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An intronic variable number of tandem repeat polymorphisms of the *cold-induced autoinflammatory syndrome 1 (CIAS1)* gene modifies gene expression and is associated with essential hypertension

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Cold-induced autoinflammatory syndrome 1 (CIAS1) gene is a member of the NALP subfamily of the CATERPILLER protein family that is expressed predominantly in peripheral blood leukocytes, which is to regulate apoptosis or inflammation through the activation of NF- κ B and caspase. Recent genetic analyses suggested an association between inflammation and oxidative stress-related genes in the development of hypertension. This is the first genetic study indicating an association between the *CIAS1* gene and susceptibility to essential hypertension (EH). The frequency of subject with the homozygote of 12 repeat allele was significantly higher in patients with hypertension compared with control subjects (987 cases, 924 controls) ($P=0.030$; odds ratio = 1.24) at a novel VNTR polymorphism of *CIAS1* intron 4 loci. We also found that the mean of systolic blood pressure of homozygotes of 12 repeat allele was 6.4 mmHg higher than those of homozygotes of non-12 repeat allele in male random population ($P=0.009$). The frequency of six SNPs spanning of the *CIAS1* gene was not significantly between patients and controls. The real-time PCR analysis showed that among healthy young adults, 12-12 subjects expressed *CIAS1* mRNA in peripheral leukocytes significantly more abundantly than homozygote of non-12 repeat alleles subjects ($P<0.05$). Reporter gene assay of the *CIAS1*-VNTR in HL60 stimulated by lipopolysaccharides showed that the intronic sequence involving 12 repeat increased the expression of luciferase compared with 9, 7, and 6 repeats.

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Received 6 October 2005; revised 29 May 2006; accepted 16 June 2006; published online 26 July 2006

Thus, we propose here the *CIAS1* is associated with EH through the dominant expression of transcripts, which may depend on the *CIAS1*-VNTR genotype.

European Journal of Human Genetics (2006) **14**, 1295–1305. doi:10.1038/sj.ejhg.5201698; published online 26 July 2006

Keywords: association study; *CIAS1*; hypertension; VNTR polymorphisms

Essential hypertension (EH [MIN1455]) is a complex disease that is thought to be influenced by polygenic and multiple environmental factors. Blood pressure is known to be a heritable trait, but the inherited genetic factors appear to have an influence on blood pressure and hypertension only ~50% of the time. Recently susceptibility loci for EH and a healthy variation in blood pressure have been mapped on several human chromosomes by genome-wide scans.^{1–9} Although several dozen candidate genes for hypertension have been proposed, understanding the genetic influences on this disease is difficult because, when combining data from different studies, there is a lack of replication of an identified locus.

Recent biochemical and morphological studies suggest that remodeling of large and small arteries is involved in the development and associated complications of hypertension.^{10,11} In the past decade, inflammation and oxidative stress have emerged as major players in vascular remodeling of hypertension in addition to known mechanisms, such as salt sensitivity, insulin resistance, and an imbalance in the renin-angiotensin system.^{12–15} Polymorphonuclear leukocytes (PMNL) are the main producer of reactive oxygen species (ROS) and have been shown to be involved in hypertension in both human and animal models. There is evidence supporting the presence of activated PMNL in humans and rats with hypertension. In a hypertension model using Sabra hypertension-prone rats, superoxide release from PMNL and inflammation anteceded the onset of hypertension (brought on by salt loading).^{16,17} Results indicated that genetic factors contributed to the activation of PMNL and production of ROS. The p22phox is a major component of NADPH oxidase and is essential for O²⁻ generation. A polymorphism in the promoter region of the p22phox gene, -930A/G, has been shown to be associated with EH.¹⁸ The present study was designed to identify a novel hypertensive-susceptibility locus using a single-candidate-gene approach on a gene involved in the activation of PMNL.

The CATERPILLER family gene (CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine protein family that is expressed predominantly in peripheral blood leukocytes^{19–25} in human. In addition, the rat orthologue of a member in CATERPILLER family gene, PYPAF5 (PYRIN-containing apoptotic protease-activating factor -1-like proteins 5), encodes angiotensin II/vasopressin dual receptor as a splicing isoform product²⁶ and the variation cosegregate with salt-sensitive hypertension in Dahl salt-

sensitive hypertension rat model.²⁷ Then, we focused the relation between hypertension and the CATERPILLER family gene. The *CIAS1* (MIN 606416; *cold-induced auto-inflammatory syndrome 1*) gene mapped 1q44¹⁹ encodes cryopyrin/NALP3/PYPAF1 (NACHT-LRR-PYD-containing protein-3/PYRIN-containing Apaf1-like protein-1) proteins, a member of the NALP subfamily of the CATERPILLER. The function of the proteins within the subfamily is thought to involve the regulation of apoptosis or inflammation through the activation of (nuclear factor) (NF)- κ B and caspase. Thus far, many missense mutations in the *CIAS1* gene have been identified in hereditary systemic auto-inflammatory disorders.²⁸ However, an association between the *CIAS1* gene and hypertension has not been reported. We used a case-control study using a single-candidate-gene approach to determine whether the *CIAS1* gene was associated with hypertension.

Methods

Subjects for genotyping

Population of panels 1 (100 cases and 109 controls) and 2 (987 cases and 924 controls) for case-control study were from the Tochigi prefecture, Ehime prefecture, Hiroshima prefecture, Osaka, or Kanagawa prefecture, Tokyo, Hokkaido in Japan (the study group from the Group of Millennium Genome Project for Hypertension). All subjects gave informed consent, and the study was approved by the ethics committee of Jichi Medical School. The hypertension group included patients who were diagnosed before 60 years of age; hypertension was defined as a blood pressure (systolic/diastolic) of 160/100 mmHg or higher. We excluded hypertensive individuals who had diabetes, intrinsic renal disease, a self-reported history of secondary hypertension that was corroborated by a family physician, or coexisting illness. We aimed to recruit hypertensive individuals with a body mass index (BMI) less than 25 to exclude the patient including obesity. In addition, the hypertensive individuals were selected with at least one first-degree family history because we considered that the patients with family history much received the genetic influence of hypertension compare to the patients without family history. Control subjects after 50 years included those with a blood pressure of 120/80 mmHg or lower and without hypertensive first-degree relatives. Blood pressure was measured with a mercury sphygmomanometer at least three times in a sitting position in clinic.

Panel 3 from random population (268 men and 162 women) was collected from the individual who came a medical check up at the Health Examination Center in Jichi Medical School. Genotypes were compared with phenotypes; age, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), total-cholesterol, high-density lipoprotein (HDL), triglycerides, fasting blood sugar (FBS), and glycosylated hemoglobin (HbA1c).

Genotyping of SNPs and a VNTR in the *CIAS1* gene

Four single nucleotide polymorphisms (SNPs) (IMS-JST084196, IMS-JST165517, IMS-JST165520, and IMS-JST084195) selected from seven SNPs registered in the Japanese SNP database,²⁰ a SNP (rs1539019) of NCBI and a SNP (HCV26052025) of commercial database (Celera Discovery System) were selected to cover the *CIAS1* gene. Each SNP was genotyped by the Taq Man-PCR assay using the ABI PRISM 7900-HT according to the manufacture's instructions (Applied Biosystems, Foster, CA, USA). For an association study, we also used a novel variable number of tandem repeat polymorphisms (VNTR) polymorphism in intron 4 of the *CIAS1* gene that was found through resequencing. To genotype the VNTR polymorphisms, standard PCR was performed in a final reaction volume of 25 μ l of 1 $\times$ PCR buffer containing 20 ng of the genomic DNA, 0.4 μ M of a set of primer (forward: 5'-CTGACCTCC CAATGTGCCTT-3' and reverse: 5'-CAGAGCTTCTCA GATTGCA-3'), 0.2 mM concentrations of each dNTP, and 2.5 U *Taq* DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany), using the following temperature profile: 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 7 min. The PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide.

Association between VNTR polymorphisms and gene expression *in vivo*

Total RNA from leukocytes was isolated from the blood of 46 healthy young adults with different genotypes by the Aquapure RNA blood kit (Bio-Rad, CA, USA). The 46 RNA consisted of 21 homozygotes of major repeats 12, 16 heterozygotes of major and minor repeats (9, 7, and 6 repeats), and nine homozygotes of minor repeats. We further analyzed the expression of *CIAS1* in their primary monocytes after lipopolysaccharide (LPS) stimulation. Monocytes were isolated from peripheral leukocytes by standard Ficoll centrifugation procedure followed by adhesion on plastic dishes coated by auto-serum. The attached cells were stimulated with 200 ng/ml LPS (LPS 026:B6; Sigma-Aldrich, St Louis, MO) for 30 or 60 min at 37°C and lysed in Trizol reagent (Life Technologies). For real-time PCR, approximately 100 ng total RNA from each cell was first reverse transcribed (final volume of 12.5 μ l) into cDNA using the SuperScript II (Life Technologies, Gaithersburg, MD) according to the manufacture's proto-

col. RT product (0.2 μ l) was used for quantification of gene expression by TaqMan real-time RT-PCR (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 5 μ l. The cumulative fluorescence for each amplicon was normalized to that seen with VIC-labeled GAPDH (Applied Biosystems, Foster, CA, USA) amplification. A FAM-labeled Hs00918086 probe (Applied Biosystems, Foster, CA, USA) was used for *CIAS1* mRNA amplification.

In vitro expression study by reporter gene assay of individual VNTR polymorphisms in HL60

We constructed expression vectors using the pGL3 basic (Promega, Madison, WI) and pCAGGS vector. The four alleles (12, 9, 7, and 6 repeats unit) of *CIAS1* 42bp-VNTR were individually amplified from DNA of the subjects encoding the alleles using *NheI*- and *NcoI*-tailed primers (GATTACCATGGGCTAGCAATGTGCCTTGTG, and AGCACCCATGGTGGCGGCCACAGCGCCCCA), and the PCR products were digested by *NheI/NcoI* and ligated into the pGL3 basic vector. After verification of the insert VNTR fragment by sequencing, VNTR-luciferase DNA fragment was extracted from the pGL3 vector by *NheI/XbaI* digestion and inserted in the pCAGGS vector at the *XbaI* site to mimic the splicing acceptor position. Ten micrograms of the constructs were transfected into 5×10^6 HL60 cells with 0.2 μ g of pRL vector (renilla luciferase, Promega) by electroporation procedure using GenePulser II (Bio-Rad Laboratories, Hercules, CA, USA). At 24 h after transfection, the relative light units (firefly/renilla light units) were measured by a dual luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). For all transfection assays, at least three independent experiments were performed.

Statistical analysis

Values are expressed as mean $\pm$ standard error of mean (SEM). Values of $P < 0.05$ were considered statistically significant. Data were analyzed using a contingency table analysis and the Student's *t*-test. Hardy-Weinberg equilibrium was calculated by the χ^2 test. Statistical analysis included a comparison of the proportions of the prevalence of the allele in cases and controls. Odds ratios (ORs) were generated from two-by-two tables, and statistical significance was assessed using the Fisher's exact. D' and r^2 -values were analyzed to evaluate a linkage disequilibrium (LD). Statistical analysis was performed using the SNPalyze (Dynacom, Mobara, Japan) and SPSS statistical package (SPSS Japan, Tokyo).

Results

Identification and characterization of a novel VNTR in the *CIAS1* locus

A novel VNTR polymorphism was found in the process of *CIAS1* resequencing, which was identified near the 3' end

Figure 1 (a) Schema of *CIAS1* gene with position of six SNPs and an identified VNTR polymorphisms for the genetic association study. Four SNPs were selected from the IMS-JST (Institute of Medical Science-Japan Science and Technology Agency) Japanese SNP database (IMS-JST): SNP1 (IMS-JST084196), SNP2 (IMS-JST165517), SNP3 (IMS-JST165520), and SNP4 (IMS-JST084195). SNP5 (rs1539019) and SNP6 (hCV26052025) were selected from a NCBI database and a commercial database (Celera Discovery System), respectively. (b) Nine genotypes of a novel VNTR polymorphism in *CIAS1*, a:12-12, b:12-9, c:12-7, d:12-6, e:9-7, f:9-6, g:7-7, h:7-6, and i:6-6 were detected in this study as shown in the agarose gel electrophoresis. M is 100 bp molecular ladder marker. (c) The nucleotide sequences of the 12 repeat allele of the *CIAS1*-42 bp VNTR. The tandem repeat number in each allele is 12 (GenBank Ac. No AB120959), 9 (GenBank Ac. No AB120960), 7 (GenBank Ac. No AB120961), and 6 (GenBank Ac. No AB120962).

of *CIAS1* intron 4, -527 to -24 bp from the splicing acceptor site of *CIAS1* exon 5 (Figure 1a). The PCR analysis identified nine genotypes derived from four alleles (Figure 1b). Each allele consisted of the 720, 594, 510, and 468 bp nucleotide sequences, respectively. The multiple alignment analysis between each allele showed the existence of the 42 bp tandem repeat sequences in the *CIAS1* gene (*CIAS1* 42 bp-VNTR). The nucleotide sequences of the 12 repeat allele was shown in Figure 1c. This tandem repeat polymorphism consisted of the 12, 9, 7, and 6 repeats units, respectively (GenBank accession number AB120959 to AB120962). The 12 repeat alleles' largest band was similar to the published sequence of the *Homo sapiens* chromosome 1 clone RP11-433K2 in GenBank (accession number AC104335). Estimation of the allele frequencies showed that the 12, 9, 7, and 6 repeat units was 0.577, 0.008, 0.248, and 0.167 in the *CIAS1* 42 bp-VNTR locus ($n=507$). The percentage of the all genotypes was 32.9% (12-12), 1% (12-9), 30% (12-7), 18.5% (12-6), 0% (9-9),

0.2% (9-7), 0.4% (9-6), 6.5% (7-7), 6.5% (7-6), and 4% (6-6), respectively. The observed genotype frequencies of all polymorphisms did not deviate from the predictions of the Hardy-Weinberg equation.

To assess whether the *CIAS1* locus is associated with hypertension, the *CIAS1* 42 bp-VNTR was genotyped for 109 control subjects and 100 patients with EH in panel 1. The frequency of subject with the homozygote of 12 repeat allele was significantly higher in patients with hypertension compared with control subjects ($P=0.04$; odds ratio = 1.84) (Table 1). The allele frequency of 12 repeat units, major allele, also tended to increase in patients compared with controls.

Single-locus analyses and case-control haplotype analyses of the *CIAS1* gene

To reveal whether the *CIAS1* 42 bp-VNTR is in tight linkage with other polymorphisms, six SNPs were chosen from the *CIAS1* gene using a public database (JSNP, [European Journal of Human Genetics](http://</p></div><div data-bbox=)

Table 1 Genotyping data and association test of DNA polymorphisms on the *CIAS1* in panel 1(100 cases and 109 controls)

	SNP1	SNP2	SNP3	SNP4	VNTR ^a	SNP5	SNP6
<i>Patients</i>							
Major allele	0.830	0.585	0.585	0.825	0.635	0.560	0.510
Minor allele	0.170	0.415	0.415	0.175	0.365	0.440	0.490
Major homozygous	68	31	31	67	40	28	22
Heterozygous	30	55	55	31	47	56	58
Minor homozygous	2	14	14	2	13	16	20
<i>Controls</i>							
Major allele	0.830	0.638	0.638	0.812	0.525	0.555	0.561
Minor allele	0.170	0.362	0.362	0.178	0.475	0.445	0.439
Major homozygous	74	47	47	72	29	37	35
Heterozygous	33	45	45	33	56	47	52
Minor homozygous	2	17	17	4	24	25	22
χ^2 (P)							
Major homozygous vs others	0.0003 (0.986)	3.274 (0.070)	3.274 (0.070)	0.021 (0.885)	4.231 (0.039)	0.860 (0.353)	2.687 (0.101)
Minor homozygous vs others	0.007 (0.930)	0.105 (0.746)	0.105 (0.746)	0.521 (0.470)	2.91 (0.087)	1.59 (0.207)	0.001 (0.974)

^aMajor allele is 12 repeat and minor alleles are 9, 7 and 6 repeat.

Table 2 Frequency of the major haplotype constructed from SNPs 1 to 6 between 109 controls and 100 cases

Haplotypes	Controls (%)	Case (%)	P
CTACGT	32.9	31.1	NS
CTACTC	16.2	13.3	NS
CCGCTC	13.1	17.5	NS
CTACTT	12.9	10.1	NS
ACGTTC	9.6	11.2	NS
ACGTGT	5.6	2.7	NS
CCGCGT	2.9	4.7	NS
CCGTTC	1.8	2.0	NS
CCGCGC	1.3	5.9	NS

NS: not significance.

www.snp.ims.u-tokyo.ac.jp, Figure 1a)²⁹ for genotyping in panel 1 subjects. In the VNTR loci, the subjects were categorized into three genotypes: major allele homozygote (12-12), heterozygote with major and minor alleles (12-9, 12-7, and 12-6), and minor allele homozygote (9-9, 9-7, 9-6, 7-6, and 7-7), respectively. As a results, no significant association was shown between cases and controls in the six SNPs of the *CIAS1* gene (Table 1).

Table 2 showed that the difference of haplotype frequency between the cases and controls. Our set of SNPs generated 10 common haplotypes (frequency>0.01) constructed from SNPs 1 to 6, and there were no differences in haplotype frequencies between cases ($n=100$) and controls ($n=109$). We also analyzed the pairwise linkage disequilibrium (LD) by calculating D' and r^2 using genotype results (Table 3); $|D'|>0.5$ and $r^2>0.5$ were regarded as tight linkage. Among the six SNPs, SNPs 1 (5' flanking region) to 4 (intron 4) tended to be in tight linkage but the LD was not distributed across the *CIAS1* gene. The LD and case-control haplotype analysis of the *CIAS1* gene suggest that

Table 3 Pairwise LD in *CIAS1* gene, evaluated by D' and r^2 from panel 1

	SNP1	SNP2	SNP3	SNP4	VNTR	SNP5	SNP6
SNP1							
SNP2 $ D' $	0.940						
r^2	0.286						
SNP3 $ D' $	0.940	1.000					
r^2	0.286	1.000					
SNP4 $ D' $	0.912	1.000	1.000				
r^2	0.765	0.351	0.351				
VNTR $ D' $	0.715	0.145	0.145	0.841			
r^2	0.142	0.082	0.082	0.214			
SNP5 $ D' $	0.294	0.405	0.405	0.348	0.185		
r^2	0.014	0.082	0.082	0.021	0.020		
SNP6 $ D' $	0.475	0.534	0.534	0.501	0.094	0.810	
r^2	0.053	0.209	0.209	0.066	0.007	0.452	

the VNTR locus was possibly independent from other SNPs and a functional polymorphism associated with hypertension.

Case-control study of the *CIAS1* 42bp-VNTR for hypertension in a large sample

To confirm the possibility that the homozygote of 12 repeat allele is a primary variant that determines susceptibility to hypertension, genotyping of *CIAS1* 42bp-VNTR was performed in panel 2 (987 cases and 924 controls). The results confirmed the statistical significance between the *CIAS1* 42bp-VNTR and EH (Table 4). The significant difference was $P=0.030$ (Odds 1.24) in the comparison of 12-12 vs 12-X + X-X and $P=0.006$ (Odds 1.39) in 12-12 + 12-X vs X-X. These results led us the homozygote of 12 repeat alleles was an association with risk of EH. The frequency of the 12-12 subjects was 0.348 and 0.301 in panel 2. The most significant association was shown in the

Table 4 Association of the *CIAS1*-VNTR genotype with essential hypertension in panel 2

Panel 2	Controls	Patients	χ^2	P	Odds
<i>Genotype distribution</i>					
12-12	278 (30.1%)	343 (34.7%)			
12-X	466 (50.4%)	498 (50.5%)			
X-X	180 (19.5%)	146 (14.8%)			
<i>Allele frequency</i>					
12	0.553	0.600			
X	0.447	0.400			
<i>Genotype comparison</i>					
12-12 vs 12-X+X-X			4.735	0.030	1.24
12-12+12-X vs X-X			7.413	0.006	1.39

Table 5 Clinical characterization of random population of panel 3 to *CIAS1* 42 bp-polymorphism

Panel 3 (Men and Women)	12-12	Genotype 12-X	X-X	P
N (%)	138 (32.1%)	218 (50.7%)	74 (17.2%)	NS
Age	51.4±8.7	52.1±9.5	53.5±8.6	NS
Height (cm)	163.0±6.8	163.2±8.8	163.3±8.5	NS
Weight (kg)	63.7±9.1	63.1±11.2	62.5±10.2	NS
BMI (kg/m ²)	24.0±2.7	23.8±3.4	23.3±2.6	NS
SBP (mmHg)	127.7±14.2	124.3±16.4	122.4±13.3	0.018*
DBP (mmHg)	78.6±9.7	77.6±11.1	76.3±9.8	NS
Total-cholesterol (mg/dl)	205.2±34.4	205.6±35.8	198.6±32.3	NS
HDL (mg/dl)	61.8±13.9	60.9±18.2	61.9±14.3	NS
Triglyceride (mg/dl)	109.3±53.8	103.0±51.7	96.2±43.1	NS
Fasting blood glucose (mg/dl)	100.5±8.7	98.9±20.2	101.4±15.8	NS
HbA1c (%)	5.0±0.3	5.1±0.4	5.1±0.4	NS

NS; not significant.

T-Chol, total cholesterol; HDL-Chol, HDL cholesterol; TG, triglyceride; FBS, Fasting blood sugar; and HbA1c, glycohemoglobin, A-1-C.

*12-12 vs 12-X+X-X.

comparison of 12-12 vs X-X ($\chi^2 = 13.048$, $P = 0.0003$, odds ratio = 1.60) (data not shown). These genetic results indicate that the *CIAS1* locus contributes to the susceptibility to EH.

Association between *CIAS1* 42bp-VNTR genotype and random population in blood pressure

We further examined the association between the *CIAS1* gene and healthy variations in blood pressure. Panel 3 from random population (268 men and 162 women) was collected at the Health Examination Center in Jichi Medical School, which was independent of panels 1 and 2. Genotypes were compared with phenotypes; age, BMI, SBP, DBP, total-cholesterol, HDL, triglycerides, FBS, and HbA1c. Table 5 showed the genotype effect in three groups of 12-12, 12-X, and X-X to clinical characteristics in *CIAS1* gene. We found the significant difference of the SBP in three genotype groups by one-way ANOVA study in panel 3 ($P = 0.018$). The association between the genotype and the SBP was revealed in men ($P = 0.028$, Table 6) but not in women (Table 7). The mean SBP in

12-12 subjects ($n = 86$) was 6.4 mmHg higher than those of X-X subjects ($n = 44$) in men. The *CIAS1* 42bp-VNTR genotype was not associated with BMI, DBP, total-cholesterol, HDL, triglycerides, fasting blood glucose, and HbA1c levels. To exclude the influence of age on BP or population stratification among generation, age was put into covariate and analysis of covariance test (ANCOVA) was carried out. After this correction significant difference of SBP was observed again among three genotypes in men ($P = 0.028$), and the significance became distinct ($P = 0.011$), when over 55 years old men were analyzed. In 162 women, we did not find a significant difference of blood pressure among the three groups in ANOVA or ANCOVA study even after age correction, but the mean SBP of 12-12 subjects was slightly higher than that of other groups (Table 7).

Genotype and the expression level of *CIAS1* transcript

The transcript from *CIAS1* was predominantly expressed in peripheral blood leukocytes compared with total RNA from bone marrow, brain, fetal liver heart, kidney, lung,

Table 6 Clinical characterization of random population of panel 3 to *CIAS1* 42 bp-polymorphism in Men

<i>Panel 3 (Men)</i>	12-12	Genotype 12-X	X-X	<i>P</i>
N (%)	86 (32.1%)	138 (51.5%)	44 (16.4%)	NS
Age	53.5 ± 8.5	52.6 ± 9.3	54.1 ± 7.2	NS
Height (cm)	167.0 ± 6.8	167.9 ± 6.1	168.7 ± 5.1	NS
Weight (kg)	67.7 ± 9.3	68.4 ± 8.6	67.2 ± 8.6	NS
BMI (kg/m ²)	24.2 ± 2.7	24.2 ± 2.3	23.6 ± 2.7	NS
SBP (mmHg)	131.7 ± 14.2	126.8 ± 16.0	125.4 ± 13.6	0.028
DBP (mmHg)	80.6 ± 9.7	79.7 ± 11.1	79.0 ± 9.6	NS
Total-cholesterol (mg/dl)	207.3 ± 34.4	206.4 ± 35.1	197.9 ± 32.9	NS
HDL (mg/dl)	58.7 ± 13.9	55.8 ± 13.3	56.8 ± 13.9	NS
Triglyceride (mg/dl)	127.6 ± 53.8	119.1 ± 53.7	112.2 ± 44.9	NS
Fasting blood glucose (mg/dl)	104.2 ± 8.7	103.0 ± 11.5	102.8 ± 11.7	NS
HbA1c (%)	5.0 ± 0.3	5.1 ± 0.4	5.0 ± 0.6	NS

NS; not significant.

T-Cho, total cholesterol; HDL-Cho, HDL cholesterol; TG, triglyceride, FBS, Fasting blood sugar; and HbA1c, glycohemoglobin, A-1-C.

Table 7 Clinical characterization of random population of panel 3 to *CIAS1* 42 bp-polymorphism in Women

<i>Panel 3 (Women)</i>	12-12	Genotype 12-X	X-X	<i>P</i>
N (%)	52 (32.1%)	80 (49.4%)	30 (18.5%)	NS
Age	47.9 ± 7.8	51.7 ± 9.8	49.5 ± 7.8	NS
Height (cm)	156.3 ± 5.115	5.1 ± 6.7	155 ± 5.7	NS
Weight (kg)	57.1 ± 7.8	54.0 ± 9.1	55.5 ± 8.1	NS
BMI (kg/m ²)	23.5 ± 3.5	23.1 ± 4.7	22.9 ± 2.4	NS
SBP (mmHg)	121.0 ± 13.5	120.1 ± 16.4	118.1 ± 11.5	NS
DBP (mmHg)	75.3 ± 8.8	74.0 ± 10.2	72.5 ± 8.7	NS
Total-cholesterol (mg/dl)	201.8 ± 27.5	204.2 ± 37.0	199.8 ± 31.4	NS
HDL (mg/dl)	67.0 ± 15.6	67.9 ± 15.6	69.2 ± 11.3	NS
Triglyceride (mg/dl)	79.2 ± 49.0	75.6 ± 33.5	72.3 ± 26.5	NS
Fasting blood glucose (mg/dl)	94.3 ± 10.3	92.0 ± 28.5	99.4 ± 20.2	NS
HbA1c (%)	5.0 ± 0.4	5.1 ± 0.3	5.1 ± 0.4	NS

NS; not significant.

T-Cho, total cholesterol; HDL-Cho, HDL cholesterol; TG, triglyceride, FBS, Fasting blood sugar; and HbA1c, glycohemoglobin, A-1-C.

placenta, prostate, salivary gland, skeletal muscle, spleen, testis, thyroid gland, trachea, uterus, colon, small intestine, spinal cord, and stomach packaged in the master panel (BD Biosciences, CA, USA) by RT-PCR analysis (data not shown). We next tested the genotype effect of *CIAS1* 42 bp-VNTR locus to the expression level of *CIAS1* transcript using the total leukocyte RNA extracted from blood samples in healthy young volunteers with different genotypes. Real-time PCR analysis showed that the expression level of the *CIAS1* transcript was different in subjects with 12-12 ($n=21$), 12-X ($n=16$), and X-X ($n=9$) VNTR genotypes (Figure 2). The *CIAS1* transcript was expressed significantly more in the 12-12 subjects compared with subjects with the X-X genotype. Real-time PCR analysis showed that the expression level of the *CIAS1* transcript showed significant difference in subjects with among three genotype groups ($P=0.009$) by One-way ANOVA tests.

(Figure 2). Among the three genotype groups, the *CIAS1* transcript from 12-12 subjects was much expressed than that of 12-X and X-X subjects.

Expression of the transcript from *CIAS1* locus in monocytes after LPS stimulation

The *CIAS1* expression level in total leukocytes showed remarkably high SD in both 12-12 and 12-X subjects (Figure 2), which was estimated to be dependent on the activated state of the leukocytes. To rule-out the possibility that the deviations simply resulted from the condition of donors or preparation procedure of leukocytes, the transcript level was also analyzed in monocytes under stimulation of LPS. As shown in Figure 3, transcript level in monocytes before stimulation of 12-12 subjects was higher than other genotype groups. One-way ANOVA tests showed the expression level of *CIAS1* mRNA on monocyte

Figure 2 The *CIAS1* mRNA levels in peripheral leukocytes among the three genotype groups of 12-12 (homozygote of 12 repeat alleles), 12-X (heterozygote of 12 and non-12 repeat alleles), and X-X (homozygote of non-12 repeat alleles) in *CIAS1* 42bp-VNTR. The *CIAS1* expression was analyzed by real-time PCR. The relative amount of *CIAS1* mRNA normalized by GAPDH is shown as fold volume. n: number of subjects. One-way ANOVA tests showed that expression level of the *CIAS1* transcript was significant difference in subjects among three genotype groups ($P=0.009$).

was the significant difference on 30 min ($P=0.01$) and 60 min ($P=0.0001$) after LPS stimulation. These results indicated that the 12 repeat allele had an additive effect on the expression level of this gene.

Functional assay of *CIAS1* VNTR in HL 60

To investigate whether *CIAS1* 42 bp-VNTR directly affected gene expression, we next examined the reporter gene assay. We constructed the pCAGGS-based expression plasmid, containing the *CIAS1* 42bp-VNTR alleles (12, 9, 7, and 6 repeat units) linked to the luciferase gene at the splicing acceptor site (Figure 4a). The pCAGGS promoter activity of the 12 VNTR repeat construct was higher than other repeat constructs before LPS stimulation in HL60 cells, although the difference was not significant (Figure 4b). The difference, however, became significant after 1 or 2 h of LPS stimulation. These results may confirmed the additive effect of 12 repeat allele on the expression level of *CIAS1* gene (Figure 3) in the functional assay.

Figure 3 The effect of stimulation of LPS on *CIAS1* mRNA levels in monocytes in different genotypes. Monocytes were isolated from peripheral leukocytes by Ficoll centrifugation followed by adhesion on a plastic dish. Cells were treated with 200 ng/ml LPS for 30 or 60 min and lysed. The relative amount of *CIAS1* mRNA normalized by GAPDH is shown. Black bars show the transcripts with X-X genotype (homozygote of non-12 repeat alleles), hatched bars indicate the transcripts with 12-X genotype (heterozygote of 12 repeat allele and non-12 alleles), and gray bars show the transcript with 12-12 genotype (homozygote of 12 repeat allele). One-way ANOVA tests showed the expression level of *CIAS1* mRNA on monocyte was the significant difference on 30 min ($P=0.01$) and 60 min ($P=0.0001$) after LPS stimulation. Three individuals with each genotypes were tested.

Discussion

A single-candidate-gene approach for hypertension focusing on the CATERPILLER protein family has not been demonstrated. This is the first genetic study indicating an association between the *CIAS1* gene and susceptibility to EH. Our study of 987 cases and 924 controls showed that subjects with 12-12 genotype of the *CIAS1* 42bp-VNTR experienced hypertension more frequently than controls, with a significant association ($P=0.006$). Furthermore, the

Figure 4 Functional assay of VNTR in HL60 cells. (a) The genomic region including the VNTR repeats, 12 (720 bp), 9 (594 bp), 7 (510 bp), and 6 (468 bp), was inserted in pGL3 vector. The VNTR-luciferase inserts were directionally subcloned in the PCAGGS vector. (b) HL60 cells were co-transfected with the pRL vector (renilla luciferase) by electroporation procedure. At 24 h after the transfection, HL60 cells were stimulated by LPS. The relative light units (firefly/renilla light units) were measured by dual luciferase reporter assay system. PCAGGS-VNTR-12 (●), PCAGGS-VNTR-9 (○), PCAGGS-VNTR-7 (■), and PCAGGS-VNTR-6 (□). The relative luciferase activity with 12 repeat allele was significantly enhanced compared with the activity with 9, 7, and 6 repeat until more than 2 h after LPS.

comparison with genotype and clinical data in a random population showed that SBP was significantly higher in 12-12 subjects compared with 12-X and X-X subjects, although there was no difference in other variables. These results lead us to question whether the *CIAS1* 42bp-VNTR in intron is a true functional polymorphism in hypertension. We showed that the transcript level of *CIAS1* in peripheral leukocytes and monocytes of healthy young adults was more abundant in 12-12 subjects than in those with the other genotypes. Furthermore, our reporter assay for *CIAS1* 42bp-VNTR showed that the 12 repeat units

increased the expression of luciferase in the *CIAS1* gene. These results may indicate that the *CIAS1* 42bp-VNTR directly affects gene expression. Thus, we believe that increased expression of *CIAS1* in leukocytes dependent on the *CIAS1* 42bp-VNTR length may contribute to the development of EH.

CIAS1 is a member of the recently discovered NALP/PYPAF subfamily of the CATERPILLER protein family, thought to function in apoptotic and inflammatory signaling pathways.²⁷ *CIAS1*/NALP3/PYPAF1 protein is composed of three domains; the N-terminal Pyrin domain, the central a specific nucleotide binding (NACHT) domain, and the C-terminal tandem copy of Leucine-rich repeats (LRRs). A large number of nucleotide substitutions have been found in the NACHT domain¹⁹ in patients with autosomal-dominant autoinflammatory disorders; Muckle-Wells syndrome, familial cold urticaria, and chronic infantile neurologic cutaneous and articular syndrome.^{19,27} It has been proposed that the missense mutation in the NACHT domain weakens the self-regulation via its interaction with the LRRs and enables spontaneous activation by releasing NACHT domain from LRRs. The activated form recruits an apoptosis-associated speck-like protein containing a CARD (ASC) and Cardinal (TUCAN, CARD8, NDDP1). The caspase recruit domain (CARD) of ASC and Cardinal interact with that of pro-caspase-1. The resulting macrocomplex, called inflammasome, is available to convert pro-IL-1 β into matured IL-1 β . Moreover, ASC and Cardinal in the inflammasome have been shown to be associated with IKK (I κ B kinase) complex and activate NF- κ B. The phenotypic heterogeneity (cold sensitivity, sensorineural hearing loss, severe neurologic involvement) has been observed in autoinflammatory syndromes. Although, the phenotypic heterogeneity has been mainly attributed to the mutation position in the NACHT domain, the amount of mutant *CIAS1* mRNA might also contribute to the development of the syndrome.

The physiological function of the wild type *CIAS1* is, however, still under investigation. Opposite results about NF- κ B activation have been reported from overexpression analysis of *CIAS1* in culture cell lines. NF- κ B was activated by the overexpression of *CIAS1* and ASC in HEK293 cells.²¹ The *CIAS1* suppressed the activation in HeLa cells after TNF stimulation and suggested that a molecular balance of *CIAS1* with ASC is critical for the inflammatory response.^{23,24} Recently *Cias1*-knockout mice are developed in two independent research groups. Mariathasan *et al* showed that Cryopyrin produced from *CIAS1* activates the inflammasome in response to toxins and ATP.³⁰ Kanneganti *et al* reports that bacteria RNA and small antiviral compounds activate caspase 1 through cryopyrin produced from *CIAS1* gene.³¹ We propose here the *CIAS1* is associated with hypertension through the dominant expression of transcripts, which may depend

on the *CIAS1*-VNTR genotype. To prove this hypothesis, an animal model overexpressing *CIAS1* in leukocytes is desirable, which may also resolve the matter of whether *CIAS1* alone activates or suppresses NF- κ B signaling.

A recent study showed that chronic inflammation is crucial for the development of hypertension. Plasma C-reactive protein has been shown to be an independent risk factor for hypertension,³² and PMNL count in peripheral blood and increased superoxide release contributes to hypertension.³³ In the animal model, the activation of NF- κ B is increased in numerous tissues in stroke-prone spontaneous hypertension (SHRsp) rats compared with Sprague-Dawley (SD) rats.³⁴ NF- κ B promotes proinflammatory genes or induces apoptosis in many organs. In leukocytes, NF- κ B can induce iNOS, cell adhesion molecules, IL-6, and other cytokines and chemokines.³⁵ The induced inflammation generates large amount of oxidative agents, and the oxidative stress activates NF- κ B.^{36,37} Thus, we predict that *CIAS1* might play an important role in the development of hypertension based on inflammation brought on by NF- κ B activation.

Acknowledgements

The study was supported by a grant in aid (No.13204075) from Japanese Ministry of Education, Culture, Sports, Science and Technology. We thank Oyamada T and Nagashima K for technical assistance. We thank Drs Kario, Hoshida S, Sugimoto K, Ishikawa K, Sakamoto A, (Jichi Medical School), Sekiguchi T, and Hara K (Nasu minami) Ohishi T (Ohishi medical clinic) for sample correction.

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Appendix

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