Table 3. 5-HT2A Genotype Frequency in Hypertensives and Normotensives According to ET-1 Genotype

ET-1 genotype	5-HT2A genotype	Genotype frequency (*n* (%)) Normotensive (*n*=1,866)	Hypertensive (*n*=1,102)	*p* value	OR	95% CI
GG	CC	256 (26.4)	139 (24.7)			
	CT	460 (47.4)	258 (45.8)			
	TT	255 (26.3)	166 (29.5)	0.20	1.10	0.95–1.26
GT+TT	CC	211 (23.6)	159 (29.5)			
	CT	439 (49.1)	259 (48.1)			
	TT	245 (27.4)	121 (22.4)	0.0056	0.81	0.70–0.94

ET-1, endothelin-1; 5-HT2A, serotonin 2A; OR, odds ratio; CI, confidence interval.

Table 4. ET-1 Genotype Frequency in Hypertensives and Normotensives According to 5-HT2A Genotype

5-HT2A genotype	ET-1 genotype	Genotype frequency (*n* (%)) Normotensive (*n*=1,866)	Hypertensive (*n*=1,102)	*p* value	OR	95% CI
CC	GG	256 (54.8)	139 (46.6)			
	GT+TT	211 (45.2)	159 (53.4)	0.028	1.39	1.04–1.86
CT	GG	460 (51.2)	258 (49.9)			
	GT+TT	439 (48.8)	259 (50.1)	0.65	1.05	0.85–1.31
TT	GG	255 (51.0)	166 (57.8)			
	GT+TT	245 (49.0)	121 (42.2)	0.064	0.76	0.57–1.02

ET-1, endothelin-1; 5-HT2A, serotonin 2A; OR, odds ratio; CI, confidence interval.

association of the ET-1 G/T polymorphism with hypertension in 5-HT2A TT homozygotes was also marginally significant (p=0.064).

Interaction between the 5-HT2A T102C and ET-1 G/T Polymorphisms in Association with Blood Pressure

Given the marginally significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension, we next analyzed the possible interactions between these polymorphisms in their association with blood pressure in the combined group of populations 1 and 2. This analysis showed a marginally significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with SBP (p=0.045). This interaction was also marginally significant after adjustment for sex and age (p=0.045), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG (p=0.058). Moreover, there was a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with DBP (p=0.0013). This interaction was also significant after adjustment for sex and age (p=0.0018), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG (p=0.0023). Table 5 shows the opposite directions of the association of the 5-HT2A T102C polymorphism with blood pressure between the ET-1 genotypes. The association of the 5-HT2A T102C polymorphism with SBP in ET-1 T allele carriers was marginally significant (p=0.054), and the association of the 5-HT2A T102C polymorphism with DBP in ET-1 T allele carriers was significant (p=0.021). Table 6 again shows the opposite directions of the association of the ET-1 G/T polymorphism with blood pressure between the 5-HT2A genotypes. The association of the ET-1 G/T polymorphism with DBP in 5-HT2A CC homozygotes was significant (p=0.0013).

Discussion

Given the biological evidence for a relation of the 5-HT2A receptor to blood pressure, a functional polymorphism (T102C) of the 5-HT2A receptor gene has been investigated in relation to hypertension. An initial study showed that increased frequency of the 102C allele was significantly associated with hypertension in female UK residents (*3*). A subsequent study failed to show a significant association between the 5-HT2A T102C polymorphism and hypertension in a Chinese population (*4*). Consistent with the results of the latter study, the present study failed to show a significant association, although increased frequency of the 102C allele was non-significantly associated with hypertension in the combined group of populations 1 and 2, in line with the results of the former study.

This failure could be attributable to racial difference. How-

Table 5. Blood Pressure for 5-HT2A Genotype According to ET-1 Genotype

BP	ET-1 genotype	5-HT2A genotype			*p* value	
		CC	CT	TT	For regression	For interaction
SBP (mmHg)	GG	129.9±18.2	129.3±19.1	130.4±17.7	0.61	
	GT+TT	132.0±18.0	130.3±18.1	128.8±17.9	0.054	0.045
DBP (mmHg)	GG	77.4±10.6	77.7±11.7	78.5±10.6	0.33	
	GT+TT	79.9±11.2	77.6±10.9	77.4±10.7	0.021	0.0013

Data are mean±SD. 5-HT2A, serotonin 2A; ET-1, endothelin-1; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP.

Table 6. Blood Pressure for ET-1 Genotype According to 5-HT2A Genotype

BP	5-HT2A genotype	ET-1 genotype		*p* value	
		GG	GT+TT	For regression	For interaction
SBP (mmHg)	CC	129.9±18.2	132.0±18.0	0.11	
	CT	129.3±19.1	130.3±18.1	0.29	
	TT	130.4±17.7	128.8±17.9	0.21	0.045
DBP (mmHg)	CC	77.4±10.6	79.9±11.2	0.0019	
	CT	77.7±11.7	77.6±10.9	0.98	
	TT	78.5±10.6	77.4±10.7	0.14	0.0013

Data are mean±SD. ET-1, endothelin-1; 5-HT2A, serotonin 2A; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP.

ever, genetic effects are usually consistent across human populations (*22*). Therefore, the failure might be rather attributable to gene–environmental and/or gene–gene interactions, because such interactions could modify or mask associations. In this respect, the present study revealed a statistically significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. Consequently, increased frequency of the 5-HT2A 102C allele is significantly associated with hypertension in ET-1 T allele carriers, consistent with the initial study (*3*). However, it should be noted that this interaction was significant in population 1 and in the combined group of populations 1 and 2, but not in population 2, despite the fact that the OR for the interaction were very similar between the two populations. This implies that studies with modest sample sizes can fail to detect interactions, and a combination of samples will be required to achieve adequate statistical power.

Moreover, the present study showed a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with blood pressure. In particular, the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with DBP was significant in both populations 1 and 2 (data not shown), constituting strong evidence in favor of the existence of this interaction.

A genetic interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms is also supported by persistent biological evidence for the existence of interactions between the serotonin and endothelin systems. For example, subthreshold concentrations of ET-1 amplify the vasoconstrictor effect of serotonin in human arteries (*6*, *7*, *9*, *10*) and in the guinea pig trachea (*11*). Preincubation of a platelet suspension with ET-1 has been shown to inhibit the serotonin-mediated platelet response (*8*). ET-1 has been shown to inhibit serotonergic amplification of epinephrine-induced aggregation of platelets (*8*). Pre-treatment of rabbit platelets with ET-1 has been shown to enhance serotonin-promoted protein tyrosine phosphorylation (*12*). In the rabbit platelet membrane, ET-1 has been shown to enhance serotonin binding and inhibit its internalization (*12*). On the other hand, serotonin also potentiates ET-1–induced vascular smooth muscle cell proliferation (*13*).

The T102C polymorphism is located in the coding sequence in exon 1 of the 5-HT2A receptor gene and does not change any amino acid, and thus it is a silent polymorphism in that both nucleotides result in a codon that encodes Ser at amino acid position 34 (*23*). Nevertheless, the T102C polymorphism results in a differential gene expression (*24*). This functionality of the 5-HT2A T102C polymorphism also increases the plausibility of a genetic interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms.

In conclusion, the present study revealed a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in the pathogenesis of hypertension in two large Japanese populations. This interaction was supported by several lines of molecular biological evidence. Nevertheless, association studies are often irreproducible, warranting further studies in large populations to investigate the interactions between the serotonin and endothelin systems, with consideration of various gene–environment and gene–gene interactions.

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Mutation of the Follicle-Stimulating Hormone Receptor Gene 5′-Untranslated Region Associated With Female Hypertension

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Abstract—Inactivating mutations in the follicle-stimulating hormone receptor (FSHR) gene have been reported to cause hereditary hypergonadotropic ovarian failure. It has been found recently that the FSHR knockout mouse exhibits hypertension. The aim of the present study was to investigate the association between polymorphisms in the human FSHR gene and essential hypertension (EH) by using single nucleotide polymorphisms (SNPs). We selected 5 SNPs in the gene (rs1394205, rs2055571, rs11692782, rs1007541, and rs2268361) and performed 2 genetic case–control studies in different populations. A confirmative case–control study was performed using 1035 EH patients and 1058 age-matched controls. Transcriptional activities were measured with a luciferase assay system. The first case–control study found that the A allele of rs1394205 was significantly higher in EH females (P=0.010). In addition, in the confirmative case–control study, there was a significant difference for this SNP between female normotensive subjects (44.5%) and EH patients (50.7%) (P=0.043). Multiple logistic regression analysis in female subjects also revealed a significant association of subjects with the A allele of rs1394205 with EH (P=0.033), with the odds ratio calculated as 1.68 (95% CI: 1.04 to 2.73). Transcriptional activity of the A allele was 56±8% (mean±SD) of that observed for the G-type allele (P=0.001). Serum estradiol levels were significantly lower in patients with the A/A genotype than in patients without the A/A genotype (P=0.004). The SNP in the 5′-untranslated region of the FSHR gene affects levels of transcriptional activity and is a susceptibility mutation of EH in women. (***Hypertension*. 2006;48:512-518.**)

Key Words: hypertension, essential ■ hormones ■ case–control studies

It is likely that essential hypertension (EH) is a polygenic disorder that results from the inheritance of a number of susceptibility genes. The causal genes identified may contribute from 30% to 50% of the variations in blood pressure seen among individuals.[1] These genetic determinants interact with environmental factors, such as dietary salt, to produce the final disease phenotype. Despite significant recent progress in genomic and statistical tools, the genetic dissection of human EH remains a major challenge.[2]

The effects of follicle-stimulating hormone (FSH) are mediated by its interaction with specific receptors and the activation of G_s, the stimulatory guanine nucleotide binding protein, which stimulates the enzyme adenylyl cyclase.[3] The FSH receptor (FSHR) belongs to the superfamily of G protein–coupled receptors that are characterized by the common structural feature of 7 transmembrane domains. They differ structurally from other G protein–coupled receptors in that they contain a large extracellular domain in the amino-terminal part of the receptor protein, which is required for interaction with complex glycoprotein hormones.[4–6] The human FSHR gene is localized to 2p21 to p16 and is composed of 10 exons.[7] The 5′-flanking region of the gene has neither a TATA nor a CCAAT box and exhibits promoter-type features that are seen in housekeeping genes.[8]

Inactivating mutations in the FSHR gene have been reported to cause hereditary hypergonadotropic ovarian failure in women.[9] These mutations have been shown to be associated with a recessive inheritance pattern, and all of the affected subjects have homozygous or compound heterozygous mutations in the coding region of the gene. The phenotype in men for

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this particular FSHR mutation is less clear, with homozygous men found to have normal masculinization and circulating testosterone, normal or slightly elevated luteinizing hormone (LH), moderately elevated FSH, and slightly to severely reduced testicular volume.[10] Although men with inactivating mutations have abnormal semen parameters that range from severe-to-moderate oligozoospermia, normal sperm concentrations with low volumes, or teratozoospermia (reported in 1 individual), none have been found to be azoospermic, and there are reports of such individuals fathering children. These results suggest that FSHR mutations have a greater consequence in women than in men.

The FSHR knockout mice have ovarian insufficiency, low estrogen levels with functionally responsive estrogen receptors, and increased testosterone levels.[11–13] They also exhibit the same changes seen in postmenopausal women, such as osteoporosis, hypercholesterolemia, and weight gains. In 2003, Javeshghani et al[14] reported that these mice have increased blood pressure, indicating that there is vascular remodeling.

There have been no previous studies on the association between the FSHR gene and EH. The aim of the present study was to investigate the association between the human FSHR gene and EH through the use of single nucleotide polymorphisms (SNPs) in the human FSHR gene.

Methods

First Case–Control Study

Patients and control subjects from the northern area of Tokyo were recruited for the first case–control screening study. A total of 235 patients were diagnosed with EH according to the following criteria: seated systolic blood pressure (SBP) >160 mm Hg and/or diastolic blood pressure (DBP) >100 mm Hg on 3 occasions within 2 months after the first medical examination. None of the patients were using antihypertensive medications, and subjects diagnosed with secondary hypertension were excluded. We also included 237 normotensive (NT) healthy individuals as controls. None of the NT participants had a family history of hypertension, and all had SBP and DBP <130 and 85 mm Hg, respectively. A family history of hypertension was defined as a previous diagnosis of hypertension in grandparents, uncles, aunts, parents, or siblings. Informed consent was obtained from each individual as per a protocol approved by the Human Studies Committee of Nihon University.[15]

Confirmative Case-Control Study: Subgroup Collaboration Study With the Hypertensive Section of the Japanese Millennium Project

For confirmation of a possible SNP association, we used a second set of 1035 EH patients and 1058 age-matched control subjects, who were recruited through a subgroup collaboration study with the hypertensive section of the Japanese Millennium Project. Six medical institutes took part in the collaborative study and collected data on hypertensive cases and controls. Hypertensive patients were defined as having SBP ≥140 mm Hg or DBP ≥90 mm Hg or were patients with chronic antihypertensive medication. To increase the statistical power of the present study, hypertensive subjects additionally had to meet the following criteria: age <60 years old or onset of hypertension <50 years of age, a family history of hypertension, and without obesity (body mass index <26 kg/m^2). The NT criteria were as follows: SBP/DBP <130/85 mm Hg, without a family history of hypertension, and without obesity. In this study, both groups were recruited from throughout Japan, and informed consent was obtained from each individual as per the protocol approved by each institutions' human studies committee.

Biochemical Analysis

Serum concentrations of total cholesterol, triglyceride, high-density lipoprotein cholesterol, uric acid, creatinine, and γ-glutamyl transpeptidase were measured using the methods of the clinical laboratory department of each hospital or institution.

Genotyping

Using information about allelic frequencies of SNPs registered on the web site of the National Center for Biotechnology Information and Celera Discovery System-Applied Biosystems, 5 SNPs with minor allele frequencies >20% were selected. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic case–control studies.

We examined the association between EH and 5 SNPs in the human FSH receptor gene. All 5 of the SNPs were confirmed using the National Center for Biotechnology Information web site and Applied Biosystems-Celera Discovery System with the accession numbers, rs1394205 (C_426553_10), rs2055571 (C_246842_10), rs11692782 (C_228130_10), rs1007541 (C_9561251_1_), and rs2268361 (C_11813031_1_; Figure 1 a). Genotypes were determined using Assays-on-Demand kits (Applied Biosystems) together with TaqMan PCR.[16] When allele-specific fluorogenic probes hybridize to the template during the PCR, the 5′ nuclease activity of *Taq* polymerase can discriminate alleles. Cleavage results in increased emission of a reporter dye that otherwise is quenched by the dye minor groove binder (MGB). Each 5′ nuclease assay requires 2 unlabeled PCR primers and 2 allele-specific probes. Each probe is labeled with a reporter dye (VIC [a proprietory dye from Applied Biosystems] and 6-carboxyfluorescein [FAM]) at the 5′ end and MGB at the 3′ end. Amplification by PCR preceded using TaqMan Universal Master Mix (PE Biosystems) in a 25-μl reaction volume containing 50 ng of DNA, 700 nM primer, and 100 nM probe final concentrations. Thermal cycling conditions consisted of 95°C for 10 minutes and then 40 cycles of 92°C for 15 s and 60°C for 1 minute in a GeneAmp 9700 system. Fluorescence levels of the PCR products were measured using an ABI PRISM 7700 Sequence Detector (Applied Biosystems), which resulted in 3 genotypes of 2 alleles being clearly identified. We analyzed *cis* elements in the 5′ upstream region of the gene using AliBaba2 software that was obtained from the Biological Databases web site (http://www.gene-regulation.com/pub/programs.html#alibaba2).

Linkage Disequilibrium Analysis

SNPAlyze version 3.2 (DYNACOM Co, Ltd) was used for determining haplotype and linkage disequilibrium (LD) analyses and is available online at http://www.dynacom.co.jp/products/package/snpalyze/index.html (Figure 1b).

Preparation of Plasmids Including the G-Type or A-Type DNA Fragment

G-type and A-type rs1394205 reporter constructs were made. Because it has been reported that the −250-bp upstream region of this gene has a core promoter activity, 2 oligonucleotide primers, 5′-CCTTAGGTCAGGGTGTAAGAAACCC-3′ (bases −202 to −226) and 5′-GGCCATAATTATGCATCCATCCACC-3′ (bases +6 to −19), were designed (Figure 1c).[8] These primers contain the *Kpn*I and *Hin*dIII restriction sites, respectively. After digestion with *Kpn*I and *Hin*dIII, PCR products were subcloned into the *Kpn*I and *Hin*dIII sites of the luciferase reporter gene vector pGV-B2 (Tokyo Ink). The G-type and A-type constructs were verified by sequencing.

Measurement of Transcriptional Activities of G-Type and A-Type Alleles

For transfection of Chinese hamster ovary (CHO) cells with FSHR gene promoter constructs, cells (60% to 70% confluent) were preincubated in OptiMEM medium (Lipofectamine, Gibco BRL) for 30 minutes at 37°C. FSHR gene promoter plasmids (1 μg) and a plasmid containing TK-driven pRL (Toyo Ink, 200 ng, used to normalize for transfection efficiency) were mixed with liposome suspension (Lipofectamine,

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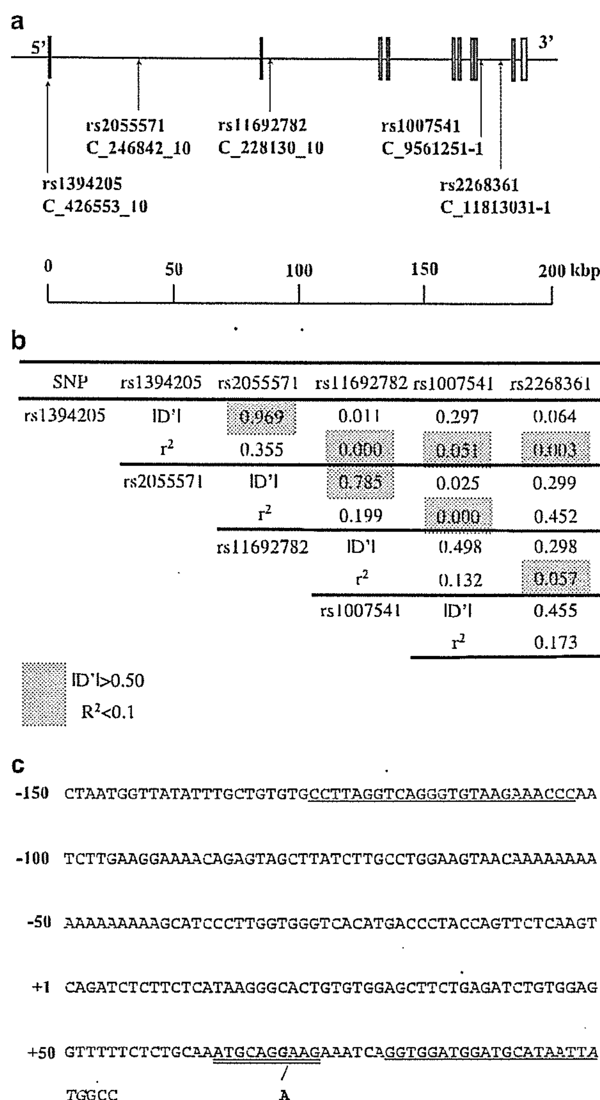

Figure 1. a, Organization of the human FSH receptor gene and location of the SNPs used for the present case–control study. ▩, exons, and —, indicate introns. b, Pairwise LD in the FSH receptor gene, as evaluated by D′ and r^2. Pairwise LD among the 6 marker pairs studied in the FSH receptor gene were computed, and pairs in LD (D′>0.5 or r^2<0.1) are shown as gray-shaded values. c, Nucleotide sequence of the 5′ upstream region of the FSH receptor gene. →, the major transcriptional initiation site, and the first nucleotide upstream of the transcriptional initiation site is designated −1. The G/A in bold type indicates the SNP of rs1394205. The italicized ATG indicates the start codon. Underlined nucleotide sequences indicate the PCR primers for making the insert of the plasmid that was used to measure transcriptional activities. The double underline indicates the *cis* element of the transcription factor Ets-1.

Gibco BRL, 6 μl/well) and incubated for 20 minutes at room temperature. The lipid-coated DNA was then added to each well containing 0.8 mL of OptiMEM media. After 3 hours, the medium was removed and replaced with complete medium for an additional 48 hours. CHO cells were then lysed (400 μL), and extracts were centrifuged to remove intact cells and debris. Extracts (50 μL) were used for measurement of luciferase activity. Luciferase activity was measured ≥3 times in duplicate with a double luciferase assay system (PicaGene Dual SeaPansy, Toyo Ink,) and a luminometer (LB-9507, Berthold). All of the data were normalized as relative light units/pRL-TK activity.[17]

Serum Levels of Estradiol

We measured serum levels of estradiol in female EH patients. Seventeen subjects in the female EH group were selected randomly. All of the subjects were confirmed to be postmenopausal by their own testimony. Six patients had the A/A genotype, and 12 patients had the G/A or G/G genotypes. Serum estradiol levels were measured with the Electro Chemil Luminescence Immunoassay (Elecsys Systems Immunoassay, Roche Diagnostics), as reported previously.[18]

Statistical Analysis

Data are shown as mean±SD. All of the statistical analyses were conducted using StatView 5.0 (SAS Inc) and Dr. SPSS II (SPSS Inc). Hardy–Weinberg equilibrium was assessed by a χ^2 analysis. The overall distributions of the genotypes or alleles were analyzed by χ^2 analysis using 2×3 or 2×2 contingency tables between EH patients and NT controls. To assess the quantitative effects of covariates, multiple logistic regression analysis was performed using SPSS II. Statistical significance was established at $P<0.05$.

Results

First Case–Control Study

Table 1 shows the distribution of genotypic and allelic frequencies of the 5 SNPs in each group. The observed and expected genotypic frequencies of each of the SNPs in the total subjects, male and female, of the NT group were in good agreement with the predicted Hardy–Weinberg equilibrium values (data not shown). The overall distribution of genotype and allele of all 5 of the SNPs did not significantly differ between men for the EH and NT groups. However, among women, the genotypic and allelic frequency of rs1394205 and the allelic frequency of rs2055571 showed a significant difference between the 2 groups. For the total subjects (male and female) the genotypic and allelic frequency of rs1394205 and the genotypic frequency of rs2268361 were significantly different between the EH and NT subjects. These data indicate that the distribution of rs1394205 in women can affect the overall analysis in the total subject group. Therefore, this could be used as a genetic marker of EH in women, because the probability value for the allelic distribution of this SNP was very significant ($P=0.003$).

Patterns of LD in the FSHR gene are illustrated by their D′ and r^2 values (Figure 1b). LD analysis for the NT group and the D′ values indicate that rs1394205 and rs2055571 are located in one haplotype block, whereas the other 3 SNPs are located in another block. Because the r^2 values were beyond 0.1 in rs1394205 to rs2055571, it is not advantageous to use these combinations to isolate susceptibility haplotypes.

Based on these results, rs1394205 was selected for the large-size case–control study. We were also interested in the fact that the rs1394205 may influence the transcriptional activity of the gene, because the SNP is located in the 5′ upstream region.

Confirmative Case–Control Study: Subgroup Collaboration Study With the Hypertensive Section of the Japanese Millennium Project

Table 2 shows the clinical features of the EH patients and NT controls. These 2 groups were age-matched for the total, male, and female groups. We performed the confirmative case–control study for the rs1394205 SNP using 1035 EH patients and 1058 NT controls.

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TABLE 1. Genotype Distribution in NTs and Patients With EH of First Screening Analysis

	Total Subjects			Male Subjects			Female Subjects		
Parameters	NT	EH	*P*	NT	EH	*P*	NT	EH	*P*
No. of participants	235	237		152	158		83	79	
Variants									
rs1394205									
Genotype									
GG	64 (0.272)	45 (0.190)		34 (0.224)	30 (0.190)		30 (0.361)	15 (0.190)	
GA	123 (0.524)	125 (0.527)		81 (0.533)	84 (0.532)		42 (0.506)	41 (0.519)	
AA	48 (0.204)	67 (0.283)	0.040*	37 (0.243)	44 (0.278)	0.673	11 (0.133)	23 (0.291)	0.010*
Allele									
G	251 (0.534)	215 (0.454)		149 (0.490)	144 (0.456)		102 (0.614)	71 (0.449)	
A	219 (0.466)	259 (0.546)	0.013*	155 (0.510)	172 (0.544)	0.391	64 (0.386)	87 (0.551)	0.003*
rs2055571									
Genotype									
TT	121 (0.515)	140 (0.591)		82 (0.540)	93 (0.589)		39 (0.470)	47 (0.594)	
TC	93 (0.396)	84 (0.354)		59 (0.388)	55 (0.348)		34 (0.410)	29 (0.367)	
CC	21 (0.089)	13 (0.055)	0.156	11 (0.072)	10 (0.063)	0.683	10 (0.120)	3 (0.038)	0.090
Allele									
T	335 (0.713)	364 (0.768)		223 (0.734)	241 (0.763)		112 (0.675)	123 (0.778)	
C	135 (0.287)	110 (0.232)	0.053	81 (0.266)	75 (0.237)	0.404	54 (0.325)	35 (0.222)	0.036*
rs11692782									
Genotype									
AA	54 (0.23)	63 (0.266)		33 (0.217)	39 (0.247)		21 (0.253)	24 (0.304)	
AT	121 (0.515)	108 (0.456)		77 (0.507)	74 (0.468)		44 (0.530)	34 (0.430)	
TT	60 (0.255)	66 (0.278)	0.426	42 (0.276)	45 (0.285)	0.761	18 (0.217)	21 (0.266)	0.446
Allele									
A	229 (0.487)	234 (0.494)		143 (0.470)	152 (0.481)		86 (0.518)	82 (0.519)	
T	241 (0.513)	240 (0.506)	0.843	161 (0.530)	164 (0.519)	0.791	80 (0.482)	76 (0.481)	0.987
rs1007541									
Genotype									
GG	100 (0.426)	80 (0.338)		59 (0.388)	53 (0.335)		41 (0.494)	27 (0.342)	
GA	104 (0.442)	122 (0.514)		72 (0.474)	84 (0.532)		32 (0.386)	38 (0.481)	
AA	31 (0.132)	35 (0.148)	0.143	21 (0.138)	21 (0.133)	0.569	10 (0.120)	14 (0.177)	0.138
Allele									
G	304 (0.647)	282 (0.595)		190 (0.625)	190 (0.601)		114 (0.687)	92 (0.582)	
A	166 (0.353)	192 (0.405)	0.101	114 (0.375)	126 (0.399)	0.544	52 (0.313)	66 (0.418)	0.051
rs2268361									
Genotype									
GG	48 (0.204)	53 (0.224)		30 (0.197)	34 (0.215)		18 (0.217)	19 (0.241)	
GA	102 (0.434)	123 (0.519)		67 (0.441)	82 (0.519)		35 (0.422)	41 (0.518)	
AA	85 (0.362)	61 (0.257)	0.046*	55 (0.362)	42 (0.266)	0.184	30 (0.361)	19 (0.241)	0.238
Allele									
G	198 (0.421)	229 (0.483)		127 (0.418)	150 (0.475)		71 (0.428)	79 (0.500)	
A	272 (0.579)	245 (0.517)	0.056	177 (0.582)	166 (0.525)	0.154	95 (0.572)	79 (0.500)	0.192

*Significant difference in distribution.

The observed and expected genotypic frequencies of each of the SNPs in the total subjects (P=0.553), males (P=0.853), and females (P=0.426) of the NT group were in good agreement with the predicted Hardy–Weinberg equilibrium values. The overall distribution for the alleles of the SNPs did not significantly differ between the total EH and total NT groups. However, among women, the allelic frequency of the A allele of rs1394205 was significantly higher for EH subjects than for NT subjects (P=0.042; Table 3). Although the genotype distribution of rs1394205 among women in the first case–control study showed

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TABLE 2. Characteristics of Study Participants of Subgroup Analysis of the Hypertension Team in Japanese Millennium Project

	Total			Male			Female		
Parameters	NT	EH	*P*	NT	EH	*P*	NT	EH	*P*
No. of subjects	1058	1035		792	762		266	273	
Age, y	60.4±7.9	59.7±9.5	0.052	59.6±7.3	59.1±8.7	0.269	63.0±9.1	61.3±11.1	0.051
BMI, kg/m^2	22.4±2.7	23.5±2.8	<0.001*	22.5±2.8	23.4±2.5	<0.001*	22.2±2.7	23.8±3.6	<0.001*
SBP, mm Hg	114.1±10.2	161.5±22.3	<0.001*	114.6±10.4	160.8±21.3	<0.001*	111.4±8.6	164.0±25.2	<0.001*
DBP, mm Hg	70.2±7.0	97.1±14.4	<0.001*	70.6±6.9	97.2±14.0	<0.001*	68.1±7.2	96.8±15.9	<0.001*
Total cholesterol, mg/dL	203.3±36.0	204.2±34.0	0.609	198.4±34.3	198.8±32.2	0.818	215.7±37.1	218.5±34.7	0.364
Triglyceride, mg/dL	116.5±74.2	143.7±97.9	<0.001*	117.4±7.6	146.3±102.2	<0.001*	114.3±67.7	135.2±72.7	0.002*
HDL cholesterol, mg/dL	56.8±14.8	58.4±16.5	0.043*	55.7±14.6	57.7±16.4	0.022*	59.5±15.0	60.0±16.6	0.736
Uric acid, mg/dL	5.3±2.6	5.7±2.4	0.004*	5.6±1.3	6.1±2.7	<0.001*	4.8±4.3	4.8±1.1	0.962
Creatinine, mg/dL	0.81±0.19	0.83±0.27	0.106	0.86±0.18	0.89±0.29	0.015*	0.71±0.17	0.69±0.17	0.372
γ-GTP	35.4±39.7	52.0±71.7	<0.001*	44.4±46.5	68.1±87.1	<0.001*	25.8±27.9	29.5±29.8	0.190
Smoking, %	45	46.6	0.583	66.6	62.1	0.182	4.6	7.3	0.271
Alcohol consumption, %	49.1	43.2	0.045*	62.9	74.3	0.001*	25.2	17.1	0.053

BMI indicates body mass index.
*Significant difference in distribution.

a significant difference, the genotype distribution in the confirmative study did not exhibit any significant difference. This discrepancy could have been caused by differences in sample size.

Multiple logistic regression analysis in the female subjects revealed that the significant association of A/A and G/A with EH ($P=0.033$) remained after adjustment for confounding factors (such as age, body mass index, total cholesterol, triglyceride, high-density lipoprotein cholesterol, uric acid, and creatinine), and the calculated odds ratio was 1.68 (95% CI: 1.04 to 2.73).

Transcriptional Activities

To study transcriptional activity, we transfected CHO cells with promoter constructs of the FSHR gene. The promoter activity of the A allele was 56±8% (mean±SD) of that for the G-type allele ($P=0.001$; Figure 2). The rs1394205 is located in the Ets-1 site, and when there is substitution of the A-type allele from the G-type allele, the Ets-1 site vanishes (Figure 1c).

Serum Levels of Estradiol

All of the values of the serum levels of estradiol were <32 pg/mL, because all of the subjects were postmenopausal. The values of G/G, G/A, and A/A genotypes were 19.8±2.1, 20.5±6.2, and 13.3±3.2, respectively. Because there was no difference between the G/G and G/A genotypes ($P=0.807$), Figure 3 only shows the serum levels of estradiol in postmenopausal EH patients with and without the A/A genotype. Serum estradiol levels were significantly lower in patients with the A/A genotype than in patients without the A/A genotype ($P=0.004$).

Discussion

We performed 2 genetic case–control studies in different populations for the FSHR gene and found that the SNP in the

TABLE 3. Genotype Distribution in NTs and Patients With Essential Hypertension of Subgroup Analysis of Hypertension in Japanese Millennium Project

	Total Subjects			Male			Female		
Parameters	NT	EH	*P*	NT	EH	*P*	NT	EH	*P*
No. of participants	1058	1035		792	762		266	273	
Variants									
rs1394205									
Genotype									
GG	297 (0.281)	253 (0.244)		212 (0.268)	184 (0.242)		85 (0.320)	69 (0.253)	
GA	518 (0.489)	531 (0.513)		393 (0.496)	400 (0.525)		125 (0.470)	131 (0.480)	
AA	243 (0.230)	251 (0.243)	0.169	187 (0.236)	178 (0.233)	0.431	56 (0.210)	73 (0.267)	0.139
Allele									
G	1112 (0.526)	1037 (0.501)		817 (0.516)	768 (0.504)		295 (0.555)	269 (0.493)	
A	1004 (0.474)	1033 (0.499)	0.112	767 (0.484)	756 (0.496)	0.509	237 (0.445)	277 (0.507)	0.04*

*Significant difference in distribution.

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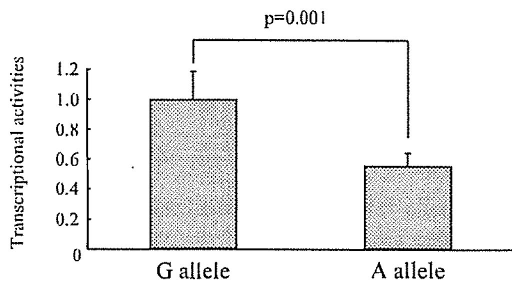

Figure 2. Transcriptional activities of the reporter constructs. The activities of the G-type constructs were considered as 100%. Values are expressed as the average of 3 independent experiments done on different days. Data are mean±SD.

5′-untranslated region of the FSHR gene affects levels of transcriptional activity and is a susceptibility mutation of EH in women. Very recently it has been reported that knockout mice can be used as a model of postmenopausal hypertension with low levels of estrogen and high levels of testosterone.[14] This report is consistent with our results that indicate that there is both an association between the polymorphism in the FSHR gene and EH and that the levels of plasma estradiol in female EH patients with the A/A genotype are significantly lower than those in female EH patients without the genotype. Furthermore, because Ets-1 has been reported to be involved in the regulation of angiogenesis and cell growth,[19,20] our findings suggest that the substitution of the A-type allele from the G-type allele of the rs1394205 leads to a decreased transcriptional activity, which is followed by a subsequent loss of the Ets-1 site. Because there have been many studies reporting that low estrogen levels are associated with hypertension in postmenopausal women,[21,22] our results also may provide evidence on the etiology of ≥1 of the causes of hypertension in postmenopausal women.

In genetic case–control studies, pseudopositive results can sometimes occur. Therefore, to improve reliability, we increased the number of samples in addition to selecting samples strictly for the purpose of removing bias associated with confounding factors. It is especially advantageous to perform case–control studies using large and different populations, because this can lead to results that have a greater likelihood of being correct. The strategy of our study was to first carry out a screening case–control study followed by a confirmative case–control study that included >1000 subjects in each group. The rs1394205 showed significant differences for both case–control studies. It should be noted that the *P* values for the associations determined in the present study are somewhat weak. However, in accordance with the guidelines issued for specific criteria for acceptability of association study results, the current study provides biological support (transient transfection finding) and plausibility based on knockout data in mice.[23] Therefore, the larger *P* value could be considered to be acceptable in this case.

Some case–control studies have identified gene variants associated with gender-specific susceptibility to EH[24,25]; however, in general, such studies do not explain the reason for the positive gender-specific association findings. Our experiment also found significant differences only for women. In contrast to previous reports, our experiment specifically examined whether the functional mutations of the gene were clearly identified in patients with a hereditary disease in a gender-specific manner. Mutations in the FSHR gene are reported to cause hereditary hypergonadotropic ovarian failure in women but not in men. To the best of our knowledge, our study is the first to report that the causal genes of gender-specific hereditary diseases are associated with EH in a gender-specific manner.

An increasing number of reports of genome-wide scans for hypertension and blood pressure variation have been seen in the past few years.[26] Some of the reports have shown that 2p, on which the FSHR gene is located, is associated with the candidate loci for EH.[27–32] However, these regions are broad, and there has been no definite susceptibility gene identified in this region. Although like our study in which the strategy for identification of susceptibility genes of EH was different between the genome-wide scans and case–control studies, the final goal for identifying the causal mutation for EH was the same. Therefore, because our current findings indicate that a gene variant is related to EH, our results are thought to be worthwhile. In conclusion, an SNP in the 5′-untranslated region of the FSHR gene affected the levels of transcriptional activity and, therefore, is a susceptibility mutation of EH in women.

Perspectives

We performed 2-step case–control studies and identified that the SNP in the 5′-untranslated region of the FSHR gene affects levels of transcriptional activity, which is a susceptibility mutation of EH in women. However, there are some limitations associated with our study. It is possible that the case–control studies sometimes exhibit pseudopositive results because of sample scales or selection of the genetic markers. Further research is required to perform familial linkage studies and transmission disequilibrium tests for the purpose of confirming the reliability of the rs1394205 SNP.

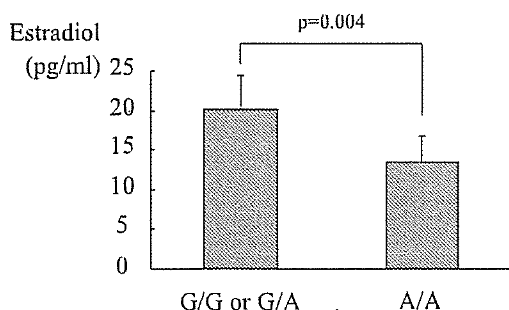

Figure 3. Serum levels of estradiol. Data are mean±SD.

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Disclosures

None.

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ARTICLE

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

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Keywords: association study; CIAS1; hypertension; VNTR polymorphis

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 O^{2-} 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 product[26] 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 autoinflammatory syndrome 1*) gene mapped 1q44[19] 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)-κB and caspase. Thus far, many missense mutations in the *CIAS1* gene have been identified in hereditary systemic autoinflammatory 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.

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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 μl of 1 × PCR buffer containing 20 ng of the genomic DNA, 0.4 μM of a set of primer (forward:5′-CTGACCTCC-CAATGTGCCTT-3′ and reverse:5′-CAGAGCTTCTTCA-GATTGCA-3′), 0.2 mM concentrations of each dNTP, and 2.5 U *Taq* DNA polymerase (Roche Dignostics, 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 homozyzotes of major repeats 12, 16 heterozygozes of major and minor repeats (9, 7, and 6 repeats), and nine homozygtes 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 μl) into cDNA using the SuperScript II (Life Technologies, Gaithersburg, MD) according to the manufacture's protocol. RT product (0.2 μl) was used for quantification of gene expression by TaqMan real-time RT-PCR (Applied Biosystems, Forster City, CA, USA) in a reaction volume of 5 μl. 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 *Nhe*I- and *Nco*I-tailed primers (GATTACCATGGGCTAGCAATGTGCCTTGTG, and AG-CACCCATGGTGGCGGCCACAGCGCCCCA), 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 *Xba*I site to mimic the splicing acceptor position. Ten micrograms of the constructs were transfected into 5 × 10^6 HL60 cells with 0.2 μg 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±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 extract. 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

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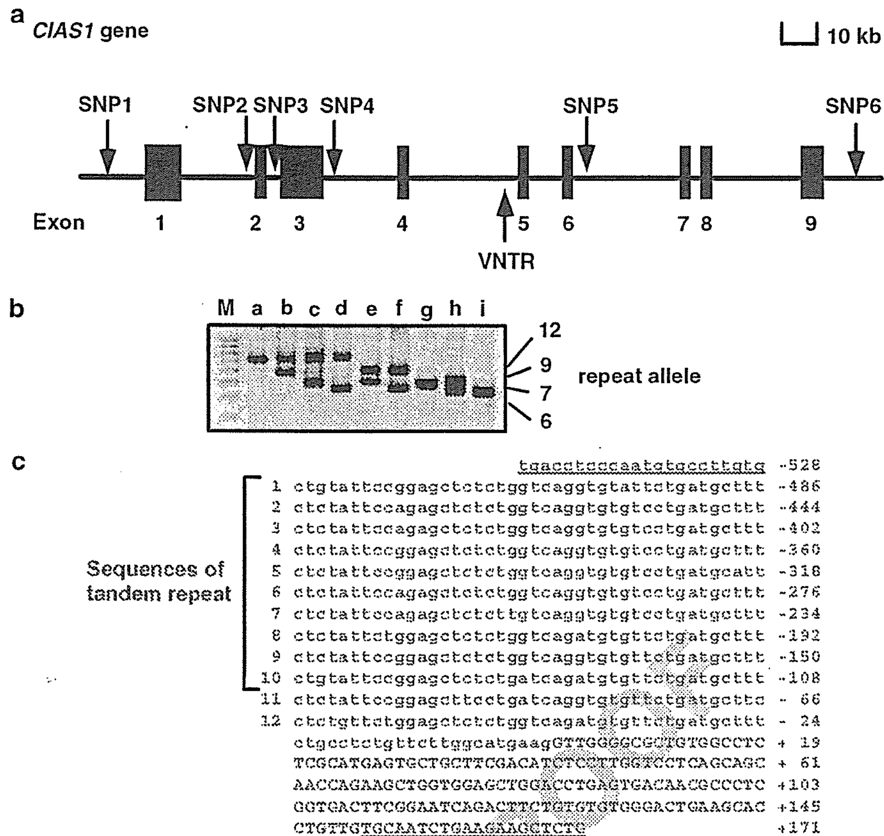

Figure 1 (a) Schema of *CIAS1* gene with position of six SNPs and an identified VNTR polymorphisms for the genetic association study. Four SNPs were selected from the IMS-JST (Institute of Medical Science-Japan Science and Technology Agency) Japanese SNP database (IMS-JST): SNP1 (IMS-JST084196), SNP2 (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* 42bp-VNTR is in tight linkage with other polymorphisms, six SNPs were chosen from the *CIAS1* gene using a public database (JSNP, http://

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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*
Patients							
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
Controls							
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)

*$P<0.05$.
[a]Major allele is 12 repeat and minor alleles are 9, 7 and 6 repeat.

Table 2 Frequency of the major haploype 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.

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

	SNP1	*SNP2*	*SNP3*	*SNP4*	*VNTR*	*SNP5*	*SNP6*
SNP1							
SNP2 $\lvert D'\rvert$	0.940						
r^2	0.286						
SNP3 $\lvert D'\rvert$	0.940	1.000					
r^2	0.286	1.000					
SNP4 $\lvert D'\rvert$	0.912	1.000	1.000				
r^2	0.765	0.351	0.351				
VNTR $\lvert D'\rvert$	0.715	0.145	0.145	0.841			
r^2	0.142	0.082	0.082	0.214			
SNP5 $\lvert D'\rvert$	0.294	0.405	0.405	0.348	0.185		
r^2	0.014	0.082	0.082	0.021	0.020		
SNP6 $\lvert D'\rvert$	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	

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

Case–control study of the *CIAS1 42 bp-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* 42 bp-VNTR was performed in panel 2 (987 cases and 924 controls). The results confirmed the statistical significance between the *CIAS1* 42 bp-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

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

Panel 2	*Controls*	*Patients*	χ^2	*P*	*Odds*
Genotype distribution					
12-12	278 (30.1%)	343 (34.7%)			
12-X	466 (50.4%)	498 (50.5%)			
X-X	180 (19.5%)	146 (14.8%)			
Allele frequency					
12	0.553	0.600			
X	0.447	0.400			
Genotype comparison					
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

		Genotype		
Panel 3 (Men and Women)	*12-12*	*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-Cho, total cholesterol; HDL-Cho, HDL cholesterol; TG, triglyceride, FBS, Fasting blood sugar; and HbA1c, glycohemoglobin, A-1-C.
*12-12 *vs* 12-X+X-X.

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frequency of the 12-12 subjects was 0.348 and 0.301 in panel 2. The most significant association was shown in the 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* 42 bp-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* 42 bp-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

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

Panel 3 (Men)	*Genotype* 12-12	12-X	X-X	*P*
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	167.9.1±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

Panel 3 (Women)	*Genotype* 12-12	12-X	X-X	*P*
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.

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bone marrow, brain, fetal liver heart, kidney, lung, 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 was the significant difference on 30 min ($P=0.01$) and 60 min ($P=0.0001$) after LPS stimulation. These results

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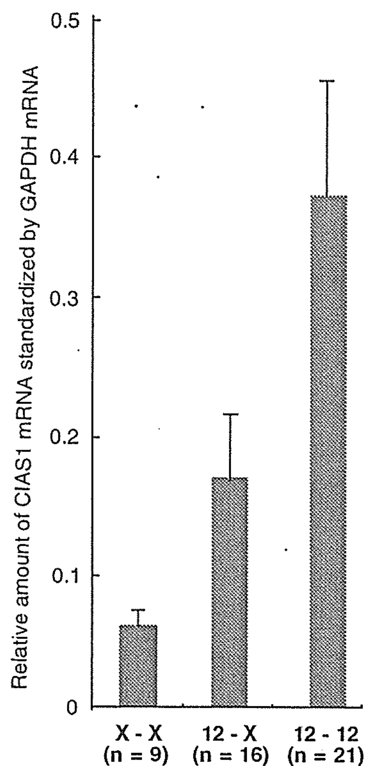

Figure 2 The CIAS1 mRNA levels in peripheral leukocytes among the three genotype groups of 12-12 (homozygote of 12 repeat alleles), 12-X (heterozygote of 12 and non-12 repeat alleles), and X-X (homozygote of non-12 repeat alleles) in *CIAS1* 42 bp-VNTR. The CIAS1 expression was analyzed by real-time 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 ($P = 0.009$).

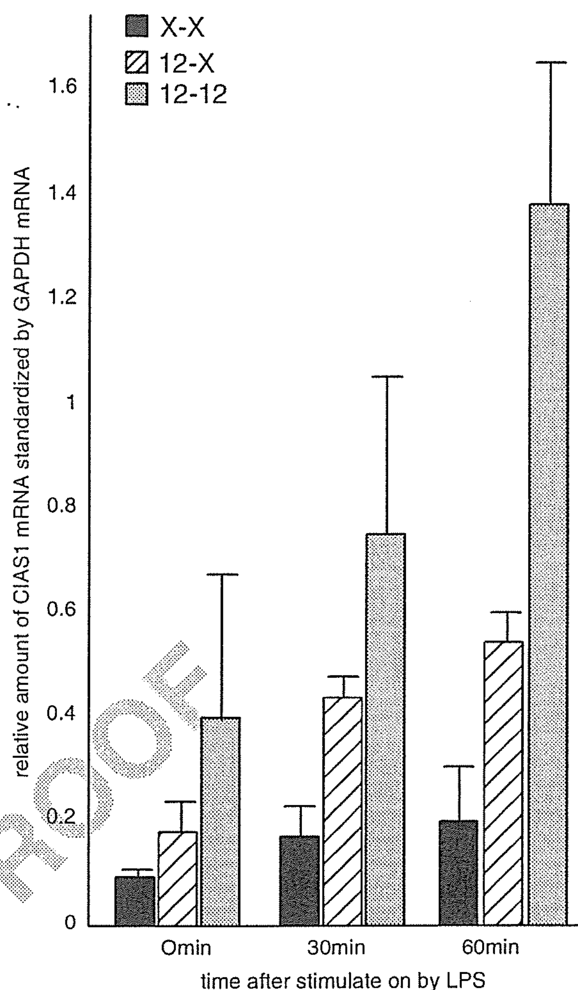

Figure 3 The effect of stimulation of LPS on CIAS1 mRNA levels in monocytes in different genotypes. Monocytes were isolated from peripheral leukocytes by Ficoll centrifugation followed by adhesion on a plastic dish. Cells were treated with 200 ng/ml LPS for 30 or 60 min and lysed. The relative amount 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.

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* 42 bp-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.

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* 42 bp-VNTR experienced hypertension more frequently than controls, with a significant association ($P = 0.006$). Furthermore, the

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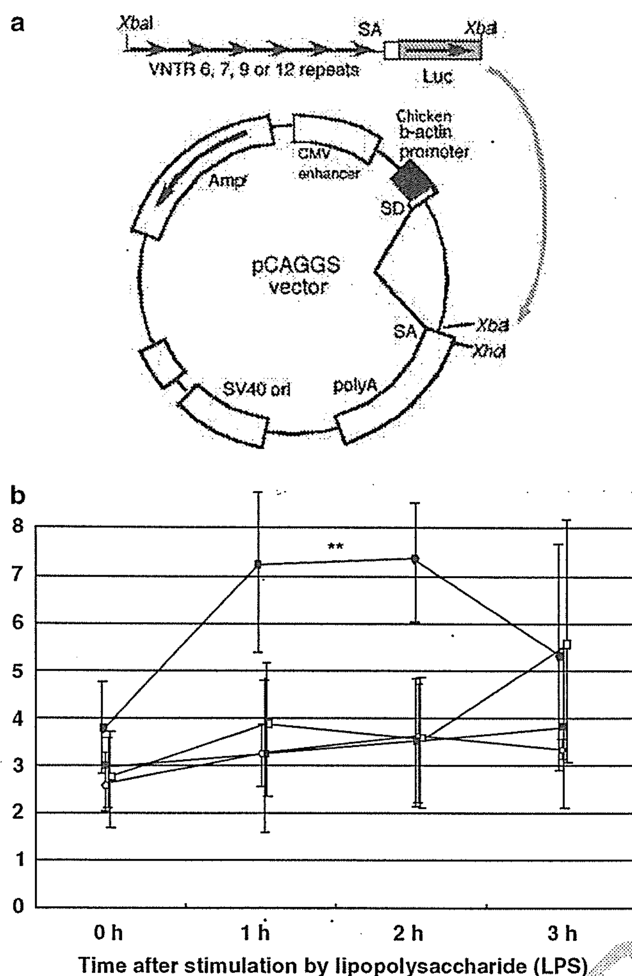

Figure 4 Functional assay of VNTR in HL60 cells. (a) The genomic region including the VNTR repeats, 12 (720 bp), 9 (594 bp), 7 (510 bp), and 6 (468 bp), was inserted in pGL3 vector. The VNTR-luciferase inserts were directionally subcloned in the PCAGGS vector. (b) HL60 cells were co-transfected with 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 (-●-) vector 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* 42 bp-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* 42 bp-VNTR showed that the 12 repeat units increased the expression of luciferase in the *CIAS1* gene. These results may indicate that the *CIAS1* 42 bp-VNTR directly affects gene expression. Thus, we believe that increased expression of *CIAS1* in leukocytes dependent on the *CIAS1* 42 bp-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 Leucin-rich repeats (LRRs). A large number of nucleotide substitutions have been found in the NACHT domain[19] in patients with autosomal-dominant autoinflammatory disorders; Muckle-Wells syndrome, familial cold ulticaria, 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-1β into matured IL-1β. Moreover, ASC and Cardinal in the inflammasome have been shown to be associated with IKK (IκB kinase) complex and activate NF-κB. 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-κB activation have been reported from overexpression analysis of CIAS1 in culture cell lines. NF-κB 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 Coas1-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* repots that bacteria RNA and small antiviral compounds activate caspase 1 through cryopyrin producted from CIAS1 gene.[31] We propose here the *CIAS1* is associated with hypertension through the dominant expression of transcripts, which may depend on the

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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 NFκB 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-κB is increased in numerous tissues in stroke-porne spontaneous hypertension (SHRsp) rats compared with Sprague-Dawley (SD) rats.[34] NF-κB promotes proinflammatory genes or induces apoptosis in many organs. In leukocytes, NF-κB 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 oxdative stress activates NF-κB.[36,37] Thus, we predict that CIAS1 might play an important role in the development of hypertension based on inflammation brought on by NF-κB activation.

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