Adrenomedullin (AM) is a hypotensive peptide produced in cardiovascular tissues such as the heart, lung, kidney, and vascular wall.1,2 Besides the potent vasodilator action, AM has also been shown to cause natriuresis in the kidney and to inhibit growth of cardiovascular cells. Moreover, a significant level of AM has been identified in human plasma, and AM is supposed to be a circulating hormone.1,3 We have previously reported that plasma AM levels are increased in patients with cardiovascular diseases such as hypertension, renal failure, and heart failure.4,5 These findings suggest that AM has implications in pathophysiology of the cardiovascular system.

We have cloned and sequenced the genomic DNA encoding human AM gene and have determined that the gene is located in the short arm of chromosome 11.3,6 Nucleotide sequencing of genomic DNA adjacent to the AM gene revealed that the 3'-end of the gene is flanked by the microsatellite marker with a variable number of cytosine adenine (CA)-repeats. This microsatellite marker is located approximately 4 kb downstream of the AM gene. Considering the possible implications of AM in the cardiovascular system, it seems of interest to elucidate whether this gene variation has any relation to the etiology of cardiovascular diseases. In the present study, we investigated the relationship between the microsatellite polymorphism adjacent to the AM gene and genetic predisposition to essential hypertension.

Study Subjects
A group of 266 case patients with essential hypertension (EH) (166 men and 100 women), who had hypertension before the age of 50 years was selected from the outpatient clinic of Dokkyo University School of Medicine Hospital. Hypertension was defined as systolic blood pressure (SBP) >140 mm Hg and/or diastolic blood pressure (DBP) >90 mm Hg on multiple visits. Secondary causes of hypertension were denied through a comprehensive checkup. The normotensive control group (NT) included 272 healthy subjects (175 men and 97 women) age 50 years. They were recruited from participants of the health-check program of Dokkyo University School of Medicine Hospital. All the control subjects had SBP <140 mm Hg and DBP <90 mm Hg on multiple occasions. Blood pressure was...
measured by a sphygmomanometer in a sitting position after a 10-minute rest. All subjects were Japanese and unrelated each other.

The study protocol was approved by the institutional review board, and informed consent was obtained from each subject.

Genotype Analysis
Genomic DNA was extracted from peripheral leukocytes using Easy DNA kit (Invitrogen). The microsatellite region containing the CA-repeats 4 kb downstream of the AM gene was amplified by PCR. The PCR was performed with 0.5 μg of genomic DNA and 25 pmol of each primer (sense, 5′-AAGGCAGCTGATCATAGAAGATTGG-3′; antisense, 5′-GCAACTCATATTTTATATCTGACAG-3′) in a final volume of 50 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 μg/mL gelatin, 0.2 mmol/L each of dNTP, 1 U of Taq DNA polymerase (Perkin-Elmer). The length of PCR product was calculated from the internal DNA size standard labeled with the red fluorescent dye ROX. The mixture was electrophoresed on 6% urea-polyacrylamide gel using an ABI 373 DNA sequencer (Perkin-Elmer). The blue fluorescent dye FAM was attached to the 5′-end of sense primer was labeled with the blue fluorescent dye 6-FAM. The DNA was amplified for 30 cycles with denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. Then, an aliquot of the PCR product was mixed with the internal DNA size standard labeled with the red fluorescent dye ROX. The mixture was electrophoresed on 6% urea-polyacrylamide gel using an ABI 373 DNA sequencer (Perkin-Elmer). The length of PCR product was calculated from the calibration curve of internal standard using GENESCAN 672 software (Perkin-Elmer).

Measurement of Plasma AM
Antecubital venous blood was collected into ice-chilled tube containing EDTA (1 mg/mL) and aprotinin (500 U/mL) in the morning after the subjects had fasted overnight and had 20 minutes of supine rest. Plasma was separated by centrifugation at 4°C and stored at −80°C until assayed. Plasma AM concentration was measured by immunoradiometric assay using an AM-RIA Shionogi kit (Shionogi & Co), which detects both amidated mature AM and glycine-extended AM.

Statistical Analysis
Data are presented as mean±SE. Clinical characteristics between the 2 groups were compared by unpaired Student’s t-test for parametric data and by χ²-test for categoric data. The allele and genotype frequencies in the 2 groups were compared by χ²-test. Differences in plasma AM among genotypes were analyzed by 1-way ANOVA followed by Dunnett’s multiple range test. A P value <0.05 was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Clinical Characteristics of Study Subjects
Table 1 lists the physical and laboratory findings of the study subjects. EH were slightly younger and had a higher body mass index compared with that of NT, whereas the gender ratio was comparable between the 2 groups. As a matter of course, SBP and DBP was far higher in EH than in NT. In laboratory findings, EH showed higher fasting blood glucose, higher serum creatinine, and higher serum uric acid compared with those of NT. With regard to the serum lipids, triglycerides were higher and HDL cholesterol was slightly lower in EH than in NT, although total cholesterol did not differ between EH and NT.

Genotype and Allele Frequencies
In the Japanese population in the microsatellite region adjacent to the AM gene, there were 4 types of alleles with different CA-repeat numbers: 11, 13, 14, and 19. The alleles with 11-, 13-, 14-, and 19-repeats yield DNA fragment lengths of 248, 252, 254, and 264 bp, respectively. Table 2 shows observed frequencies of the alleles and the genotypes of this microsatellite gene polymorphism in the study subjects. The frequencies of the 11-, 13- and 14-repeat alleles were approximately 30%, whereas the frequency of the 19-repeat allele was 13.5%. Namely, 13.5% of EH patients carried

### Table 1. Clinical Characteristics of the NT and EH patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive (n=272)</th>
<th>Hypertensive (n=266)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>57.0±5.3</td>
<td>53.3±11.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>175/97</td>
<td>166/100</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.0±2.8</td>
<td>25.0±3.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>118±10</td>
<td>171±22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>73±8</td>
<td>104±12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting blood glucose, mmol/L</td>
<td>5.31±0.50</td>
<td>5.73±0.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum total cholesterol, mmol/L</td>
<td>5.14±1.00</td>
<td>5.11±0.83</td>
<td>NS</td>
</tr>
<tr>
<td>Serum HDL cholesterol, mmol/L</td>
<td>1.40±0.34</td>
<td>1.33±0.33</td>
<td>0.034</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.47±0.92</td>
<td>1.74±0.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>77.4±14.6</td>
<td>83.3±19.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum uric acid, μmol/L</td>
<td>309±77</td>
<td>346±77</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 2. Genotype and Allele Frequencies of Microsatellite Repeat Adjacent to AM Gene in NT and EH groups

<table>
<thead>
<tr>
<th>CA-Repeat Number</th>
<th>Normotensive (n=272)</th>
<th>Hypertensive (n=266)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele frequency, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 and 11</td>
<td>24 (8.8)</td>
<td>27 (10.1)</td>
<td></td>
</tr>
<tr>
<td>11 and 13</td>
<td>52 (19.1)</td>
<td>43 (16.2)</td>
<td></td>
</tr>
<tr>
<td>11 and 14</td>
<td>54 (19.9)</td>
<td>54 (20.3)</td>
<td></td>
</tr>
<tr>
<td>11 and 19</td>
<td>3 (1.1)</td>
<td>11 (4.1)</td>
<td></td>
</tr>
<tr>
<td>13 and 13</td>
<td>31 (11.4)</td>
<td>25 (9.4)</td>
<td></td>
</tr>
<tr>
<td>13 and 14</td>
<td>57 (21.0)</td>
<td>46 (17.3)</td>
<td></td>
</tr>
<tr>
<td>13 and 19</td>
<td>9 (3.3)</td>
<td>12 (4.5)</td>
<td></td>
</tr>
<tr>
<td>14 and 14</td>
<td>37 (13.6)</td>
<td>35 (13.2)</td>
<td></td>
</tr>
<tr>
<td>14 and 19</td>
<td>5 (1.8)</td>
<td>13 (4.9)</td>
<td></td>
</tr>
<tr>
<td>19 and 19</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Allele distribution in EH vs NT: χ²=9.43, P=0.024. *Frequency of 19-repeat allele in EH vs NT: χ²=7.62, P=0.007; significance level is P<0.012 after correction for multiple comparison.
the 19-repeat allele, whereas the frequency was 6.2% in NT ($\chi^2=7.62$, $P=0.007$).

**Plasma AM Concentration**

To clarify the influence of allele types on plasma AM levels, plasma AM was measured in homozygotes of each allele type in NT. However, because there were no homozygotes of 19-repeat allele, plasma AM was also measured in heterozygotes that included this allele. Plasma concentrations of AM in 21 homozygotes of 11-repeat allele, 27 homozygotes of 13-repeat allele, 30 homozygotes of 14-repeat allele, and 17 heterozygotes carrying 19-repeat allele were 7.5±1.0, 7.0±1.3, 7.2±1.3, and 7.3±1.7 pmol/L, respectively, and the values were not significantly different between the genotypes.

**Discussion**

In the present study, we examined the microsatellite DNA polymorphism lying 3’-downstream of the AM gene in NT and EH. Among the 4 types of alleles with CA-repeat numbers of 11, 13, 14, and 19, the frequency of 19-repeat allele was higher in EH than in NT. The frequency of EH patients carrying the 19-repeat allele was more than twice that of NT subjects. This result suggests the association of microsatellite DNA polymorphism adjacent to the AM gene with the genetic predisposition to hypertension. Namely, the existence of 19-CA-repeat allele is supposed to be associated with the risk of developing hypertension. However, because the frequency of this 19-repeat allele was small, this gene variation alone is not thought to reflect the large part of genetic predisposition to essential hypertension. So far, a number of gene polymorphisms have been indicated to have relation with the predisposition to essential hypertension. Most of them are related to the genes of cardiovascular hormones and their signal transduction systems, such as angiotensinogen, $\beta_1$-adrenergic receptor, and endothelial NO synthase. As proposed by Page’s mosaic theory, multiple genes are supposed to contribute to the development of hypertension. And together with these gene variations, the DNA polymorphism examined in this study may participate, to some degree, in the genetic predisposition to essential hypertension.

Some gene polymorphisms of cardiovascular hormones have been shown to affect expression of the genes or activity of the gene products. For instance, the serum ACE activity is known to be increased in individuals carrying the deletion allele of the gene, and it has been reported that the methionine-to-threonine substitution at amino acid residue 235 (M235T) of angiotensinogen is associated with an increase in the gene expression. Because the microsatellite polymorphism examined in this study is located 3’-downstream of the AM gene, it is unlikely that the gene transcription is affected by this polymorphism. Indeed, the plasma AM levels were not significantly different among the genotypes of this polymorphism. Therefore, the association of 19-CA-repeat allele with hypertension is not likely mediated by the gene expression and the action of AM itself. It may be possible that this microsatellite polymorphism is associated with the expression of other genes. Near the location of AM gene in the short arm of chromosome 11, there exist such genes as sphingomyelinase, parathyroid hormone and lactate dehydrogenase. With regard to the parathyroid hormone gene, it is known that a high serum level of parathyroid hormone is associated with hypertension. In this context, it may be worth examining the relation of the current microsatellite polymorphism with parathyroid hormone and calcium metabolism.

Microsatellite markers, like the one examined in this study, consist of variable number of repeats of short nucleotides, and more than hundreds of such repeat markers are scattered throughout the genomic DNA. These microsatellite markers can be used to locate the genomic region responsible for hereditary diseases or traits. Until now, several diseases have been shown to be associated with such microsatellite DNA polymorphism. For instance, it has been reported that the CA-repeat polymorphism lying upstream of aldose reductase gene affects the development of nephropathy and retinopathy in type 1 diabetes mellitus. With regard to essential hypertension, a certain number of TCAT-repeat in the intron 1 of tyrosine hydroxylase gene has been shown to have positive association with essential hypertension. The intron 2 of type B natriuretic peptide receptor gene was also found to contain the GT microsatellite–repeat associated with essential hypertension. Including these and our results, accumulation of information about association of essential hypertension with various microsatellite markers may serve to understand the whole figure of the genetic predisposition to essential hypertension which is attributed to multiple genomic regions.

The results of this study revealed the association of the microsatellite CA-repeat polymorphism adjacent to the AM gene with genetic predisposition to essential hypertension in Japanese population. The frequency of 19-repeat allele was significantly increased in patients with essential hypertension. Although this DNA polymorphism is not likely to affect the AM gene transcription, the existence of 19-CA-repeat allele is supposed to reflect a certain part of the genetic predisposition to essential hypertension.

**Acknowledgments**

The authors thank Ms Yasuako Kawamura and Dr Kazumi Akimoto for technological assistance in executing the study. This study was supported in part by the grant-in-aid for scientific research (10218209) from the Ministry of Education, Science and Culture of Japan.

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Microsatellite DNA Polymorphism of Human Adrenomedullin Gene in Normotensive Subjects and Patients With Essential Hypertension

Toshihiko Ishimitsu, Kazuyoshi Hosoya, Kohju Tsukada, Junichi Minami, Yasuo Futoh, Hidehiko Ono, Masami Ohrui, Jun Hino, Kenji Kangawa and Hiroaki Matsuoka

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Expanded Methods

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