polymorphisms in CYP11B2 regions and
renin–aldosterone profiles and blood pressure values. Our
findings in study population II indicate that subjects with the
TT genotype of the T(−344)C polymorphism appear to have
higher aldosterone levels in response to PRA levels than
those with the TC or CC genotype when renin activity is

There are at least three possibilities with regard to the
aldosterone/sodium/volume/pressure relationship. First, rela-
tively higher aldosterone levels in subjects with the TT
genotype are offset by suppressing renin activity, and appro-
riate aldosterone levels are maintained. Thus, the genotype
is not associated with a tendency for volume retention and,
thus, not associated with higher blood pressure. Second,
relatively higher aldosterone levels in subjects with the TT
genotype are offset by suppressing renin activity. However,
the degree of suppression of renin activity may not be enough
to prevent sodium retention, and the TT genotype is associ-
ated with higher blood pressure. Third, the tendency for
sodium retention in subjects with the TT genotype may be
offset by the reduced intake of sodium. A similar situation
has been reported in subjects with Gitelman’s syndrome muta-
tions, who develop higher sodium intake to prevent volume
loss and low blood pressure levels.26 According to the
observation in study population II, the first possibility seems
to be unlikely, because subjects with the TT genotype had
higher aldosterone levels under low renin activity (reflecting
high sodium intake) as shown in Figure 2. To evaluate the
second and third possibilities, we should know the sodium
intake status of the study population. Therefore, the relation-
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assessed in study population I, in which sodium intake status
was assessed by spot urine. The observation in study popu-
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showed a positive correlation between SBP and urinary
sodium excretion, which supports the second hypothesis
inferred from the observation in study population II. How-
ever, urinary sodium excretion was not different among
subjects with different genotypes, which excludes the third
hypothesis (Table 1).

The present study confirmed the hypothesis that the
T(−344)C polymorphism determines salt sensitivity through the
use of 2 separate study populations with different phenotypes:
renin–aldosterone profile (study population II) and sodium–

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hypothesis (Table 1).
Previously, we reported that the T(−344)C polymorphism in CYP11B2 was unlikely to influence blood pressure levels in Japanese. However, that previous conclusion was based on observations that did not consider sodium and potassium intake in the analysis. Our present conclusion is that the relationship between the T(−344)C polymorphism and blood pressure depends on the sodium intake status. This is not inconsistent with the previous study. Only subjects with the TT genotype appear to be salt sensitive and have higher SBP under high sodium excretion, which reflects high sodium chloride intake, than those with the TC or CC genotype (Figure 3).

Study Strengths and Limitations
The strengths of the present study are as follows: (1) a large community-based sample, (2) assessment of various possible confounding factors, (3) resequencing of CYP11B1 and CYP11B2 genes in 96 subjects to catalog polymorphisms in Japanese, and (4) functional confirmation of the significance of the T(−344)C polymorphism in vitro. However, the present study also has some limitations. The mean ages of male and female subjects were 62±11 and 63±11 years, respectively. Because the phenotypes of elderly subjects are suspected to be influenced more by environmental factors than those of younger subjects, younger subjects might be more appropriate for a genetic association analysis.

Although the aldosterone levels were corrected by renin activity, several environmental factors are known to affect aldosterone levels, and these are difficult to control in an epidemiological research setting. In fact, we did not measure urine sodium excretion over a 24-hour period or serum potassium levels in this population (study population II). These 2 factors are the most important environmental factors that affect aldosterone levels.

We excluded subjects with possible renovascular hypertension or primary aldosteronism from the analysis based on the screening criteria proposed by Omura et al. However, precise diagnostic procedures were not performed on these suspected cases because of the constraints of the epidemiological research setting. Because we did not measure PAC or PRA in study population I, hypertensive subjects with possible renovascular hypertension or primary aldosteronism were not excluded from study population I. This may have obscured the influence of the T(−344)C polymorphism on blood pressure.

The estimation of sodium and potassium excretion in study population I was based on a spot urine specimen. The correlation between the estimated and measured values has been reported to be high when urine specimens are collected in the morning, and spot urine specimens were collected between 9:00 AM and 10:00 AM in the present study. We need to assess the sodium intake status more precisely in future studies.

Perspectives
The T(−344)C polymorphism may be useful for identifying hypertensive subjects to whom a low NaCl diet or diuretics should be recommended.

Acknowledgments
We express our gratitude to the participants of the present study. We thank Dr Chun-chen Shen for technical assistance.

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Disclosures
None.

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