Table 2. Polymorphism genotypic frequencies in nonobese subjects and overweight or obese subjects

	Genotype			χ^2 Test results in nonobese v overweight or obese subjects	
				For 3 genotypes	For alleles
Arg16Gly of β2-adrenoceptor gene	Arg16/Arg16	Arg16/Gly16	Gly16/Gly16	$\chi^2 = 8.82$, $P = .012$	$\chi^2 = 7.87$, $P = .005$
Nonobese subjects, $n = 206$	77 (37.4%)	88 (42.7%)	41 (19.9%)		
Overweight or obese subjects, $n = 123$	27 (22.0%)	62 (50.4%)	34 (27.6%)		
Total subjects, $n = 329$	104 (31.6%)	150 (45.6%)	75 (22.8%)		
Gln27Glu of β2-adrenoceptor gene	Gln27/Gln27	Gln27/Glu27	Glu27/Glu27	—	$\chi^2 = 5.18$, $P = .023$
Nonobese subjects, $n = 206$	192 (93.2%)	14 (6.8%)	0 (0.0%)		
Overweight or obese subjects, $n = 123$	104 (84.6%)	19 (15.4%)	0 (0.0%)		
Total subjects, $n = 329$	296 (90.0%)	33 (10.0%)	0 (0.0%)		

Values in parentheses are percentage of subjects. Nonobese subjects, body mass index (BMI)<25 kg/m^2; overweight or obese subjects, BMI≥25 kg/m^2.

Table 3. Characteristics of subjects according to Gly16 allele

Variable	All subjects		Nonobese subjects		Overweight or obese subjects	
	With Gly16 allele	Without Gly16 allele	With Gly16 allele	Without Gly16 allele	With Gly16 allele	Without Gly16 allele
Subjects (n)	225	104	129	77	96	27
Age (y)	37 ± 7	36 ± 6	37 ± 6	36 ± 6	37 ± 8	35 ± 7
BMI (kg/m^2)	25.0 ± 2.2	23.8 ± 2.9	22.7 ± 1.8	22.5 ± 2.1	28.0 ± 2.3†	27.6 ± 2.8†
Total body fat mass (kg)	18.4 ± 3.7	13.9 ± 4.0‡	13.3 ± 2.1	11.2 ± 2.0‡	25.2 ± 4.5†	21.9 ± 4.3†‡
Waist-to-hip ratio	1.00 ± 0.09	0.91 ± 0.09‡	0.91 ± 0.10	0.85 ± 0.09‡	1.12 ± 0.10*	1.07 ± 0.11†‡
Systolic BP (mm Hg)	131 ± 5	129 ± 5	127 ± 5	127 ± 5	134 ± 5*	135 ± 4*
Diastolic BP (mm Hg)	79 ± 7	75 ± 7	76 ± 6	73 ± 7	82 ± 7*	81 ± 8*
Mean BP (mm Hg)	96 ± 6	93 ± 7	93 ± 6	91 ± 6	100 ± 6*	99 ± 7*
Heart rates (beats/min)	71 ± 6	67 ± 7	68 ± 6	65 ± 6	74 ± 7*	73 ± 8*
Norepinephrine (pmol/mL)	1.60 ± 0.57	1.38 ± 0.61	1.33 ± 0.45	1.11 ± 0.38‡	1.97 ± 0.64†	2.12 ± 0.65†
Leptin (ng/mL)	5.5 ± 2.6	4.2 ± 2.0‡	5.1 ± 2.4	3.9 ± 1.8§	6.1 ± 2.5†	5.4 ± 2.0†‡

Abbreviations as in Table 1.

Data are mean ± SD.

* $P < .05$; † $P < .01$ v values in nonobese subjects; ‡ $P < .05$; § $P < .01$ v values in subjects with Gly16 allele.

Table 4. Characteristics of subjects according to Glu27 allele

Variable	All subjects		Nonobese subjects		Overweight or obese subjects	
	With Glu27	Without Glu27	With Glu27 allele	Without Glu27 allele	With Glu27 allele	Without Glu27 allele
Subjects (*n*)	33	296	14	192	19	104
Age (y)	37 ± 7	37 ± 6	37 ± 7	37 ± 6	37 ± 7	37 ± 8
BMI (kg/m^2)	26.4 ± 3.8	24.4 ± 2.7	23.5 ± 2.1	22.5 ± 1.7	28.5 ± 3.4†	27.8 ± 2.3†
Total body fat mass (kg)	23.1 ± 3.7	16.6 ± 5.1§	13.9 ± 1.3	12.3 ± 2.0‡	29.8 ± 4.1†	24.4 ± 4.5†§
Waist-to-hip ratio	1.07 ± 0.11	0.95 ± 0.10‡	0.92 ± 0.11	0.88 ± 0.09	1.18 ± 0.10†	1.09 ± 0.09†‡
Systolic BP (mm Hg)	133 ± 5	128 ± 6	132 ± 5	126 ± 5	134 ± 5	133 ± 7*
Diastolic BP (mm Hg)	79 ± 7	76 ± 8	77 ± 6	74 ± 5	81 ± 7	81 ± 8*
Mean BP (mm Hg)	97 ± 7	95 ± 6	95 ± 5	93 ± 5	99 ± 7	98 ± 8*
Heart rates (beats/min)	75 ± 8	69 ± 6	69 ± 5	67 ± 6	79 ± 9*	73 ± 7*‡
Norepinephrine (pmol/mL)	1.79 ± 0.58	1.48 ± 0.69	1.30 ± 0.14	1.19 ± 0.43‡	2.15 ± 0.64†	2.02 ± 0.75†
Leptin (ng/mL)	7.5 ± 2.5	4.8 ± 2.9‡	6.9 ± 2.4	4.6 ± 2.0‡	7.9 ± 2.2*	5.4 ± 2.7*§

Abbreviations as in Table 1.
Data are mean ± SD.
* $P < .05$; † $P < .01$ *v* values in nonobese subjects; ‡ $P < .05$; § $P < .01$ *v* values in subjects with Glu27 allele.

Variables Relating to Obesity and BP Levels

When we used multiple linear regression analysis using total body fat mass as a dependent variable, plasma NE ($P = .023$), leptin ($P < .001$), waist-to-hip ratio ($P = .009$), BMI ($P < .001$), and mean BP ($P = .037$) were significant determinant variables ($R^2 = 0.60$; $R = 39.22$; $P < .001$) in all subjects. Plasma NE ($P = .001$), total body fat mass ($P = .031$), waist-to-hip ratio ($P = .048$), but not plasma leptin ($P = .347$), were significant determinant variables for mean BP ($R^2 = 0.19$; $R = 4.77$; $P = .002$).

Discussion

The main findings in the present study show that obese normotensive subjects have higher frequencies of the Gly16 and Glu27 alleles of the β_2-adrenoceptor gene, and the subjects who carry these polymorphisms have greater total body fat mass and waist-to-hip ratios (abdominal obesity) associated with higher levels of plasma leptin. These observations demonstrate that the Gly16 and Glu27 allele of the β_2-adrenoceptor genes are related to abdominal obesity and higher leptin levels. Further, the subjects carrying the Gly16 and Glu27 allele have significantly lower slopes in linear regression between plasma leptin and NE levels. As we have shown in previous studies, a heightened sympathetic nerve activity (high mean plasma NE) and high mean plasma leptin are present in obesity.[4,5,15,16] Now we show that the blunted sympathetic nerve activity response in obesity as observed in plasma NE to high plasma leptin offers further evidence for selective leptin resistance contributing to the mechanism of obesity.

Heightened sympathetic nerve activity[2–7] and high plasma leptin levels[4,5,10,15] are well established observations in obesity. Leptin is an adipocyte-derived hormone that promotes weight loss by reducing appetite and food intake and by increasing energy expenditure through sympathetic stimulation to brown adipose tissue.[9,11] Leptin resistance defined as impaired action of leptin, accompanied by hyperleptinemia is a common feature of obesity in animal models and in human obesity.[23] Therefore, hyperleptinemia has been used as a surrogate index of leptin resistance to the adipose tissue mass.[24] Experimental results show that chronic systemic and intracerebral administration of leptin increases BP in animal models.[13,23] Leptin produces sympathetic activation to kidneys, brown adipose tissue, and adrenal glands, indicating that the obesity-associated increase in sympathetic nerve activity could be due in part to these various sympathetic effects of leptin.[10–13] Thus, several investigators have reported that leptin resistance occurs heterogeneously within the sympathetic nerve activity, and have speculated that hyperleptinemia could contribute to the control of BP through renal sympathetically mediated mechanisms.[3] In other words, leptin resistance is selective to the metabolic effects, but

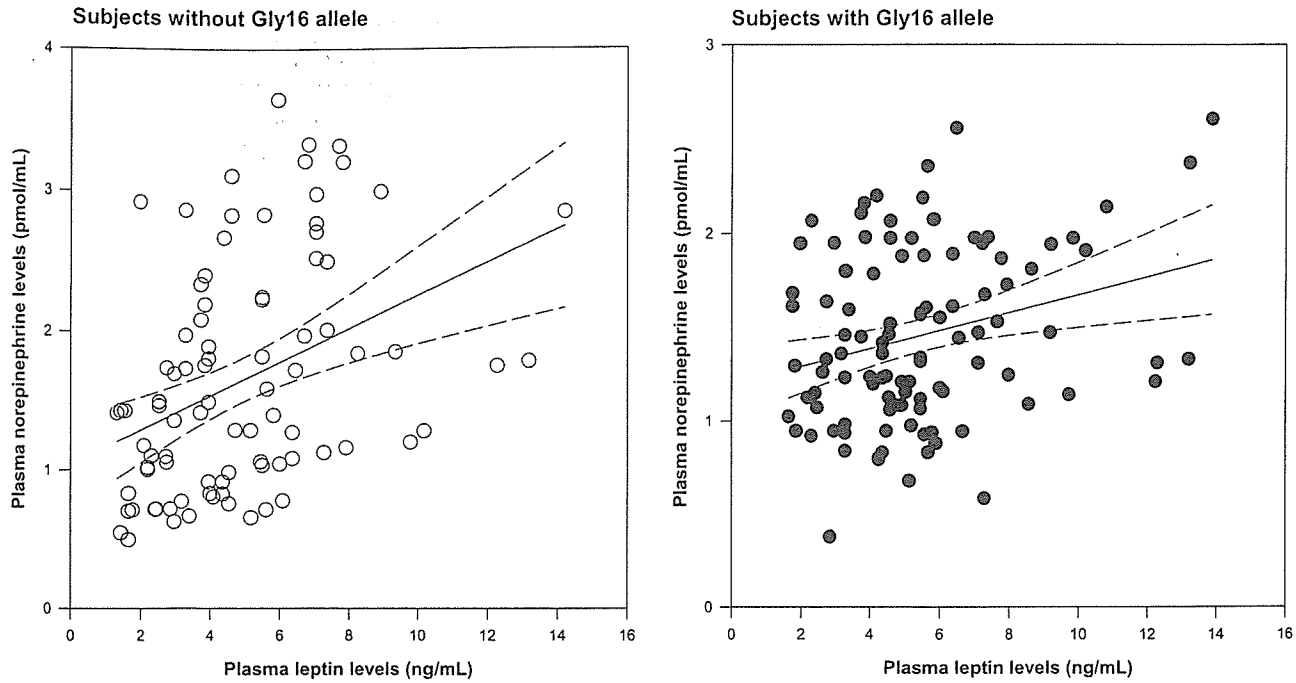

FIG. 1. Correlations between plasma leptin levels and plasma norepinephrine levels in subjects without Gly16 allele (**left panel**) and subjects with Gly16 allele (**right panel**). Dotted lines show 95% confidence interval for the linear regression slope.

sparing leptin-mediated sympathetic activation.[25] Subjects carrying the Gly16 or Glu27 allele had significantly higher level of plasma leptin and lower slopes using linear regression between plasma leptin and NE levels. This demonstrates that the subjects carrying the Gly16 or Glu27 have leptin resistance and blunted leptin-mediated sympathetic activity. In addition, in multiple regression analyses, mean BP is mostly determined by plasma NE, but not leptin, whereas both plasma NE and leptin levels in our cohort determined total body fat mass and waist-to-hip ratio (abdominal obesity). The findings show that sympathetic nerve activity as determined by plasma NE, but not leptin levels, play an important role in BP control, but that both sympathetic nerve activity and leptin levels contribute

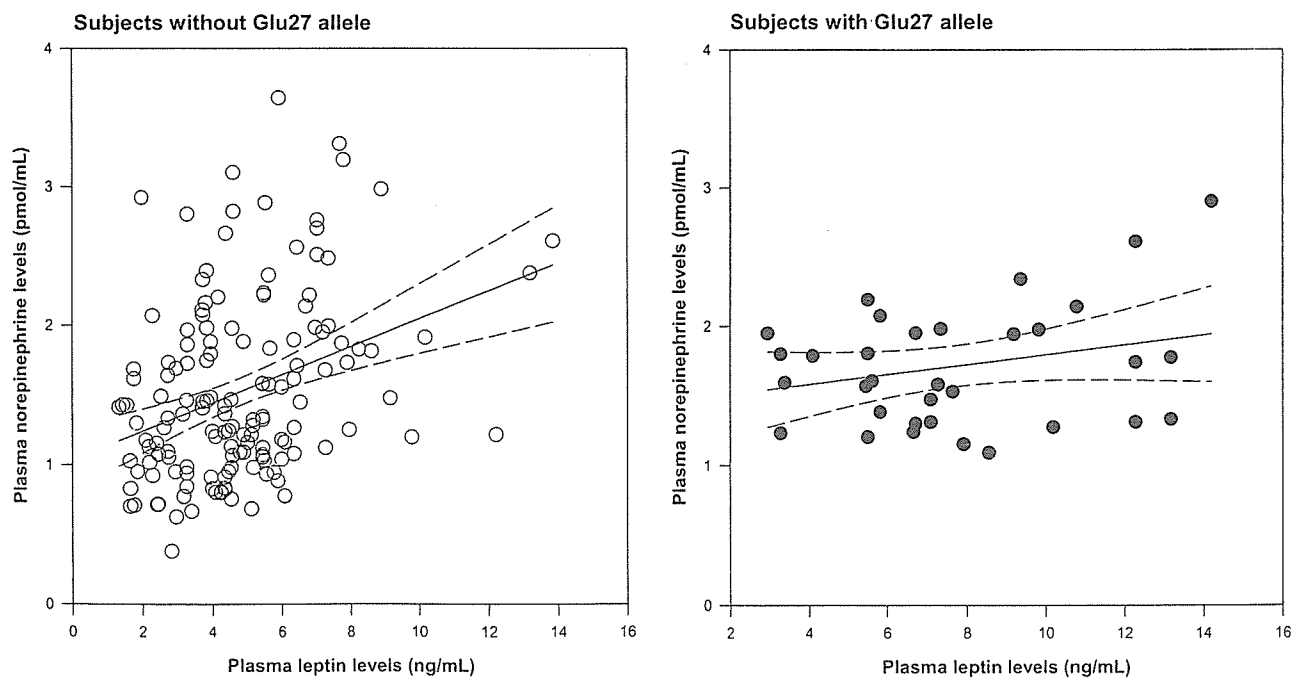

FIG. 2. Correlations between plasma leptin levels and plasma norepinephrine levels in subjects without Glu27 allele (**left panel**) and subjects with Glu27 allele (**right panel**). Dotted lines show 95% confidence interval for the linear regression slope.

to abdominal obesity. Sympathetic nerve activity dictated by plasma leptin levels and in part determined by the β_2-adrenoceptor polymorphisms might be a determinant factor for BP levels and abdominal obesity in the normotensive Japanese cohort. This observation might be important for understanding the mechanisms of obesity-related hypertension, and may help to explain why not all obese subjects have hypertension.

In the present study, we used plasma NE levels as an index of SNA. Tuck[26] and Grassi et al[27,28] reported that there are different results in SNA values in hypertensive patients depending on the method of SNA measurement including: regional spillover, muscle sympathetic nerve activity (microneurography), and plasma NE measurements. Spillover methods and microneurography are considered the gold standard for SNA measurements, but in human beings these are difficult especially in a population study such as the present study with invasive measurements. Furthermore, different values have been reported for regional sympathetic nerve activity between the kidneys and heart,[3,29] and between arms and legs.[30] Plasma NE levels are more practical for large populations,[4–6,16] but represent several different processes (secretion, clearance and reuptake of NE) making it difficult to determine whether the defect is overproduction or decreased metabolism.[29] Thus, the strong correlations between plasma leptin and NE levels in multiple regression analysis indicates a close relationship between leptin–sympathetic nerve activity axis and NE processes.

The Gly16 allele of Arg16Gly contributes lower peripheral vasodilation after infusion of a β-blocking agent.[31] The Gly16 substitution exaggerates agonist-mediated receptor down-regulation.[32,33] Rasmussen et al reported that elevated levels of less responsive β_2-adrenoceptor protein in adipose tissue may contribute to the development of obesity.[34] Thus, we could speculate the β_2-adrenoceptor polymorphisms might contribute to blunted β_2-adrenoceptor function as well as to the relatively lower plasma NE (blunted sympathetic activity) by leptin stimulation and resultant obesity.

The findings represent original observations of the effects of genetic variation on the β_2-adrenoceptor gene on the relationships between obesity, BP levels, plasma leptin levels, and plasma NE levels as an index of sympathetic nerve activity in a large cohort with a wide range of body weight. The Gly16 and Glu27 alleles of the β_2-adrenoceptor polymorphisms are associated with abdominal obesity through blunted leptin-mediated sympathetic nerve activity. The present study was a cross-sectional design, but it should be noted that body weight and BP levels in the subjects were stable over the year preceding measurements, so we could observe the variables during the maintenance of obesity and BP levels (stable body weight and BP levels). Furthermore, only normotensive men were studied to exclude the influence of hypertension and gender on body weight, body fat distributions, sympathetic nerve activity, and leptin. We have previously reported that subjects with the Gly16 or Glu27 allele of the β_2-adrenoceptor polymorphisms in association with a heightened sympathetic nerve activity could predict the future onset of obesity in originally nonobese, normotensive subjects in a longitudinal study of 5 years.[16] Taken together, the β_2-adrenoceptor polymorphisms might relate to both the onset and maintenance of obesity through abnormal leptin-mediated sympathetic stimulation.

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Original Article

Genetic Influences of β-Adrenoceptor Polymorphisms on Arterial Functional Changes and Cardiac Remodeling in Hypertensive Patients

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Three subtypes of β-adrenoceptor, β_1, β_2 and β_3, are involved in the sympathetic nervous system, which plays an important role in the development of hypertension and hypertensive complications. These complications can include left ventricular hypertrophy and arterial stiffness, which are reported risk factors for cardiovascular diseases. We designed clinical trials to clarify the association between hypertensive complications and β-adrenoceptor single nucleotide polymorphisms in essential hypertension. Using Taqman PCR methods, we detected five polymorphisms of three β-adrenoceptors: Ser49Gly and Arg389Gly for the β_1-adrenoceptor; Gly16Arg and Glu27Gln for the β_2-adrenoceptor; and Trp64Arg for the β_3-adrenoceptor. We included 300 subjects and measured pulse wave velocity, vasodilator response to hyperemia, left ventricular hypertrophy (by electrocardiogram and echocardiography), and cardiac enlargement (by chest X-ray). We found that pulse wave velocity and nitroglycerin-induced hyperemia were both closely associated with the Ser49Gly polymorphism ($p<0.05$), and Glu27Gln was found by both electrocardiogram and echocardiography to be significantly associated with left ventricular hypertrophy ($p<0.05$). These data suggested that two polymorphisms of different β-adrenoreceptor subtypes are the genetic influences on the development of arterial stiffness and left ventricular hypertrophy in essential hypertension. (*Hypertens Res* 2006; 29: 875–881)

Key Words: **β-adrenoreceptor, polymorphisms, hypertensive complications, arterial stiffness**

Introduction

The human β-adrenoceptor (βADR) is a member of the family of seven-transmembrane G-protein–coupled receptors, encoded by a gene on chromosome 5 (*1*). Previous studies have shown that sympathetic nervous activity *via* βADRs regulates numerous physiological events and modulates a wide range of physiological responses, including cardiac chronotropy and inotropy, vascular and smooth muscle tone, carbohydrate and lipid metabolism (*2*). Sympathetic nervous activity plays an important role in the development of hyper-

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Table 1. Probes and Primers for β-Adrenoceptor Polymorphism

		Probe	Primer
β_1	Ser49Gly	FAM CCAGCGAAAGCCCCGAGCC	Forward GTCGCCGCCCGCCTCGTT
		VIC CCAGCGAAGGCCCCGAGC	Reverse CCATGCCCGCTGTCCACTGCT
	Arg389Gly	FAM AGGCCTTCCAGGGACTGCTCTGCT	Forward GGCCTTCAACCCCATCATCTA
		VIC AGGCCTTCCAGCGACTGCTCTGC	Reverse CCGGTCTCCGTGGGTCGCGT
β_2	Gly16Arg	FAM CGCATGGCTTCTATTGGGTGC	Forward GGAACGGCAGCGCCTTCT
		VIC CGCATGGCTTCCATTGGGTGC	Reverse CAGGACGATGAGAGACATGACGAT
	Glu27Gln	FAM CTCGTCCCTTTGGCTGCGTGACGT	Forward GGAACGGCAGCGCCTTCT
		VIC CTCGTCCCTTTCCCTGCGTGACGT	Reverse CAGGACGATGAGAGACATGACGAT
β_3	Trp64Arg	FAM TCTCGGAGTCCAGGCGATGGCCA	Forward GGAGGCAACCTGCTGGTCAT
		VIC CTCGGAGTCCGGGCGATGGCC	Reverse CACGAACACGTTGGTCATGGT

In each probe, the polymorphic nucleotide is underlined.

tension and its complications (*3*). Three isotypes of human βADRs, β_1, β_2, and β_3, are involved in this system. The classical subdivision of βADRs defines β_1 as the subtype that stimulates cardiac muscle (*4*) and β_2 as the subtype that relaxes smooth muscle (*5*). The expression of the β_3 subtype is essentially limited to adipose tissue (*6*).

Genetic polymorphisms may influence the development of disease states (*7*). Diseases such as hypertension could be based on genetic disorders as well as environmental factors, or may occur as a secondary product of either cardiac events such as left ventricular hypertrophy (LVH) and arterial stiffness, or interactions among them (*8*). LVH has been considered to be an intermediate phenotype of hypertensive heart diseases (*9*). Recently arterial stiffness was suggested to be an independent predictor of the presence of coronary artery disease (*10*), and arterial stiffness was associated with LVH in hemodialysis patients (*11*). Therefore, arterial stiffness was considered to be an intermediate phenotype of hypertensive complications. A number of single nucleotide polymorphisms (SNPs) of βADR subtypes have recently been reported to be positional candidate genes for cardiovascular diseases (*12*, *13*). For instance, the genotype of the Arg389Gly polymorphism in the human β_1-adrenoceptor (β_1ADR) gene is reported to be associated with acute myocardial infarction (*14*) and hypertensive status (*15*), an association study (*16*) suggested that the gene encoding the β_2-adrenoceptor (β_2ADR) was associated with essential hypertension; and the Arg64 allele of the β_3-adrenoceptor (β_3ADR) gene is associated with obesity-related phenotypes (*17*), insulin resistance, hypertension, coronary artery disease and earlier age of onset of diabetes (*18*). The essential effects of the three βADRs, however, need to be further clarified.

The aim of the present hospital-based observational study was to investigate potential genetic relationships between the βADR SNPs that result in amino acid substitutions and the cardiovascular risk factors of hypertension, such as LVH and aortic stiffness. We focused on five representative β-adrenoceptor SNPs in patients with essential hypertension.

Methods

Study Population

The present clinical study was designed as a hospital-based and cross-sectional study. We identified 331 Osaka University Medical Hospital patients with essential hypertension. Hypertension was defined as systolic blood pressure (SBP) of more than 140 mmHg and/or diastolic blood pressure (DBP) of more than 90 mmHg and/or administration of antihypertensive drugs. Twelve patients were excluded because of atrial fibrillation and/or frequent ventricular premature beats (more than 5% of the day), and we excluded nine patients whose echocardiography could not be evaluated. Four patients refused to undergo venepuncture. We also excluded six patients in whom we failed to detect the five target SNPs. Finally, 300 patients with essential hypertension for whom the target SNPs could be detected were enrolled. We diagnosed essential hypertension as defined above. The protocol of this study was approved by the hospital ethics committee (permission number: 80) and written informed consent was obtained from all participants. For this study, 115 patients not receiving antihypertensive drug treatment were included. The remaining 185 patients were treated with one or more antihypertensive drugs as follows: 128 patients with a calcium antagonist, 62 patients with an angiotensin receptor blocker, 47 patients with an angiotensin-converting enzyme (ACE) inhibitor, 37 patients with a β-blocker, 25 patients with a diuretic and 10 patients with an α-blocker.

Genotyping

Total genomic DNA was extracted from leukocytes obtained from samples of whole blood, following the standard techniques. In this study, the TaqMan PCR assay as described previously (*19*) was used to perform polymorphisms analysis of the three βADRs, β_1, β_2 and β_3 (*20*). We detected two poly-

Table 2. Patients' Characteristics and β-Adrenoceptor Polymorphism

	Total	β_1					
		Ser49Gly			Arg389Gly		
		Ser/Ser	Ser/Gly	Gly/Gly	Arg/Arg	Arg/Gly	Gly/Gly
Number	300	194	96	10	213	80	7
Male/female	164/136	105/89	56/40	3/7	123/90	38/42	3/4
Age (years old)	62.0±0.7	61.8±0.9	62.3±1.1	65.3±3.0	61.9±0.8	62.0±1.2	66.4±6.9
BMI (kg/m^2)	24.2±0.2	24.2±0.2	24.1±0.4	25.7±2.3	24.2±0.2	24.4±0.3	24.5±0.3
Hyperlipidemia (%)	51.5	50.5	52.6	60.0	51.9	51.3	42.9
Diabetes (%)	21.7	17.5	27.1	50.0	23.5	17.5	14.3%
SBP (mmHg)	141.4±1.1	140.9±1.3	142.4±2.0	141.7±4.1	141.6±1.3	140.9±1.9	140.7±6.6
DBP (mmHg)	83.4±0.7	83.6±0.8	83.3±1.3	79.3±2.2	83.3±0.8	83.7±1.3	80.7±3.7
HR (bpm)	67.8±0.7	67.9±0.9	67.8±1.1	64.3±4.3	67.1±0.8	69.1±1.2	72.4±7.1
TC (mmol/l)	5.3±0.1	5.3±0.1	5.2±0.1	5.0±0.2	5.3±0.1	5.3±0.1	4.8±0.3
HDL-C (mmol/l)	1.4±0.03	1.4±0.04	1.4±0.05	1.5±0.13	1.4±0.03	1.5±0.06	1.5±0.25
FBG (mmol/l)	6.0±0.1	5.9±0.1	6.0±0.2	6.4±0.6	6.1±0.1	5.9±0.2	5.7±0.3

β_2					β_3		
Gly16Arg			Glu27Gln		Trp64Arg		
Gly/Gly	Gly/Arg	Arg/Arg	Gln/Gln	Glu/Gln	Trp/Trp	Trp/Arg	Arg/Arg
74	166	60	260	40	228	69	3
44/30	86/80	34/26	146/114	18/22	129/99	32/37	3/0
62.0±1.3	62.3±0.9	61.5±1.9	62.3±0.6	63.8±1.1	62.8±0.6	61.6±1.6	58.3±0.8
24.1±0.4	24.3±0.2	24.3±0.4	24.2±0.2	24.6±0.5	24.5±0.2	23.5±0.3	26.3±0.4*
51.4	52.7	48.3	49.4	65.0	53.3	47.8	0
20.3	21.7	23.3	21.5	22.5	22.8	18.8	0
137.9±1.8	141.4±1.5	145.9±2.8*	140.7±0.9	139.9±2.7	140.5±1.0	141.4±2.0	124.7±12.7
82.3±1.7	83.4±0.9	84.7±1.6	83.0±0.6	82.7±1.5	82.8±0.6	83.7±1.2	74.3±14.3
66.5±1.4	68.0±0.9	68.5±1.4	68.2±0.7	68.9±1.5	68.3±0.7	68.2±1.1	64.5±2.5
5.3±0.12	5.2±0.07	5.3±0.11	5.3±0.05	5.5±0.10	5.3±0.05	5.3±0.08	4.9±0.30
1.5±0.05	1.4±0.04	1.4±0.06	1.5±0.02	1.6±0.06	1.5±0.02	1.5±0.04	1.5±0.21
6.2±0.3	6.1±0.1	5.7±0.1	5.9±0.8	6.2±0.3	6.0±0.8	6.0±0.2	5.7±0.1

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose. *$p<0.05$ *vs.* other genotype.

morphisms of β_1ADR that result in serine/glycine (Ser49Gly) and arginine/glycine (Arg389Gly) amino acid substitutions at residues 49 and 389, respectively. We also detected two polymorphisms of β_2ADR that result in glycine/arginine (Gly16Arg) and glutamate/glutamine (Glu27Gln) amino acid substitutions at residues 16 and 27, respectively. Finally, we identified a polymorphism in the β_3ADR that results in a tryptophan/arginine (Trp64Arg) amino acid substitution at residue 64. The primers and probes for the five representative SNPs are shown in Table 1.

Arterial Functional Changes

We non-invasively evaluated pulse wave velocity (PWV) and vasodilator response to hyperemia as arterial functional changes. PWV was measured using a model FCP-4731 (Fukuda Denshi Co., Tokyo, Japan), which allowed on-line pulse wave recording and automatic calculation as previously reported (*21*, *22*), and the details of this procedure have been reported previously (*23*). The intra-observer coefficient of variation was 2.8±1.2%. We measured the post-ischemic vasodilator response to reactive hyperemia by strain-gauge plethysmography (EC5R; DE Horkkanson, Inc., Bellevue, USA), using a previously published protocol (*24*). To calculate the reactive hyperemia/baseline value of forearm blood flow, we used the reactive hyperemia ratio, and we used the nitroglycerin (NTG)-induced hyperemia ratio as the NTG-induced hyperemia/baseline value. The intra-observer coefficient of variation was 3.4±1.4%.

Cardiac Remodeling

To evaluate cardiac remodeling, we estimated LVH by electrocardiography and echocardiography, and also estimated cardiac enlargement using the cardio-thoracic-ratio (CTR) obtained from chest X-rays. Electrocardiographic LVH

Table 3. β-Adrenoceptor Genotypes and Arterial Functional Changes/Cardiac Remodeling

		PWV	Hyperemia		ECGLVH	LVMI	CTR
		(m/s)	Reactive	NTG	(mm)	(g/m²)	(%)
β_1	Ser49Ser	9.1±0.1	1.60±0.05	1.03±0.05	3.0±0.1	132±2.8	49.6±0.4
	Ser49Gly	9.0±0.2	1.55±0.08	0.92±0.03	2.9±0.1	134±3.7	49.0±0.6
	Gly49Gly	10.5±0.4*	1.32±0.12	0.74±0.06*	2.5±0.3	144±1.7	57.8±2.5**
	Arg389Arg	9.1±0.1	1.59±0.05	0.98±0.04	2.9±0.1	115±7.8	49.3±0.4
	Arg389Gly	9.2±0.1	1.54±0.08	1.00±0.08	2.9±0.1	126±3.8	50.5±0.5
	Gly389Gly	9.3±0.5	1.51±0.29	0.79±0.10	3.2±0.2	134±7.9	49.8±1.0
β_2	Gly16Gly	9.0±0.2	1.67±0.09	0.92±0.04	3.0±0.1	132±4.1	50.1±0.9
	Gly16Arg	9.2±0.1	1.55±0.06	0.99±0.05	2.9±0.1	131±3.2	49.7±0.4
	Arg16Arg	9.0±0.2	1.57±0.09	1.03±0.10	3.0±0.1	133±5.4	49.2±0.6
	Gln27Gln	9.3±0.2	1.54±0.04*	1.00±0.04	2.9±0.1*	129±2.4**	49.5±0.4
	Glu27Gln	9.1±0.1	1.82±0.16	0.87±0.04	3.2±0.2	147±7.0	50.5±1.0
β_3	Trp64Trp	9.2±0.1	1.56±0.05	0.95±0.03	2.9±0.1	135±2.6	50.2±4.9
	Trp64Arg	8.9±0.2	1.66±0.09	1.08±0.11	2.9±0.1	127±5.0	48.9±0.6
	Arg64Arg	8.6±0.8	1.57±0.34	1.02±0.02	2.8±0.4	158±4.8	49.9±0.4

PWV, pulse wave velocity; reactive, reactive hyperemia; NTG, nitroglycerin-induced hyperemia; ECGLVH, electrocardiographic left ventricular hypertrophy; LVMI, left ventricular mass index; CTR, cardio-thoracic-ratio. *$p<0.05$ and **$p<0.01$ *vs.* other genotype.

(ECGLVH) was determined by Sokolow-Lyon's criteria as the depth of SV_1 plus RV_5 (mV) (*25*). Left ventricular mass index (LVMI) was calculated by echocardiography following Devereux's methods (*26*). Additionally, CTR from chest X-rays was calculated as the directly measured ratio of the width of the heart to the thorax width.

Statistical Analysis

Data were analyzed using JMP ver. 4 (SAS Inc., Cary, USA) and presented as the mean±SEM. ANOVA and Student's *t*-test were used to test for significant differences among the SNPs. A value of $p<0.05$ was regarded as statistically significant.

Results

Participant characteristics stratified by genotypes are presented in Table 2. With respect to the Gly16Arg β_2ADR polymorphism, SBP in patients with the Gly16Gly or Gly16Arg polymorphism was significantly lower than that of patients with the Arg16Arg polymorphism. With respect to the Trp64Arg β_3ADR polymorphism, the body mass index of patients with the Arg64Arg or Trp64Arg polymorphism was significantly higher than that of patients carrying other genotypes.

The relationship between βADR polymorphisms and arterial functional changes is shown in Table 3. We evaluated three different parameters for arterial changes, the carotid-femoral PWV as an index of aortic stiffness, reactive hyperemia as an index of post-ischemic vasodilatation to reactive hyperemia, and NTG-induced hyperemia as an index of endothelium-independent vasodilatation. PWV in patients with the Gly49Gly genotype of the β_1ADR polymorphism was significantly elevated compared to that of patients with other genotypes ($p<0.05$). In the patients with the Gly49Gly genotype, NTG-induced hyperemia was significantly lower compared to that of patients with other genotypes; however, reactive hyperemia showed no significant differences compared to the β_1ADR Ser49Gly polymorphism. Reactive hyperemia in patients with the Glu27Gln genotype of the β_2ADR polymorphism was significantly lower compared to that of patients with Gln27Gln genotype. Our results for the LVMI, ECGLVH and CTR measurements are also shown in Table 3. In patients with the Gly/Gly genotype of the β_1ADR Ser49Gly polymorphism, CTR was significantly higher compared to the CTRs of patients with other genotypes. In patients with the Glu27Gln genotype of the β_2ADR polymorphism, both ECGLVH and LVMI were significantly higher compared to these measures in patients with the Gln27Gln genotype.

To evaluate genetic cooperation between β_1ADR Ser49Gly polymorphism and β_2ADR Glu27Gln polymorphism, we analyzed the combined influences of these two polymorphisms on arterial and cardiac phenotypes (Table 4). Although the numbers of patients with Ser49Gly/Glu27Gln and Gly49Gly/Gln27Gln were very small ($n=7$), patients with Gly49Gly/Gln27Gln showed higher PWV and CTR, but lower LVMI compared with the other combined genotypes. Patients with Ser49Ser/Glu27Gln ($n=33$) showed a hyperreactive hyperemia ratio, which meant improved vasodilator response.

Moreover, we performed haplotype-analysis for the β_1ADR and β_2ADR polymorphisms (Table 5). Although the number of patients with Gly49Gly/Arg389Arg in the β_1ADR was

Table 4. Combination Analysis of β-Adrenoceptor Genotypes and Arterial Functional Changes/Cardiac Remodeling

	n	PWV (m/s)	Hyperemia		ECGLVH (mm)	LVMI (g/m^2)	CTR (%)
			Reactive	NTG			
Ser49Ser/Gln27Gln	180	9.1±0.1	1.54±0.05	1.01±0.05	3.0±0.1	133±3.3†	49.8±0.5#
Ser49Ser/Glu27Gln	33	9.2±0.3†	1.88±0.19*	0.84±0.05	3.1±0.2	130±4.9†	50.0±1.4#
Ser49Gly/Gln27Gln	73	9.1±0.2#	1.55±0.08	1.00±0.09	2.8±0.1	126±4.3†	48.8±0.7#
Ser49Gly/Glu27Gln	7	7.8±0.5*,#	1.51±0.17	1.02±0.13	3.3±0.4	169±20.7	50.2±0.9
Gly49Gly/Gln27Gln	7	10.5±0.5*	1.51±0.29	0.79±0.10	2.6±0.3	119±8.1†	56.9±2.9

PWV, pulse wave velocity; reactive, reactive hyperemia; NTG, nitroglycerin-induced hyperemia; ECGLVH, electrocardiographic left ventricular hypertrophy; LVMI, left ventricular mass index; CTR, cardio-thoracic-ratio. *$p<0.05$ and **$p<0.01$ *vs.* Ser49Ser/Gln27Gln; #$p<0.05$ *vs.* Gly49Gly/Gln27Gln; †$p<0.05$ *vs.* Ser49Gly/Glu27Gln.

Table 5. The Haplotype Analysis of β_1- and β_2-Adrenoceptor Polymorphism

		n	PWV (m/s)	Hyperemia		ECGLVH (mm)	LVMI (g/m^2)	CTR (%)
				Reactive	NTG			
β_1	Ser-Ser/Arg-Arg	150	9.1±0.2*	1.66±0.07	1.04±0.06	3.0±0.1	134±4.1	49.3±0.7**
	Ser-Gly/Arg-Arg	56	9.0±0.2*	1.50±0.08	1.02±0.11	2.9±0.1	129±5.0	48.7±0.9**
	Gly-Gly/Arg-Arg	7	10.5±0.5	1.51±0.29	0.79±0.10	2.6±0.3	119±8.1	56.9±2.9
	Ser-Ser/Arg-Gly	71	9.3±0.2*	1.52±0.08	0.91±0.03	2.9±0.1	132±4.3	50.7±0.7**
	Ser-Gly/Arg-Gly	18	9.1±0.4*	1.66±0.22	0.95±0.07	2.8±0.3	128±10.1	49.5±1.0**
	Gly-Gly/Arg-Gly	10	9.3±0.4	1.32±0.12	0.74±0.06#	3.2±0.3	129±14.7	49.8±0.8*
β_2	Gly-Gly/Gln-Gln	59	9.1±0.2	1.66±0.10	1.02±0.10	3.1±0.1†	137±6.7	50.2±1.1
	Gly-Arg/Gln-Gln	145	9.3±0.1	1.53±0.06†	1.01±0.05	2.9±0.1	130±3.2†	49.8±0.5
	Gly-Arg/Glu-Gln	21	8.9±0.4	1.68±0.16	0.84±0.05	2.9±0.3	125±6.9†	50.6±1.4
	Arg-Arg/Gln-Gln	55	9.0±0.2	1.44±0.07†	0.93±0.05	2.8±0.1†	126±5.3†	49.0±0.8
	Arg-Arg/Glu-Gln	19	9.0±0.4	1.98±0.29	0.89±0.07	3.4±0.2	153±8.9	49.3±2.0

PWV, pulse wave velocity; reactive, reactive hyperemia; NTG, nitroglycerin-induced hyperemia; ECGLVH, electrocardiographic left ventricular hypertrophy; LVMI, left ventricular mass index; CTR, cardio-thoracic-ratio. *$p<0.05$ and **$p<0.01$ *vs.* Gly-Gly/Arg-Arg; #$p<0.05$ *vs.* Ser-Ser/Arg-Gly; †$p<0.05$ *vs.* Arg-Arg/Glu-Gln.

very small ($n=7$), patients with this haplotype showed higher PWV and CTR compared with patients having the other haplotypes. Patients with the Arg16Arg/Glu27Gln haplotype in the β_2ADR showed higher reactive hyperemia and LVMI.

Discussion

In this hospital-based study, the frequencies of five SNPs of three βADRs in a patient population were determined. With respect to the two SNPs of β_1ADR SNPs, the data for the Ser49Gly SNPs were not significantly different from those in a report from Sweden (*27*), and Arg389Gly also exhibited the same percentage of SNPs as reported by a Japanese group (*14*). In addition, the previously reported frequencies of two β_2ADR SNPs, Gly16Arg and Glu27Gln, in a Japanese (*28*) and a Taiwanese (*29*) cohort were identical to the respective frequencies determined here. Finally, the frequency of Trp64Arg for the β_3ADR reflected the findings of another Japanese group (*30*). Based on the accordance of our findings with these previous studies, our results would seem to be an accurate evaluation of the frequencies of these five βADR SNPs.

The β_1ADR are positioned at the cell membrane of cardiomyocytes. Subjects with the Gly allele in the Ser49Gly polymorphism have been reported to have a lower heart rate (*20*), but in another study, the Ser49Gly β_1ADR polymorphisms did not seem to exert a major effect on the changes in heart rate and blood pressure of patients with essential hypertension during 12 weeks of treatment with atenolol (*31*). The C allele of β_1AR 1165C>G encodes arginine at amino acid 389, whereas the G allele encodes glycine. *In vitro* studies of isoproterenol stimulation showed that the Arg-389 receptors produce higher levels of adenylyl cyclase activity than the Gly-389 receptors (*32*), resulting in enhanced cardiac sensitivity to catecholamines. In another study, the Gly16 and Glu27 polymorphisms in the β_2ADR were associated with sympathetic overactivity, as reflected by high plasma norepinephrine levels (*33*). In a study by Eisenach *et al.*, dietary sodium restriction blunted the increase in nitric oxide-mediated, β_2ADR responsiveness in Gly16 homozygotes of the forearm following administration of normal dietary sodium, while baseline CO decreased and peripheral resistance were

increased by the sodium restriction (*34*). In male twins with highly similar genetic and environmental backgrounds, the Arg64 variant of the β_3ADR polymorphism was found to be associated with insulin resistance and higher post-prandial hyperglycemia (*35*).

In the previous studies, PWV was shown to be effective as an index of stiffened vessels and decreased compliance, which was strongly correlated with increased pulse pressure and aging, and NTG-induced hyperemia was considered to be a marker of endothelium-independent vasodilatation. This type of dilatation was considered to be medial smooth-muscle-cell–related vasodilatation. Previous reports have suggested that βADR mediates smooth muscle relaxation in the small resistance arteries and large conduit arteries (*36*). In the present study, we only found an association between a genetic polymorphism in Ser49Gly of the β_1ADR and the aortic stiffness as measured by PWV, and the Gly49Gly genotype showed a genetic association with NTG-induced hyperemia. Although functional analysis is required, Ser49Gly polymorphism of the β_1ADR might influence arterial functional changes.

We also performed a combined analysis of the various polymorphism and haplotype results. This analysis revealed several statistically significant parameters in regard to the intermediate phenotype; however, we were unable to reach a definitive conclusion about these findings due to the small number of subjects studied. Although the present study had several limitations, it nonetheless underscored the possibility of a relationship between βADR SNPs and intermediate phenotypes, such as functional arterial changes and cardiac remodeling. Clinically, these findings might be important when considering the relationship between autonomic nervous system responses and hypertensive complications in patients with essential hypertension.

Study Limitations

Our study was a hospital-based, cross-sectional research that included patients with essential hypertension and had several limitations. First, a large number of clinical studies will be required to analyze the relationship between polymorphisms and intermediate phenotypes, such as LVH and arterial stiffness. Moreover, for the haplotype and combined analyses, the number of subjects in this study was too small to provide definitive results, and the statistical power was low. Second, subjects showed heterogeneity of clinical background, which influences cardiovascular events, with some patients having taken medicines and some patients having been sick for a longer or shorter period of time. Third, to strictly evaluate the genetic influences of βADR genes, a cohort study would be much better. Fourth, as our study was a hospital-based study, there may have been a selection-bias. In general, it is better to use a population-based study for analyzing genetic influences, in order to avoid such a bias. Nonetheless, although there were several important limitations and although a larger, population-based cohort study might be required, the present study showed an association between βADR gene polymorphisms and arterial or cardiac remodeling in hypertensive patients.

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Genetics

Association Study Between C-Reactive Protein Genes and Ischemic Stroke in Japanese Subjects

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Background: C-reactive protein (CRP) is reported to be involved in the development of atherosclerosis. Elevated CRP levels are considered to be a predictor of ischemic stroke (IS) in elderly individuals. Some single-nucleotide polymorphisms (SNP) are reportedly associated with elevated CRP levels. The aims of this study were to genotype some of the SNP in the human CRP gene and to assess the association between the CRP gene and IS.

Methods: Japanese patients with IS (72.4 ± 8.2 years of age, n = 152) and elderly Japanese subjects without IS (78.0 ± 4.2 years of age, n = 304) were genotyped for four SNP of the human CRP gene: rs1341665, rs1800947, rs1130864, and rs1205. Each genotyping was performed using the TaqMan SNP genotyping assay. The haplotype-based association study was assessed with a permutation test.

Results: The genotype rs1800947 was statistically significant between patients with IS and control subjects (CC+GC versus GG variant, P = .016 by multiple logistic regression analysis). This analysis revealed that the CC+GC variant of rs1800947 was an independent risk factor of IS. All four SNP were located in one haplotype block. The haplotype was constructed using rs1341665, rs1800947, and rs1130864, in that order. There was a significant association between IS and the C-C-C haplotype (P = .015).

Conclusions: The rs1800947 SNP and the C-C-C haplotype in the CRP gene appear to be prognostic markers of ischemic stroke and this polymorphism could be a useful genetic marker. Am J Hypertens 2006;19: 593–600 © 2006 American Journal of Hypertension, Ltd.

Key Words: C-reactive protein, polymorphism, single-nucleotide polymorphism, haplotype, association study.

Ischemic cerebrovascular disease is known to be a multifactorial disorder. Various factors such as aging, hypertension,[1] diabetes mellitus,[2] hyperlipidemia,[3] low levels of plasma high-density lipoprotein,[4] and smoking[5] are reportedly established risk factors. Atherosclerosis is one of the major causes of ischemic stroke (IS) and is considered to be an inflammatory disease.[6] C-reactive protein (CRP), widely known to be an inflammatory marker, was detected in atherosclerotic plaques,[7] and was recently reported to be involved in the development of atherosclerosis.[8] Elevated plasma CRP levels may be an atherothrombotic biomarker that provides additive prognostic information about not only coronary heart disease[9] but also peripheral arterial disease, along with standard lipid measures.[10] Moreover elevated plasma CRP levels could be a marker that predicts the risk of IS in the elderly.[11]

The human CRP gene is located on chromosome 1q21 to 1q23, spanning approximately 1.9 kb and containing two exons. In the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP), 29 SNP were recorded (accessed October 20, 2004). Nonsense mutations in the coding region were not recorded and there was one silent mutation named rs1800947.[12] The SNP rs1130864 was reported to be associated with elevated basal CRP levels, whereas some SNP (ie, rs1205 and rs1800947) were reportedly associated with reduced levels.[13–16] The SNP rs2794521 has been associated with type 2 diabetes mellitus.[17] The SNP rs1205 has been

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associated with systemic lupus erythematosus (SLE) and antinuclear autoantibody production.[13] The SNP rs1130864 has been associated with coronary heart disease requiring surgical treatment with an artery bypass graft.[14] However there have been no reports assessing the relationship between the variants of the CRP gene and IS.

The aims of this study were to genotype some of the SNP in the human CRP gene in Japanese subjects and to assess the association between the CRP gene and IS.

Subjects and Methods

Subjects

The participants in whom IS was diagnosed were recruited at Nihon University Itabashi Hospital in Tokyo, Japan, and the other neighboring hospitals during a decade (1993 to 2003). We selected 152 patients with IS diagnosed by neurologic examination and the findings of computed tomography (CT) or magnetic resonance imaging (MRI) or both, and who had neurologic deficit ratings greater than grade 3 on the modified Rankin Scale.[18] Patients with hemorrhage stroke diagnosed by CT or MRI or both were excluded in this study group. Patient age ranged from 60 to 99 years (mean ± standard deviation [SD], 72.4 ± 8.2 years). Japanese subjects (n = 304) were enrolled as control subjects. Control subject age ranged from 66 to 94 years (mean ± SD, 78.0 ± 4.2 years). Because the mean age of the control group was older than that of the IS group, the control group was regarded as a super–control group.[19] They were members of the New Elder Citizen Movement in Japan who lived in Tokyo and the suburbs of Tokyo and who had vascular risk factors such as hypertension, hypercholesterolemia, or diabetes mellitus but no history of IS. They were confirmed as having grade 0 on the modified Rankin Scale. In this study group, participants with cancer or autoimmune disease, including antiphospholipid antibody syndrome, were excluded. The number of participants with a history of atrial fibrillation was 14 in the IS group. Patients with cerebral embolism caused by atrial fibrillation, diagnosed by anamnesis and the findings of electrocardiography, echocardiography, CT, or MRI were excluded. However patients in whom cerebral thrombosis was diagnosed were included. No participant had a history of peripheral arterial occlusive disease. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University.

Genotyping

According to the information on the NCBI SNP database or the Applied Biosystems–Celera Discovery System (ABI-CDS, http://www.appliedbiosystems.com) database, the SNP that had a minor allele frequency >18% or those established by previous articles were chosen. We selected four SNP in the human CRP gene for determination of the genetic association between patients with IS and control subjects. In the present experiment, four SNP were named SNP1 to SNP4 in order of their position from the 5′ end of this gene. The SNP named SNP1, located in the 5′ flanking region, was rs1341665. SNP2, located in the intron 2 region, was

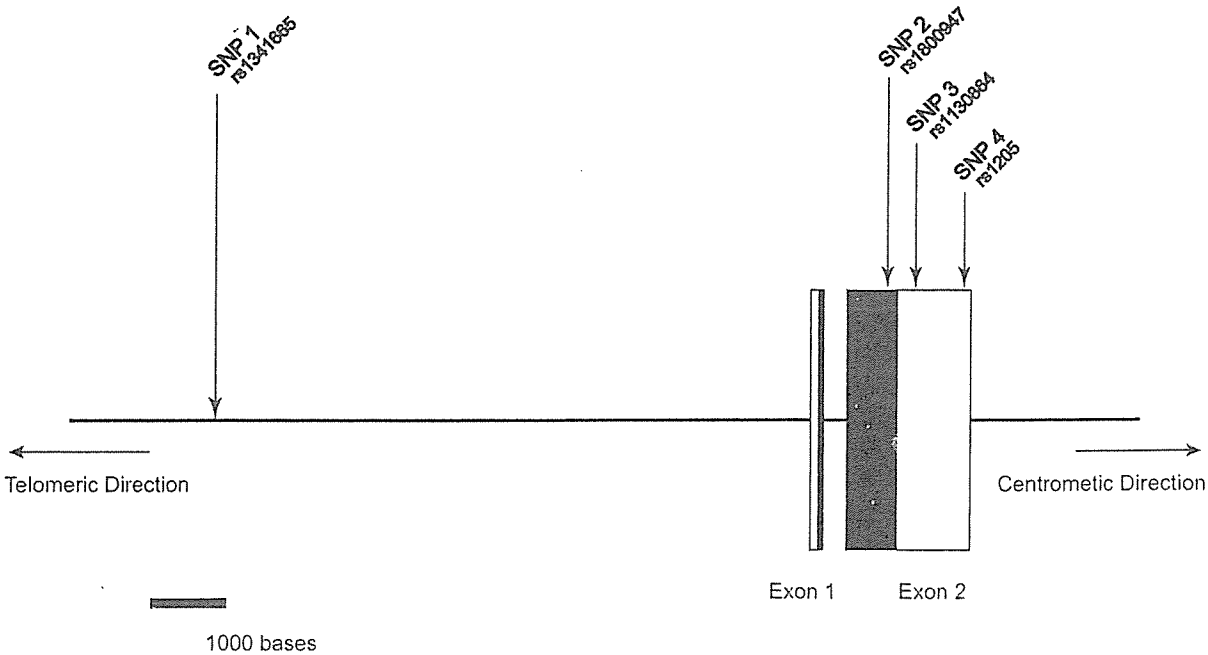

FIG. 1. Structure of the human C-reactive protein (CRP) gene. This gene consists of two exons separated by a single intron. The orientation of this gene in the centromeric and telomeric directions is marked by **arrows. Open boxes** indicate exons, and **lines** indicate introns and intergenic regions. **Filled boxes** indicate coding regions. **Arrows** mark the locations of single nucleotide polymorphisms. The **bar** marks the length of 1000 base pairs.

rs1800947. SNP3, also known as rs1130864, and SNP4, also known as rs1205, were located in the 3′ untranslated region of the gene (Fig. 1).

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes by extraction with phenol and chloroform.[20]

Each genotyping, except that of rs1205, was performed using the TaqMan SNP Genotyping Assay, which was commercially supplied by Applied Biosystems Inc. (ABI, Foster City, CA). SNP rs1205 was genotyped by using a Custom TaqMan SNP Genotyping Assay, which was also commercially supplied by ABI.

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

Polymerase chain reaction amplification was accomplished by using 6 μL of TaqMan Universal Master Mix, No AmpErase UNG (2×) (ABI) in 12 μL final reaction volumes, with 2 ng DNA, 0.22 μL TaqMan SNP Genotyping Assay Mix (20× or 40×) containing 900 nmol/L primers, and 200 nmol/L final concentrations of the probes. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 62°C for 1 min. Thermal cycling was performed on GeneAmp 9700 systems.

Each 96-well plate contained 80 samples of an unknown genotype and four reactions with reagents but no DNA (control). The control samples without DNA were necessary for the Sequence Detection System (SDS) 7700 signal processing as outlined in the TaqMan Allelic Discrimination Guide (ABI). These plates were read on the SDS 7700 instrument with the end-point analysis mode of the SDS version 1.6.3 software package (ABI). Genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X-Y scatter plot of the SDS software. Genotypes were also determined automatically by the signal processing algorithms in the software. Results of each scoring method were saved in two separate output files for later comparison.[21]

Biochemical Analysis

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

Statistical Analysis

All continuous variables were expressed as mean ± SD. Continuous variables of IS patients and control subjects were assessed with Mann-Whitney *U* test. Categorical variables were assessed with the Fisher's exact test. Genotypes were classified into three distinct groups: additive, dominant, and recessive. The distribution of the additive, dominant, and recessive models of genotypes was examined with Fisher's exact test. The contingency table including 0 was not examined. In addition, multiple logistic regression analyses were performed to assess the contribution of confounders (history of hypertension, diabetes, hyperlipidemia, ischemic heart disease, and atrial fibrillation). Based on the genotype data of the genetic variations, linkage disequilibrium (LD) analysis, and a haplotype-based case-control study were performed using the expectation maximization (EM) algorithm,[22] with the SNPAlyze software program, version 3.2 (Dynacom Co., Ltd., Yokohama, Japan). The pairwise LD analysis of this study was performed using four SNP pairs. The D′ values >0.5 were used to assign SNP locations to one haplotype block. Tagged SNP were selected by omitting one SNP from a SNP pair showing r^2 >0.5 for each haplotype block. In this haplotype-based case control study, haplotypes with a frequency <0.02 were excluded. The frequency distribution of the haplotypes was calculated by a permutation test using the bootstrap method. Statistical significance was established at $P < .05$. Statistical analyses were performed with SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL).

Results

We genotyped four SNP in 152 patients with IS and 304 control subjects. Table 1 shows the clinical features of the two study populations. The mean age was significantly higher in the control group than in the IS group. body mass index, systolic blood pressure, diastolic blood pressure, and pulse rate were significantly higher in the patient group, except for the body mass index and diastolic blood pressure in women. Serum creatinine was significantly higher in the IS group. However there was no significance between men and women in the two groups. Plasma concentration of total cholesterol was significantly higher in the control group. Incidence of of hypertension, diabetes, ischemic heart disease, and atrial fibrillation were significantly higher in the IS group. Incidence of hyperlipidemia was significantly higher in the control group, except in the male control subjects. Alcohol consumption was signifi-

Table 1. Characteristics of study participants

Characteristic	Total group			Men			Women		
	IS	Control	*P* value	IS	Control	*P* value	IS	Control	*P* value
Number of subjects	152	304		87	149		65	155	
Age (y)	72.4 ± 8.2	78.0 ± 4.2	<.001*	70.2 ± 7.5	78.2 ± 4.7	<.001*	75.3 ± 8.4	77.8 ± 3.8	.01*
BMI (kg/m^2)	23.5 ± 3.9	22.6 ± 2.9	.04*	23.0 ± 3.5	22.8 ± 2.8	.96	24.6 ± 4.5	22.5 ± 3.0	.003*
SBP (mm Hg)	149 ± 26	136 ± 16	<.001*	149 ± 26	136 ± 15	<.001*	149 ± 26	136 ± 17	.002*
DBP (mm Hg)	84 ± 15	79 ± 11	<.001*	85 ± 16	79 ± 10	.002*	81 ± 14	78 ± 12	.07
Pulse (beats/min)	76 ± 15	70 ± 11	<.001*	75 ± 15	69 ± 12	.002*	77 ± 16	71 ± 10	.02*
Creatinine (mg/100 mL)	1.02 ± 0.68	0.85 ± 0.23	.02*	1.13 ± 0.66	0.96 ± 0.22	.13	0.88 ± 0.68	0.75 ± 0.18	.35
Total cholesterol (mg/100 mL)	190 ± 47	218 ± 43	<.001*	180 ± 39	205 ± 31	<.001*	202 ± 53	231 ± 49	<.001*
Hypertension (%)	62	8	<.001*	65	9	<.001*	58	7	<.001*
Diabetes (%)	21	3	<.001*	19	4	<.001*	25	2	<.001*
Hyperlipidemia (%)	31	50	<.001*	22	34	.07	43	65	.003*
Ischemic heart disease (%)	16	4	<.001*	15	3	.002*	17	4	.002*
Atrial fibrillation (%)	14	0	<.001*	15	0	<.001*	12	0	<.001*
Alcohol consumption (%)	56	37	.005	79	46	.001*	28	25	.82
Smoking (%)	52	30	.001*	66	45	.01*	34	12	.04*

BMI = body mass index; DBP = diastolic blood pressure; IS = ischemic stroke; SBP = systolic blood pressure.

Continuous variables were expressed as mean ± standard deviation. Categorical variables were expressed as percentage. *P* value of continuous variables was calculated by Mann-Whitney *U* test. The *P* value of categorical variables was calculated by Fisher's exact test.

* $P < .05$.

Table 2. Genotype distribution in patients with ischemic stroke and control subjects

SNP	Genotype	IS patients (n = 152)	Control subjects (n = 304)	*P* value: Fisher's exact test	*P* value: Multiple logistic regression
SNP1					
rs1341665	Additive				
	CC	73	144	.089	.225
	CT	70	123		
	TT	9	37		
	Dominant				
	CC+CT	143	267	.047*	.188
	TT	9	37		
	Recessive				
	CC	73	144	.921	.515
	TT+CT	79	160		
SNP2					
rs1800947	Additive				
	GG	140	293	—	—
	GC	12	11		
	CC	0	0		
	Dominant				
	GG+GC	152	304	—	—
	CC	0	0		
	Recessive				
	GG	140	293	.067	.016*
	CC+GC	12	11		
SNP3					
rs1130864	Additive				
	CC	132	265	1.00	.253
	CT	19	38		
	TT	1	1		
	Dominant				
	CC+CT	151	303	1.00	.098
	TT	1	1		
	Recessive				
	CC	132	265	1.00	.858
	TT+CT	20	39		
SNP4 rs1205	Additive				
	GG	72	137	.184	.237
	GA	68	125		
	AA	12	42		
	Dominant				
	GG+GA	140	262	.067	.173
	AA	12	42		
	Recessive				
	GG	72	137	.690	.623
	AA+GA	80	167		

IS = ischemic stroke.

The *P* value of genotypes was calculated by Fisher's exact test. The *P* value of multiple logistic regression was calculated by multiple logistic regression analysis with adjustment for the history of hypertension, diabetes hyperlipidemia, ischemic heart disease, and atrial fibrillation.

* $P < .05$.

cantly higher in the male IS group. Smoking prevalence was significantly higher in the IS group.

Table 2 shows the distribution of the genotypes of the four SNP. The genotype distribution of each SNP did not show significant differences from the Hardy-Weinberg equilibrium values (data not shown). The Fisher's exact test showed that the distribution in the dominant model of SNP1 (CC+CT versus TT) was significantly different between the two groups (P = .047). However this distribution did not show a significant difference by multiple logistic regression analysis (P = .188; 95% confidence interval [CI], 0.73 to 5.0).

On the other hand, multiple logistic regression analysis showed that the distribution in the recessive model of SNP2 (GG versus CC+GC) was statistically significant (P = .016; 95% CI, 1.3 to 10.9) (Table 2). The effects of confounding factors such as hypertension, diabetes, hyperlipidemia, ischemic heart disease, and atrial fibrillation are shown in Table 3. Hypertension ($P < .001$; 95% CI, 9.4 to 30), diabetes (P = .002; 95% CI, 1.8 to 12), hyperlipid-

Table 3. Effect of confounders on prevalence of ischemic stroke and control groups by multiple logistic regression analysis

	β	SE	χ^2	*P* value	OR	95% CI
SNP2 C/C and G/C genotypes	1.32	0.55	5.83	.016*	3.75	1.3–10.9
Hypertension	2.82	0.29	92.6	<.001*	16.7	9.4–30
Diabetes	1.52	0.49	9.69	.002*	4.59	1.8–12
Hyperlipidemia	−0.68	0.28	6.07	.014*	0.51	0.29–0.87
Ischemic heart disease	1.01	0.51	3.91	.048*	2.74	1.009–7.4
Atrial fibrillation	8.83	11.6	0.58	.45	6850	0–5.2 × 10^{13}

β = regression coefficient; CI = confidence interval; OR = odds ratio; SE = standard error.
* $P < .05$.

emia ($P = .014$; 95% CI, 0.29 to 0.87), and ischemic heart disease ($P = .048$; 95% CI, 1.009 to 7.4) were also estimated as independent predictors of IS.

Patterns of linkage disequilibrium in the CRP gene are shown with their D′ and r^2 values (Table 4). All SNP were located in one haplotype block. Because the r^2 of SNP1-SNP4 was large, we constructed the haplotype-based association study using SNP1, SNP2, and SNP3. Four possible haplotypes were predicted and each had a frequency >0.02 (Table 5). The overall distribution of these haplotypes was statistically significant (χ^2= 24.7, permutation $P = .046$). In the H3 haplotype (C-C-C), statistical significance was shown (χ^2= 23.4, permutation $P = .015$).

Discussion

Atherosclerosis, one of the major causes of IS, is considered to involve the inflammatory system.[6] C-reactive protein, a major inflammatory marker, has been detected in atherosclerotic plaques[7] and may be involved in the development of atherosclerosis.[8] An elevated plasma CRP level may be an atherothrombotic biomarker that provides additive prognostic information about not only coronary heart disease[9] but also peripheral arterial disease.[10] Moreover an elevated plasma CRP level could be a marker that predicts the risk of IS in elderly individuals.[11]

Some SNP in the human CRP gene were reported to be associated with elevated basal CRP levels, while some were associated with reduced levels.[13–16] Case control studies have shown that some SNP are associated with coronary heart disease or type 2 diabetes mellitus. However the basal CRP level was easily elevated with an inflammatory stimulus such as bacterial infection, other inflammatory disease, malignancy, and necrosis. In fact patients with IS may have had complications such as pneumonia and deep venous thrombosis that made the basal CRP level difficult to assess. Thus we examined the SNP that were already known to be associated with variable basal CRP level.

In the present study, we genotyped four SNP of the CRP gene in Japanese subjects and assessed the association between this gene and IS. In this association study, we used a super-control group, consistent with aged and unaffected subjects. Healthy elderly subjects are more suitable than young or middle-aged subjects for the determination of phenotypes of cardiovascular and cerebrovascular diseases related to aging, because many such diseases occur late in life. Ischemic stroke is an age-influenced disease; therefore it was better to use the super–control group than the age-matched control group. In this study, the level of total cholesterol and the number of subjects with hyperlipidemia were significantly higher in the control group, especially in women. In the control group, many subjects had diagnosed hyperlipidemia and were treated with diet therapy without antihyperlipemic drugs.

Table 4. Pairwise linkage disequilibrium (D′ above diagonal and r^2 below diagonal) for the four single nucleotide polymorphisms

		D′								
		IS patients				Control subjects				
	SNP	SNP1	SNP2	SNP3	SNP4	SNP	SNP1	SNP2	SNP3	SNP4
r^2	SNP1		**0.999**	**1**	**1**	SNP1		**0.999**	**1**	**1**
	SNP2	0.017		**1**	**0.999**	SNP2	0.009		**0.680**	**0.999**
	SNP3	0.182	0.003		**1**	SNP3	0.147	0.001		**1**
	SNP4	**0.951**	0.018	0.171		SNP4	**0.915**	0.009	0.134	

IS = ischemic stroke.
Value of D′ > 0.5 and values of r^2 > 0.5 are boldface.

Table 5. Haplotype frequency estimates

Haplotype	CRP polymorphism			Frequency		χ^2	*P* value*
	SNP1	SNP2	SNP3	IS patients	Control subjects		
H1	Mj C	Mj G	Mj C	0.67	0.67	0.004	.96
H2	Mn T	Mj G	Mj C	0.22	0.26	2.08	.17
H3	Mj C	Mn C	Mj C	0.04	0.00	23.4	.015†
H4	Mn T	Mj G	Mn T	0.07	0.07	0.02	.88

IS = ischemic stroke; Mj = major allele; Mn = minor allele. Mj and Mn indicate haplotypes with major and minor frequencies, respectively. Haplotypes with frequencies >0.02 were estimated using SNPAlyze software.

* *P* value was calculated by permutation test using bootstrap method. Overall distribution of these haplotypes was statistically significant ($\chi^2 = 24.7$, $P = .046$); † $P < .05$.

The multiple logistic regression analysis adjusted by the confounders hypertension, diabetes, hyperlipidemia, ischemic heart disease, and atrial fibrillation showed significant differences in the distribution of the recessive model (GG versus CC+GC) of SNP2. The result indicated that the risk of IS could be increased in subjects with the C allele of SNP2.

In general, high CRP levels reportedly promoted a proatherosclerotic and proinflammatory phenotype.[8] The relationship between high CRP levels and the progression of atherosclerosis has been well documented.[23] Although the SNP2 had no function because of its location in the intron, the SNP was reported to be associated with reduced basal CRP levels. However there was no association between SNP2 and the risk of arterial thrombosis.[15] It has been described that in coronary artery bypass graft patients, stimulated CRP levels were found in patients with the TT genotype of SNP3. However the genotype of SNP3 did not influence the basal CRP level.[14] It was considered that the stimulated CRP level could be another predictor of atherosclerosis in addition to the high basal CRP level. Ischemic stroke is a multifactorial disorder and this SNP was only one of its risk predictors. The genetic and environmental factors each importantly contributed to the vascular risk associated with inflammation. The association between a high stimulated CRP level and IS has not yet been reported and may be a subject for future study. In the multiple logistic regression analysis, hyperlipidemia was evaluated as a protective factor of IS. Hypertension and diabetes were described to be strong risk factors of IS.[2,24] However hypercholesteremia was not an established risk factor of IS in the Japanese population.[25] Moreover in the present study, many control subjects, especially women, had hyperlipidemia that might influence other confounders.

The distribution of the dominant genotypes of SNP1 was statically significant between the IS patients and control subjects in the Fisher's exact test. However in the multiple logistic regression analysis, there was no statistical significance. The confounders might affect the significant difference of SNP1. This SNP is located in the 5′ flanking region. Nevertheless this SNP is unlikely to influence transcriptional activity because it is far from the transcriptional initiation site, at approximately 7200 nucleotides.

The genotype SNP3 was reportedly associated with an elevated CRP level[13,14] and the genotype SNP4 was reportedly associated with SLE and antinuclear autoantibody production.[13] In the present study, none of the participants had a history of collagen diseases including SLE. Anti–phospholipid antibody syndrome (APS), which is often complicated with SLE, was known to be a major risk factor for IS and related to the progression of atherosclerosis and the pathogenesis of thrombosis.[26–28] A significant positive correlation was found between the presence of anticardiolipin antibody, one of the antiphospholipid antibodies, and an elevated CRP level.[29] However in the present study there was no statistical significance by the the Fisher's exact test and the multiple logistic regression analysis.

In genes with multiple susceptibility alleles, a haplotype-based association study could have advantages over analysis based on individual SNP, particularly when LD between SNP was weak.[30] In the present study, haplotypes were constructed using three SNP (SNP1, SNP2, and SNP3), based on results of LD analysis and a statistical significance was shown in the H3 haplotype (C-C-C). An association study using each SNP showed that the CC+GC variant of SNP2 could be a predictor of IS. This result was consistent with the result of a haplotype-based association study. The frequency of the H3 haplotype (C-C-C) was found in only 4% of the IS group. However this was no surprise given the low frequency of the susceptibility haplotype because IS is thought to be a multifactorial disorder.

In conclusion, the SNP2 and the H3 haplotype (C-C-C) of the CRP gene are genetic markers for IS. Further studies are needed to clarify the causal and susceptibility mutation of IS in the CRP gene or neighboring genes.

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