Association Between the Calcitonin-Related Peptide α (CALCA) Gene and Essential Hypertension in Japanese Subjects

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Background: Calcitonin-related peptide α (CALCA) is a neuropeptide that is a very potent vasodilator. It has been reported that CALCA knockout mice have a significantly elevated systolic blood pressure (BP). The aims of this study were to discover novel polymorphisms or mutations in the 5′ flanking region of the human CALCA gene in Japanese subjects and to assess the association between this gene and essential hypertension (EH).

Methods: Japanese patients with EH (50.1 ± 6.6 years old, n = 274) and age-matched Japanese subjects without EH (51.1 ± 6.6 years old, n = 225) were recruited. The 5′ flanking region of the human CALCA gene was searched to identify novel polymorphisms in the 20 EH patients using polymerase chain reaction (PCR) and a direct sequencing method. These novel polymorphisms, as well as the known single nucleotide polymorphisms (SNPs), were used for genotyping.

Results: We discovered a novel 2-bp microdeletion polymorphism in intron 1. The only three participants with 2-bp microdeletion polymorphism were found in the EH group. None of the subjects without EH had a 2-bp microdeletion polymorphism. The genotype and allele distribution of the 4 SNPs were not significantly different between the groups. All five polymorphisms were located in one haplotype block. The haplotype was constructed using, in order, rs1553005, 2-bp microdeletion polymorphism, and rs5241. There was a significant association between EH and the C-AGins-A haplotype (P = .00031).

Conclusions: A novel 2-bp microdeletion polymorphism was discovered in the CALCA gene. Based on the results of the haplotype-based case control study, the CALCA gene could be the susceptibility gene for EH. Am J Hypertens 2007;20:527–532 © 2007 American Journal of Hypertension, Ltd.

Key Words: Calcitonin-related peptide α (CALCA), polymorphism, SNP, haplotype, association study.

Calcitonin-related peptide α (CALCA) is a 37-amino-acid vasoactive neuropeptide. In mammals, CALCA is distributed in the central and peripheral nervous systems. The calcitonin/calcitonin gene-related peptide gene complex encodes a small family of peptides: calcitonin, katacalcin, and CALCA. CALCA is produced by tissue-specific alternative splicing of the primary transcript of the calcitonin/calcitonin gene-related peptide gene complex and is synthesized almost exclusively in neuronal tissues.1 CALCA is a potent vasodilator, which is from 100 to 1000 times more potent than other vasodilators, such as adenosine, substance P, or acetylcholine.2 The systolic blood pressure (BP) of CALCA knockout mice has been reported to be significantly elevated.3

Hypertension affects 25% of most adult populations and is a major risk factor for death from stroke, myocardial infarction, and congestive heart failure.4–8 The largest group of hypertensive patients has essential hypertension (EH). Essential hypertension is considered to be a multifactorial disease.9 Several reports have shown that there are EH susceptibility genes, including the genes for angiotensinogen10 and angiotensin-converting enzyme.11

The human CALCA gene is located on chromosome 11p15.2-p15.1, spans approximately 3.8 kb, and contains five exons (Fig. 1). In the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/projects/SNP), 28
SNPs have been recorded (accessed on April 6, 2005). In the coding region, four mutations have been recorded: two missense mutations (rs5241 and rs13306224 located on exon 4) and two silent mutations (rs5239, located on exon 3, and rs2644689, located on exon 4). Nonsense mutations in the coding region were not recorded.

A 16-bp microdeletion polymorphism on intron 1 has been reported in a family with multiple cases of unipolar or bipolar depressive disorder; however, this polymorphism has not been formally registered. There have been no reports dealing with the relationship between the CALCA gene variants and EH.

The aims of this study were to discover novel polymorphisms or mutations in the 5’ flanking region of the CALCA gene in Japanese subjects and to assess the association between this gene and EH using novel genetic markers and known SNPs.

**Genotyping**

We sequenced from −1 to −2000 nucleotides upstream ATG as the start codon of the human CALCA gene to find novel polymorphisms or mutations in the 20 EH patients using the polymerase chain reaction (PCR) and the direct sequencing method. In addition, based on the information obtained from the NCBI SNP database and the Applied Biosystems–Celera Discovery System (ABI-CDS, http://www.appliedbiosystems.com) database, SNPs that had a minor allele frequency of more than 20% or had a known function were selected.

Blood samples were collected from all participants and genomic DNA was extracted from the peripheral blood leukocytes by extraction with phenol and chloroform. Genotyping was done using the TaqMan SNP Genotyping Assay (Applied Biosystems Inc., Foster City, CA), PCR and single-stranded conformation polymorphism (PCR-SSCP) analysis, and PCR and electrophoresis.

The TaqMan SNP Genotyping assays were done using the method of Taq amplification. In the 5’ nuclease assay, discrimination occurs during PCR, as the allele-specific fluorogenic probes, when they are hybridized to the template, are cleaved by the 5’ nuclelease activity of Taq polymerase. The probes contain a 3’ minor groove-binding group (MGB) that hybridizes to single-stranded targets with increased sequence specificity compared to ordinary DNA probes. This reduces nonspecific probe hybridization and results in low background fluorescence during the 5’ nuclease PCR assay (TaqMan, ABI). Cleavage results in the increased emission of a reporter dye. Each 5’ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe was labeled with two reporter dyes at the 5’ end. In this study, VIC and FAM were used as the reporter dyes. The primers and probes in the TaqMan SNP Genotyping Assay (ABI) were chosen from the information available on the ABI web site (http://my-science.appliedbiosystems.com).

The PCR amplification was done using 6 μL of TaqMan Universal Master Mix, No AmpErase UNG (2×) (ABI) in 12-μL final reaction volumes, with 2 ng of DNA, 0.22 μL of TaqMan SNP Genotyping Assay Mix (20× or 40×) containing 900 nmol/L primers, and a 200-nmol/L final concentration of the probes. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and finally 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system.

Each 96-well plate contained 80 samples of an unknown genotype and 4 samples with no DNA but with reagents (control). The control samples without DNA were necessary in the Sequence Detection System (SDS) 7700 for signal processing as outlined in the TaqMan Allelic Discrimination Guide (ABI). These plates were read on the SDS 7700 instrument using the end point analysis mode of the sequence detection system (SDS) version 1.6.3 software package (ABI). The genotypes were deter-

**Methods**

**Subjects**

From 1993 to 2003, subjects diagnosed as having EH were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo. There were 274 EH patients (male/female ratio = 1.84). Essential hypertension was diagnosed based on the following criteria: seated systolic BP >160 mm Hg or diastolic BP >100 mm Hg on three occasions within 2 months after the first BP reading. None of the EH patients used antihypertensive medications. Patients diagnosed as having secondary hypertension were excluded. A total of 225 normotensive (NT) age-matched healthy individuals (male/female ratio = 2.6) served as control subjects. None of the control subjects had a family history of hypertension, and all had systolic BP <130 mm Hg and diastolic BP <85 mm Hg. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.
analyzed on 5% nondenaturing acrylamide gels. The PCR products were denaturation at 96°C for 3 min, followed by 60 cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were separated on 6% polyacrylamide sequencing gel.

Genotyping of the 2-bp microdeletion polymorphism was performed using PCR and electrophoresis. The PCR primer was designed as follows: sense primer, 5′-CCCGCCTGT-3′; and antisense primer, 5′-AGAGCTGGAGGAGCGATCCTAGAGGGA-3′. The PCR conditions included initial denaturation at 96°C for 3 min, followed by 60 cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were analyzed on 5% nondenaturing acrylamide gels.

Genotyping of rs13306225 was done using PCR-SSCP analysis. The PCR primer was designed as follows: sense primer, 5′-CAGGTTCTGGAAGCATGAGGGTGACGC-3′; and antisense primer, 5′-CGACTGCTCTTATTC-CGCCGCTGT-3′. The PCR conditions were initial denaturation at 96°C for 3 min, followed by 60 cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were analyzed on 5% nondenaturing acrylamide gels.

**Biochemical Analysis**

The plasma total cholesterol concentration and the serum creatinine concentration were measured using standard methods in the Clinical Laboratory Department of Nihon University Hospital.

**Statistical Analysis**

All continuous variables are expressed as mean ± standard deviation. Continuous variables were assessed using Mann-Whitney’s U test. Categorical variables were assessed with Fisher’s exact test. The genotype and allele distributions were examined with Fisher’s exact test. The contingency table including zero was not examined. Multiple logistic regression analyses were done to assess the contribution of confounders (gender, body mass index [BMI], history of diabetes, and hyperlipidemia). Differences in continuous variables between groups were analyzed by one-way ANOVA or t test after normal distribution and homogeneity of variance were confirmed. Based on the genotype data of the genetic variations, a linkage disequilibrium (LD) analysis and a haplotype-based case-control study were done using the expectation maximization (EM) algorithm of the SNPAlalyze software program, version 3.2 (DYNACOM Co., Ltd., Yokohama, Japan). A pair-wise LD analysis was done using four SNP pairs. D’ values >0.5 were used to assign SNP locations to one haplotype block. Tagged SNPs were selected by omitting one SNP from an SNP pair showing an r2 >0.5 for each haplotype block. In this haplotype-based case-control study, haplotypes with a frequency <0.03 were excluded. The distribution of the haplotypes’ frequency was calculated using the χ2 test. Statistical significance was established at P < .05. Statistical analyses were done using SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL).

**Results**

We discovered a novel 2-bp microdeletion polymorphism GenBank accession number AB259617 located in intron 1 and –374 nucleotides upstream ATG as the start codon. The four SNPs were already registered and were named rs3781719 (–1784 T/C), rs1553005 (–1750 C/G), rs13306225 (–1218 C/T), and rs2644690 (–1036 G/A). rs3781719 and rs1553005 were completely linked. The T allele of rs3781719 was linked to the C allele of rs1553005, and the C allele of rs3781719 was linked to the G allele of rs1553005. In addition, we added three SNPs, rs7948017 (A/C), rs5241 (C/A), and rs2956 (A/T), that were referenced in the database. rs7948017 was located at –7644 bp upstream from the start codon. rs5241, which was a missense mutation, was located on exon 4. rs2956 was located at 120 bp from the end of exon 5. Thus, in total, we genotyped six SNPs and one microdeletion in the human CALCA gene to examine genetic differences between EH patients and control subjects.

Table 1 shows the clinical features of the two study populations. The BMI, systolic and diastolic BP, pulse, and the rate of hyperlipidemia were significantly higher in the EH group than in the control group. The total cholesterol plasma concentration and the rate of diabetes were significantly higher in the total EH group and the male EH group.

rs2644690 was excluded because there was no heterogeneity; all of the participants were genotyped as A/A. rs13306225 was also excluded because the genotype distribution of rs13306225 did not agree with the Hardy-Weinberg equilibrium. In the present study, rs7948017 was labeled as SNP1, rs1553005 as SNP2, rs5241 as SNP3, rs2956 as SNP4, and the 2-bp microdeletion polymorphism was labeled as DEL. The genotype distribution of each polymorphism did not show a statistically significant difference from the Hardy-Weinberg equilibrium values (data not shown).

Table 2 shows the distribution of the genotypes and the alleles of the four SNPs and the 2-bp microdeletion polymorphism. There were no statistically significant differences between the groups using Fisher’s exact test and multiple logistic regression analysis (the results of multiple logistic regression analysis are not shown). The three subjects with DEL were all from the EH group. In the EH group, there was a significant difference in the diastolic BP between the two genotypes of DEL using Student t test (with DEL, 124.3 ± 23.8; without DEL, 105.5 ± 14.0; P = .023).
Table 1. Characteristics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EH Patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>56.9</td>
<td>60.1</td>
<td>.14</td>
</tr>
<tr>
<td>Continuous variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/100 mL)</td>
<td>214.8</td>
<td>221.1</td>
<td>.015</td>
</tr>
<tr>
<td>HDL cholesterol (mg/100 mL)</td>
<td>51.2</td>
<td>53.3</td>
<td>.16</td>
</tr>
<tr>
<td>Uric acid (mg/100 mL)</td>
<td>7.0</td>
<td>7.4</td>
<td>.02</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>56%</td>
<td>39%</td>
<td></td>
</tr>
</tbody>
</table>

Continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as percentage. The p value of categorical variables was calculated by Fisher's exact test. The p value of continuous variables was calculated by Student's t-test. BMI - body mass index; DPP - diastolic blood pressure; EH - essential hypertension; HDL - high density lipoprotein; SBP - systolic blood pressure.

Table 2. Genotype and allele distributions in patients with EH and control subjects

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>EH Patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>310</td>
<td>307</td>
<td>.85</td>
</tr>
<tr>
<td>C/G</td>
<td>690</td>
<td>695</td>
<td>.54</td>
</tr>
<tr>
<td>G/G</td>
<td>930</td>
<td>932</td>
<td>.56</td>
</tr>
</tbody>
</table>

The p values of genotype and allele were calculated by Fisher's exact test. DEL, and SNP5. Three possible haplotypes (H1, H2, and H3) were predicted, and each had a frequency of more than 0.03 (Table 4). The overall distribution of these haplotypes was statistically significantly different between the groups (Table 4). The p value of categorical variables was calculated by Fisher's exact test.
CALCA has been well documented as one of the major vasodilators. It has been reported that plasma immunoreactive CALCA levels are significantly higher in spontaneously hypertensive rats (SHR). In addition, neuronal levels of CALCA mRNA have been reported to be increased in rats with mineralocorticoid salt-induced hypertension. Furthermore, the systolic BP of CALCA gene knockout mice was significantly higher than in wild-type mice, in both male mice (knockout, 160 ± 6.1 mm Hg) and wild, 125 ± 4.8 mm Hg) and female mice (knockout, 163 ± 33 mm Hg). Furthermore, the renin-angiotensin system was found to be activated in CALCA gene knockout mice. Based on this information, we concluded that the human CALCA gene could be a candidate gene for EH.

In the present study, we genotyped seven polymorphisms of the CALCA gene in Japanese subjects and then assessed the association between the CALCA gene and EH. There were no significant differences between any of the CALCA gene polymorphisms and EH. However, all of the participants with a 2-bp microdeletion polymorphism (DEL) belonged to the EH group. Given the small number of subjects, it was not possible to assess the relationship between DEL and EH using statistical techniques. In the EH group, there was a significant difference in diastolic BP between the two genotypes of DEL. Given this finding, a study with more subjects needs to be done to further assess the relationship between DEL and EH.

Since the draft sequence of the human genome was completed in 2001, the methodology and strategy of doing genetic research have changed dramatically. The SNPs are now used for the positional cloning of susceptibility genes by doing whole genome-wide scanning. Most of all, haplotype analysis has changed. Recent studies have shown that the human genome has a haplotype block structure that can be divided into discrete blocks of limited haplotype diversity. In each block, a small fraction of SNPs, referred to as tag SNPs, can be used to distinguish a large fraction of the haplotypes. These tag SNPs have the potential to be extremely useful for association studies, as they could make it unnecessary to genotype all of the SNPs. Haplotype-based analysis is considered to be much more powerful than a marker-by-marker analysis.

### Table 3. Pairwise linkage disequilibrium (D' below diagonal and $r^2$ above diagonal) for the five polymorphisms

<table>
<thead>
<tr>
<th></th>
<th>SNP1</th>
<th>SNP2</th>
<th>DEL</th>
<th>SNP3</th>
<th>SNP4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D'</strong></td>
<td>1.00</td>
<td>0.001</td>
<td>0.103</td>
<td>0.810</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>0.000</td>
<td>0.071</td>
<td>0.878</td>
</tr>
<tr>
<td><strong>$r^2$</strong></td>
<td>0.248</td>
<td>0.248</td>
<td>0.011</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>0.279</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.989</td>
<td>0.989</td>
<td>0.933</td>
<td>0.700</td>
<td></td>
</tr>
</tbody>
</table>

EH = essential hypertension. Values of $D' > 0.5$ and values of $r^2 > 0.5$ are shaded.

### Table 4. Haplotype frequency estimates

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>EH patients</th>
<th>Control subjects</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mj-Mj-Mj</td>
<td>0.721</td>
<td>0.755</td>
<td>1.36</td>
<td>.24</td>
</tr>
<tr>
<td>G-AG-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn-Mj-Mj</td>
<td>0.242</td>
<td>0.245</td>
<td>0.02</td>
<td>.90</td>
</tr>
<tr>
<td>C-AG-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn-Mj-Mn</td>
<td>0.037</td>
<td>&lt;0.001</td>
<td>16.13</td>
<td>.000059*</td>
</tr>
<tr>
<td>C-AG-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EH = essential hypertension; Mj = major allele; Mn = minor allele. Overall distribution of these haplotypes was statistically significant ($\chi^2 = 16.2, P = .00031$). Haplotypes with frequencies more than 0.03 were estimated using SNPAlalyze software. Mj and Mn indicate haplotypes with major and minor frequencies, respectively. * $P < .05$. 

#### Discussion

CALCA has been well documented as one of the major vasodilators. It has been reported that plasma immunoreactive CALCA levels are significantly higher in spontaneously hypertensive rats (SHR). In addition, neuronal levels of CALCA mRNA have been reported to be increased in rats with mineralocorticoid salt-induced hypertension. Furthermore, the systolic BP of CALCA gene knockout mice was significantly higher than in wild-type mice, in both male mice (knockout, 160 ± 6.1 mm Hg) and wild, 125 ± 4.8 mm Hg) and female mice (knockout, 163 ± 33 mm Hg). Furthermore, the renin-angiotensin system was found to be activated in CALCA gene knockout mice. Based on this information, we concluded that the human CALCA gene could be a candidate gene for EH.

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haplotype-based association study has advantages over an analysis based on individual polymorphisms. In the present study, the haplotypes were constructed using three polymorphisms (SNP2, DEL, and SNP3), based on the results of the LD analysis. The H3 haplotype (C-AGins-A) was statistically significantly more frequent in the EH group than in the control group. This result was not found by using a haplotype-based association study but only by using an association study that examined each polymorphism. The frequency of the H3 haplotype in the EH group was only 3.7%. However, this is not surprising given the low frequency of the susceptibility haplotype, as EH is thought to be a multifactorial disorder.

In conclusion, a novel 2-bp microdeletion polymorphism was discovered in intron 1 of the CALCA. The haplotype-based case-control study showed that the CALCA gene could be the susceptibility gene of EH. Further studies are needed to clarify the causal/susceptibility mutation of the CALCA gene or neighboring genes in EH.

Acknowledgments
We thank Naoyuki Sato and Kaoru Sugama for their excellent technical assistance.

References