Table 6a Logistic regression analysis of baPWV and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	0.99	(0.544–1.804)	0.9747	—	—	—
(AA-GG)	2.353	(1.110–4.989)	0.0256	—	—	—
ECE1- rs212528 (TT-TC)	—	—	—	1.833	(1.058–3.176)	0.0307
(TT-CC)	—	—	—	0.541	(0.146–2.003)	0.3575
Age	1.105	(1.070–1.142)	<0.0001	1.11	(1.075–1.147)	<0.0001
Height	0.937	(0.889–0.986)	0.0132	0.936	(0.889–0.985)	0.0118
Weight	1.006	(0.975–1.038)	0.7156	1.002	(0.970–1.034)	0.9137
Mean BP	1.071	(1.042–1.101)	<0.0001	1.071	(1.042–1.100)	<0.0001
HR	1.022	(0.993–1.052)	0.1323	1.023	(0.995–1.052)	0.1032

Abbreviations: baPWV, brachial-ankle pulse wave velocity; BP, blood pressure; ET-1, endothelin-1; HR, heart rate.
The average baPWV of male patients was 1756 cm/s. Rapid group, ⩾baPWV 1756 cm/s; slow group, <1756 cm/s.

Table 6b Logistic regression analysis of plaque scores and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	3.255	(0.898–11.802)	0.0725	—	—	—
(AA-GG)	5.017	(1.308–19.239)	0.0187	—	—	—
ECE1- rs2038089 (AA-AG)	—	—	—	0.334	(0.132–0.844)	0.0205
(AA-GG)	—	—	—	0.356	(0.076–1.666)	0.1895
Age	1.09	(1.032–1.152)	0.0020	1.097	(1.038–1.160)	0.0010
Height	0.984	(0.907–1.068)	0.7006	0.988	(0.912–1.071)	0.7748
Weight	0.962	(0.906–1.021)	0.2047	0.956	(0.901–1.014)	0.1333
DBP	1.031	(0.986–1.077)	0.1843	1.039	(0.994–1.086)	0.0911

Abbreviations: DBP, diastolic blood pressure; ET-1, endothelin-1.
The severe atherosclerotic group of male patients refers to PS⩾10.1. Rapid group, ⩾PS 10.1; slow group, <10.1.

Table 6c Logistic regression analysis of mean-IMT and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRA- rs5333 (TT-TC)	1.066	(0.655–1.737)	0.7966	—	—	—
(TT-CC)	2.328	(0.846–6.406)	0.1018	—	—	—
EDNRB- rs5351 (AA-AG)	—	—	—	0.770	(0.449–1.319)	0.3409
(AA-GG)	—	—	—	1.349	(0.686–2.653)	0.3861
Age	1.049	(1.022–1.076)	0.0003	1.052	(1.025–1.081)	0.0002
Height	1.008	(0.969–1.048)	0.6995	1.008	(0.969–1.049)	0.6853
DBP	0.989	(0.965–1.014)	0.3835	0.992	(0.967–1.017)	0.5110
HbA_{1c}	1.188	(0.872–1.619)	0.2735	1.270	(0.932–1.730)	0.1301
HDL-CHOL	0.976	(0.958–0.995)	0.0115	0.979	(0.959–0.996)	0.0150

Abbreviations: ET-1, endothelin-1; IMT, intima-media thickness.
The average mean-IMT in male patients was 0.86 mm. Severe group, ⩾mean-IMT 0.86 mm; mild group, <0.86 mm.

ET-1 level or on the interaction of several hormonal systems. The negative vascular effects of ET-1 may contribute to the pathogenesis of hypertension and its complications in black patients.[27] Unfortunately, we did not have enough data regarding serum ET-1 levels to analyze the relationship between serum levels and ET-1 family gene polymorphisms.

It is also important to examine the influence of menopause on atherosclerosis in female subjects. However, most female subjects in the present study were older than 60 years, so it was impossible to clarify the influence of menopause in this study.

Any synergetic effects of polymorphisms on baPWV and PS should also be evaluated. In male subjects, baPWV values were slower in those with TT than with TC+CC of *ECE1*-T/C-rs212528, and were also slower in those with AA than with AG+GG of *EDNRB*-A/G-rs5351. We therefore compared baPWVs of subjects with both TT of *ECE1*-rs212528 and AA of *EDNRB*-rs5351 to those of subjects with both CC of *ECE1*-rs212528 and GG of *EDNRB*-rs5351. However, we did not obtain a stronger correlation for combined gene types than single genotypes (data not shown). We obtained similar results by analyzing IMT and PS.

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What is known about this topic

- ET-1 is thought to play important roles in the development of atherosclerosis through endothelial dysfunction and proliferation of vascular smooth muscle cells.
- There have been various studies of the relationship between polymorphisms of ET-1 and BP.

What this study adds

- EDNRB-rs5351 in exon6 might contribute to the progression of atherosclerosis in male patients with EHT.
- In future, an evaluation of these polymorphisms may be valuable for the treatment of hypertensive and/or atherosclerotic patients.

Abbreviations: ET-1, endothelin-1; BP, blood pressure; EHT, essential hypertension.

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Original Article

Association of Genetic Polymorphisms of Endothelin-Converting Enzyme-1 Gene with Hypertension in a Japanese Population and Rare Missense Mutation in Preproendothelin-1 in Japanese Hypertensives

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Endothelin-1 (EDN1), a 21-amino acid peptide, is a potent vasoconstrictor with various pharmacological responses. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. Endothelin-converting enzyme (ECE) cleaves a Trp73–Val74 peptide bond in big-EDN1 to give rise to mature EDN1. In this study, we examined the possible association of genetic variations in *ECE1* with hypertension in a general Japanese population and searched for missense mutations in and around the EDN1 polypeptide. We genotyped 5 single nucleotide polymorphisms (SNPs) in the *ECE1* gene in 1,873 individuals from a general Japanese population and identified one SNP associated with hypertension in women (rs212528: TT *vs.* TC+CC: odds ratio=1.40; 95% confidence intervals: 1.04–1.89; p=0.026), after adjusting for confounding factors. The systolic blood pressure in women with the CC genotype was 6.44 mmHg higher than that in those with the TT genotype (p=0.007), after adjusting for the same factors. Next, to identify the missense mutations that may influence the biological activity of EDN1, we sequenced the genomic region that encodes EDN1 in 942 Japanese hypertensive patients. We identified a novel missense mutation, G36R, in one hypertensive patient, but no mutations were observed in EDN1. A gene polymorphism in *EDN1*, Lys198Asn, has been reported to be associated with hypertension in obese subjects. Taken together, these findings reveal that the EDN-ECE pathway is an important system involved in essential hypertension in Japanese. (*Hypertens Res* 2007; 30: 513–520)

***Key Words*: endothelin, endothelin-converting enzyme, gene variants, hypertension, general population**

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Introduction

The endothelin (EDN) system is comprised of 4 active EDNs, with EDN1 being the predominant isoform in the cardiovascular system (*1*). Because of the potent vasoconstricting and mitogenic effects of EDN1 and its involvement in various cardiovascular diseases, biosynthesis of EDN1 has received considerable attention. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. In the first step, preproEDN1 is cleaved by a signal peptidase, resulting in the formation of proEDN1, which is then cleaved by a furin-like enzyme to yield the 38-amino acid protein known as big-EDN1 (amino acids 53–92) or other intermediates. Big-EDN1 is subsequently cleaved by a unique type II metalloprotease, EDN-converting enzyme-1 (ECE1), to yield EDN1 (amino acids 53–73) (*2*).

The EDN system is a promising target for the genetic analysis for hypertension. The missense mutation Lys198Asn has been identified in preproEDN1, and several reports have described that this polymorphism showed a positive association with blood pressure elevation in overweight people (*3–5*), although no significant difference in the EDN1 levels between the Asn-type and Lys-type transfectant was observed in an expression analysis (*6*). As for *ECE1*, an association between the −338C>A polymorphism in *ECE1* and blood pressure levels in women but not in men has recently been reported (*7*). This C>A polymorphism is associated with increased promoter activity, as demonstrated in a promoter assay analysis (*8*).

Complex traits such as hypertension, diabetes mellitus, and hyperlipidemia are suggested to be caused by common sequence variants that may have a small to moderate phenotypic effect (*9–11*). On the other hand, accumulating data has shown that most Mendelian disorders are caused by a set of different mutations that often reside in coding regions. These rare variants tend to have strong phenotypic effects. Several recent studies have shown that rare genetic variations in *ABCA1*, *APOA1*, and *LCAT* collectively contribute to the variation in plasma levels of high-density lipoprotein (HDL) cholesterol in the general population (*12, 13*). We hypothesized that rare genetic variations in hypertension candidate genes could collectively contribute to hypertension. To investigate this hypothesis, we have been identifying such mutations in Japanese hypertensive subjects; to date, we have identified missense mutations in the β- or γ-subunit of the amiloride-sensitive epithelial sodium channel encoded by *SCNN1B* and *SCNN1G* (*14*), a causative gene for pseudohypoaldosteronism type II encoded by serine-threonine kinase *WNK4* (*15*), the regulator of G-protein signaling 2 (*RGS2*) (*16*), and the mineralocorticoid receptor encoded by *NR3C2* (*17*). As the next hypertension candidate gene, we have begun to sequence the *EDN1* gene and to search for missense mutations (*18*).

In present study, we genotyped the genetic polymorphisms of one of the EDN-converting enzymes, the *ECE1* gene, in a general Japanese population to examine whether the *ECE1* gene is a susceptibility gene for hypertension. Secondly, to evaluate the EDN system in essential hypertension in Japanese, we re-sequenced the EDN1 polypeptide in the *EDN1* gene in Japanese hypertensive patients to identify missense mutations that may deleteriously affect EDN1 function.

Methods

General Population

The selection criteria and design of the Suita study have been described previously (*19, 20*). Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. In this study, the genotypes of 1,873 samples were determined. The characteristics of the 1,873 participants (863 men and 1,010 women) are shown in Table 1. Routine blood examinations that included total serum cholesterol, HDL cholesterol, triglyceride, and glucose levels were performed. A physician or nurse interviewed each patient regarding smoking and alcohol drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke. Blood pressure was measured after at least 10 min of rest in a sitting position. Systolic and diastolic blood pressures (SBP and DBP) were the means of two measurements by well-trained doctors (recorded >3 min apart). Hypertension was defined as SBP of ≥140 mmHg, DBP of ≥90 mmHg, or the current use of antihypertensive medication (*20*). Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/L (126 mg/dL), non-fasting plasma glucose ≥11.1 mmol/L (200 mg/dL), current use of antidiabetic medication, or HbA1c ≥6.5%. Hyperlipidemia was defined as total cholesterol ≥5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared.

Hypertensive Subjects

A total of 942 hypertensive subjects (518 men and 424 women; average age: 65.1±10.5 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center. Ninety-two percent of study subjects (870 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension, including renal hypertension (36 subjects), renovascular hypertension (23 subjects), primary aldosteronism (11 subjects) and hypothyroid-induced hypertension (2 subjects) (*14–17*). The hypertension criteria were blood pressure above 140 and/or 90 mmHg or the use of antihypertensive agents. Blood pressure was the average of three measurements taken in a sitting position after at least 5 min of rest on each occasion. About one-third of the hypertensive subjects had hypertensive cardiovas-

Table 1. Basic Characteristics of Subjects in Japanese General Population (Suita Study)

	Women (n=1,010)	Men (n=863)
Age (yeasr old)	63.3±11.0	66.3±11.1*
Systolic blood pressure (mmHg)	128.0±19.6	131.9±19.5*
Diastolic blood pressure (mmHg)	76.6±9.8	79.7±10.7*
Body mass index (kg/m^2)	22.3±3.2	23.3±3.0*
Total cholesterol (mmol/L)	5.57±0.79*	5.10±0.78
HDL-cholesterol (mmol/L)	1.67±0.40*	1.42±0.36
Current smokers (%)	6.3	30.1†
Current drinkers (%)	29.3	67.0†
Present illness (%)		
Hypertension	38.2	47.4†
Hyperlipidemia	55.2†	27.4
Diabetes mellitus	5.2	12.6†

Values are mean±SD or percentage. Hypertension: systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or antihypertensive medication; hyperlipidemia: total cholesterol ≥220 mg/dL or antihyperlipidemia medication; diabetes: fasting plasma glucose ≥126 mg/dL or non-fasting plasma glucose ≥200 mg/dL or HbA1c ≥6.5% or antidiabetic medication. *$p<0.05$ between women and men by Student's t-test. †$p<0.05$ between women and men by χ^2 test. HDL, high-density lipoprotein.

Table 2. General Characteristics of Patients with Hypertension and/or Renal Failure

Number	942
Age (years)	65.1±10.5
Gender (M/F)	518/424
Body mass index (kg/m^2)	24.2±3.3
Systolic blood pressure (mmHg)	145.5±19.2
Diastolic blood pressure (mmHg)	84.8±13.4
Essential hypertension	870
Secondary hypertension	72
Renal hypertension	36
Renovascular hypertension	23
Primary aldosteronism	11
Hypothyroid-induced hypertension	2
Renal impairment*	110
Ischemic heart disease	102
Stroke**	145

Values are expressed as mean±SD. *Patients who had serum creatinine ≥1.4 mg/dL. **Silent cerebral infarction was included. M, male; F, female.

cular complications. The clinical features of the patients in this study are summarized in Table 2.

All of the participants for the genetic analysis in the present study gave their written informed consent. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Genotyping of Mutations of Single Nucleotide Polymorphisms of the *ECE1* Gene in the General Population

We obtained genetic polymorphisms in the *ECE1* gene using the database of Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/) (*21, 22*) and genotyped the following 5 single nucleotide polymorphisms (SNPs) by the TaqMan-PCR system: rs212548-TC (IMS-JST017298 in intron 4), rs212528-TC (IMS-JST004319 in intron 5), rs212526-CT (IMS-JST009090 in intron 6), rs2038090-AC (IMS-JST004325 in intron 17), and rs2038089-AG (IMS-JST004324 in intron 17). The primers and probes of the TaqMan-PCR system are available on request. Hereafter, SNPs are described according to the RS nomenclature system.

Screening of Mutations in Exon 2 of the *EDN1* Gene

Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). The region of exon 2 was amplified by polymerase chain reaction (PCR) using a pair of specific primers, 5′-CTGATGGCAGGCTGTGTGCTT-3′ and 5′-CCCCATCAG ATGCCACTGTGA-3′, which flank the 612-bp region containing exon 2. The PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) as described previously (*23, 24*). The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection (*25*).

Statistical Analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis. Association analyses between genotypes and blood pressure in each sex were performed through logistic regression analysis with consideration for potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with the 95% confidence intervals. The relationship between genotypes and risk of hypertension was expressed in terms of the odds ratios adjusted for possible confounding effects, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios were calculated as a measure of the association between each genotype

Table 3. Odds Ratio of Polymorphisms in *ECE1*

SNP	Sex	Genotype	*n*	Odds ratio	(95% CI)	*p*	Genotype	*n*	Odds ratio	(95% CI)	*p*
rs212548	Women	TT	328	1	(reference)		TT+TC	821	1	(reference)	
		TC+CC	686	1.28	(0.94–1.74)	0.116	CC	193	1.21	(0.85–1.72)	0.293
	Men	TT	275	1	(reference)		TT+TC	692	1	(reference)	
		TC+CC	590	1.10	(0.82–1.50)	0.520	CC	173	0.98	(0.69–1.40)	0.924
rs212528	Women	TT	663	1	(reference)		TT+TC	980	1	(reference)	
		TC+CC	347	1.40	(1.04–1.89)	0.026	CC	30	1.63	(0.74–3.58)	0.227
	Men	TT	528	1	(reference)		TT+TC	827	1	(reference)	
		TC+CC	335	0.83	(0.62–1.11)	0.198	CC	36	0.75	(0.37–1.53)	0.428
rs212526	Women	CC	734	1	(reference)		CC+CT	996	1	(reference)	
		CT+TT	280	0.76	(0.55–1.05)	0.099	TT	18	0.77	(0.25–2.35)	0.650
	Men	CC	615	1	(reference)		CC+CT	842	1	(reference)	
		CT+TT	251	0.95	(0.70–1.30)	0.751	TT	24	1.40	(0.58–3.38)	0.455
rs2038090	Women	AA	774	1	(reference)		AA+AC	999	1	(reference)	
		AC+CC	239	1.17	(0.84–1.64)	0.348	CC	14	1.05	(0.30–3.61)	0.939
	Men	AA	676	1	(reference)		AA+AC	856	1	(reference)	
		AC+CC	189	1.00	(0.71–1.40)	0.989	CC	9	3.32	(0.67–16.45)	0.142
rs2038089	Women	AA	414	1	(reference)		AA+AG	880	1	(reference)	
		AG+GG	598	1.19	(0.89–1.59)	0.240	GG	132	1.21	(0.80–1.84)	0.358
	Men	AA	380	1	(reference)		AA+AG	788	1	(reference)	
		AG+GG	486	1.12	(0.84–1.49)	0.450	GG	78	1.33	(0.81–2.18)	0.264

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; CI, confidence interval.

Table 4. Association of Genotypes with Blood Pressure Variation

SNP	Genotype	Women					Men				
		n	DBP (mmHg)	*p**	SBP (mmHg)	*p**	*n*	DBP (mmHg)	*p**	SBP (mmHg)	*p**
rs212528	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43		131.94±0.75	
	TC	317	76.55±0.53		129.21±0.93		299	79.48±0.57		131.18±1.00	
	CC	30	77.57±1.72	0.698	133.33±3.02	0.007	36	80.93±1.66	0.931	133.83±2.89	0.941
	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43		131.94±0.75	
	TC+CC	347	76.63±0.51	0.823	129.56±0.89	0.016	335	79.64±0.54	0.840	131.47±0.94	0.698
	TT+TC	980	76.51±0.30		127.64±0.53		827	79.67±0.34		131.66±0.60	
	CC	30	77.57±1.72	0.545	133.33±3.02	0.066	36	80.93±1.66	0.458	133.83±2.89	0.463
rs212526	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40		131.67±0.69	
	CT	262	76.90±0.58		127.51±1.03		227	80.15±0.66		131.39±1.15	
	TT	18	70.08±2.19	0.344	120.04±3.87	0.175	24	84.13±2.06	0.048	138.16±3.59	0.422
	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40		131.67±0.69	
	CT+TT	280	76.45±0.56	0.874	127.02±0.99	0.371	251	80.52±0.63	0.135	132.03±1.09	0.780
	CC+CT	996	76.65±0.30		127.92±0.52		842	79.61±0.34		131.59±0.59	
	TT	18	70.08±2.19	0.003	120.04±3.87	0.044	24	84.13±2.06	0.030	138.16±3.59	0.071

Values are mean±SEM. *Conditional logistic analysis, adjusted for age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; DBP, diastolic blood pressure; SBP, systolic blood pressure.

and hypertension under the assumption of a dominant (with scores of 0 for patients homozygous for the major allele and 1 for carriers of the minor allele) or recessive (with scores of 0 for carriers of the major allele and 1 for patients homozygous

Table 5. Haplotype Frequency (Freq) of *ECE1* Gene in Hypertensives (HT) and Normotensives (NT)

Haplotype		All				Men				Women			
		Freq (%)	χ^2	p Asymptotic	p Permutation	Freq (%)	χ^2	p Asymptotic	p Permutation	Freq (%)	χ^2	p Asymptotic	p Permutation
H1 T/T/C/A/A	Overall	19.2	1.278	0.258	0.327	19.0	0.040	0.841	0.893	19.3	2.954	0.086	0.127
	NT	19.8				18.8				20.4			
	HT	18.4				19.2				17.3			
H2 C/C/C/A/A	Overall	16.2	1.305	0.253	0.284	17.5	0.193	0.661	0.669	15.1	2.991	0.084	0.091
	NT	15.5				17.9				14.0			
	HT	16.9				17.1				16.9			
H3 T/T/C/A/G	Overall	14.3	0.122	0.727	0.769	14.7	0.231	0.631	0.695	14.2	0.060	0.807	0.825
	NT	14.1				14.4				14.0			
	HT	14.5				15.2				14.4			
H4 C/T/C/A/A	Overall	11.8	0.181	0.670	0.716	11.9	0.033	0.857	0.867	11.8	0.250	0.617	0.699
	NT	12.0				12.1				12.1			
	HT	11.5				11.8				11.4			
H5 T/T/T/A/A	Overall	10.7	8.254	0.004	0.015	10.9	0.421	0.516	0.575	10.6	11.865	0.001	0.003
	NT	12.0				11.4				12.4			
	HT	9.0				10.4				7.5			
H6 T/T/C/C/G	Overall	8.3	0.317	0.574	0.618	7.8	0.327	0.568	0.624	9.0	0.001	0.974	0.978
	NT	8.1				8.1				9.0			
	HT	8.7				7.3				9.0			
H7 C/T/C/A/G	Overall	7.8	0.133	0.715	0.775	6.2	1.115	0.291	0.402	8.8	2.071	0.150	0.192
	NT	7.6				5.5				8.2			
	HT	7.9				6.7				10.0			

Haplotypes (rs212548/rs212528/rs212526/rs2038090/rs2038089) with frequencies of more than 5% are shown. One hundred thousand replicates were used for permutation test for all, men and women. Numbers of haplotypes in Overall, NT, and HT are 3,736, 2,150, 1,586 for All; 1,730, 914, 816 for men; and 2,030, 1,254, 776 for women, respectively.

for the minor allele) mode of inheritance. The p values were adjusted by Bonferroni correction. SAS statistical software (release 6.12; SAS Institute Inc., Cary, USA) was used for the statistical analyses. The data of linkage disequilibrium, haplotype blocks and coverage of HapMap SNPs were downloaded from the HapMap Consortium (http://www.hapmap.org). Haplotypes and permutation analyses were calculated using SNPAlyze version 4.0 software (DYNACOM Co., Mobara, Japan).

Results

Association between SNPs in the *ECE1* Gene and Hypertension

Five genetic polymorphisms in the *ECE1* gene were genotyped in 1,873 individuals. The genotype frequencies for each polymorphism were as follows: rs212548-T>C, 0.563/0.437; rs212528-T>C, 0.800/0.200; rs212526-C>T, 0848/0.152; rs2038090-A>C, 0.880/0.120; rs2038089-A>G, 0.655/0.345. None of the genotype frequencies were significantly different from those expected from the Hardy-Weinberg equilibrium ($p>0.05$). Multiple logistic regression analysis after adjusting for confounding factors of age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that one polymorphism, rs212528, in intron 5 was significantly associated with hypertension in women (rs212528-T>C: TT *vs.* TC+CC; odds ratio=1.40; 95% confidence interval: 1.04–1.89; $p=0.026$) (Table 3). The SBPs in women with the TT, TC, and CC genotypes were 126.89±0.64 mmHg ($n=663$), 129.21±0.93 mmHg ($n=317$), and 133.33±3.02 mmHg ($n=30$) ($p=0.007$), after adjusting for the same confounding factors (Table 4). Thus, the difference in SBP was 6.44 mmHg between women with the CC genotype and those with the TT genotype. This association was still significant even after the Bonferroni correction.

Another polymorphism, rs212526, was associated with a significant difference in DBP: women having the CC+CT genotype had a DBP of 76.65±0.30 mmHg ($n=996$) and those with the TT genotype had a DBP of 70.08±2.19 mmHg ($n=18$) ($p=0.003$) after adjusting for the same confounding factors (Table 4). This polymorphism was also significantly associated with the SBP in women (CC+CT: 127.92±0.52 mmHg, $n=996$; TT: 120.04±3.87 mmHg, $n=18$; $p=0.044$). However, this polymorphism did not show a significant association with hypertension. In men, this polymorphism was

Table 6. List of 5 Polymorphisms and Their Allele Frequency in Exon 2 of *EDN1* Identified by Direct Sequencing of 942 Hypertensive Japanese

Allele 1 > allele 2	Amino acid change	region	Allele frequency		Flanking sequence	rs ID
			Allele 1	Allele 2		
1753G>A	G36R	exon 2	1.000	0.000	TGAGAACGGC[G/A]GGGAGAAACC	
1910G>T		intron 2	0.473	0.527	TGTAACCCTA[G/T]TCATTCATTA	rs2070699
1918T>A		intron 2	0.999	0.001	TAGTCATTCA[T/A]TAGCGCTGGC	
2008G>A		intron 2	0.999	0.001	GTGCCTCAGT[G/A]GGGACAGTTT	
2107G>A		intron 2	0.999	0.001	TACTCATGAT[G/A]GGACAAGCAG	

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (*28*). The nucleotide number was according to the reference sequences GenBank Accession ID: NT_007592.

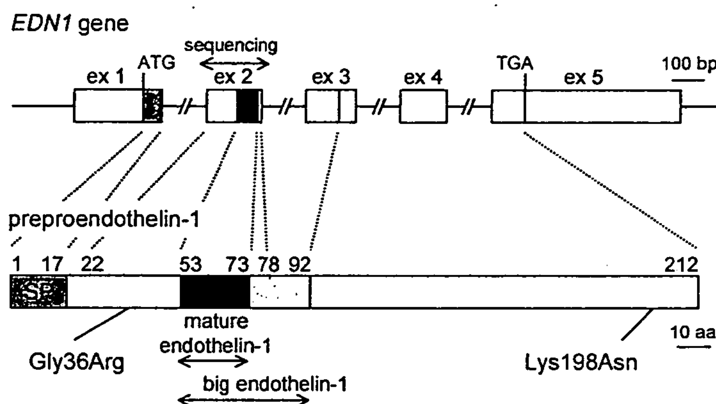

Fig. 1. *Genome and domain structure of human endothelin 1. Two missense mutations in endothelin-1, Gly36Arg (G36R) and Lys198Asn (K198N), are shown. The G36R mutation in preproendothelin-1 was identified in this study.*

significantly associated with DBP (CC+CT: 79.61±0.34 mmHg, n=842; TT: 84.13±2.06 mmHg, n=24; p=0.030).

The haplotypes composed of the 5 SNPs genotyped in this study are shown in Table 5. Seven inferred haplotypes with frequencies of more than 5% were examined to determine their association with hypertension in all patients and in two sub-populations (men and women). In women, the frequency of haplotype H5 in the hypertensive group was significantly lower than that in the normotensive group.

A Novel Missense Mutation in the preproEDN1 Polypeptide in Japanese Hypertensives

We sequenced the region of exon 2 of *EDN1* in 942 hypertensive patients with strong genetic background and secondary hypertension. The results are shown in Table 6. In this study, we were not able to detect any missense mutations within the mature EDN1 region. However, we identified one novel missense mutation, G36R, in *EDN1* in a heterozygous form in a male patient. The prevalence of this mutation was 0.05% in our Japanese hypertensive population. We tried to screen this missense mutation, G36R in *EDN1*, in our general population by the TaqMan-PCR method, but this genotyping failed due to technical problems.

Discussion

In this study, we used two different approaches to reveal the contribution of the EDN system to hypertension in two different populations, a general population and a hypertensive population, both from the Osaka region in Japan.

We genotyped 5 SNPs in *ECE1* and identified rs212528 as the hypertension/blood pressure susceptibility genetic variant. We used the currently available HapMap data from CHB-JPN to assess the coverage of haplotype blocks across the *ECE1* gene by 5 SNPs. The *ECE1* gene consisted of 6 haplotype blocks, in which rs212548 was present in block 2, two SNPs, rs212528 and rs212526, were present in block 3, and two SNPs, rs2038090 and rs2038089, were present in block 6, and the genotyped SNPs were estimated to cover approximately 90% of the haplotypes in block 2, 30% of those in block 3, and 90% of those in block 6, respectively. Two SNPs, rs212528 and rs212526, in block 3 had an r^2 of 0.031 and LOD score of 0.43, and rs2038090 and rs2038089 in block 6 had an r^2 of 0.163 and LOD score of 2.33.

In this study, the rs212528-T>C polymorphism in *ECE1* in

women was identified as the SNP conferring susceptibility for hypertension and blood pressure change. It is well known that the incidence of coronary artery disease shows a gender difference that may in part be related to the female sex hormones estrogen and progesterone. The literature provides evidence that estrogen inhibits EDN1 production (*26*). Furthermore, estrogen inhibits ECE-1 mRNA expression (*27*). These findings may explain the gender difference of *ECE1* polymorphisms for hypertension. The mean age of women in our population was 63.3 years. Despite the relatively advanced age of this population, we identified a contribution of the rs212528 polymorphism to hypertension and blood pressure change, while haplotypes containing the rs212528-C allele were not clearly associated with normotension or hypertension. The association might have been stronger if we had used a younger female population.

Another polymorphism, rs212526-C>T in intron 6, was associated with a blood pressure change in women and men. The mean DBP of the 996 women with the CC+CT genotype was 6.57 mmHg higher than that of the 18 women with the TT genotype ($p=0.003$), and the SBP change also showed the same trend—that is, women with the CC+CT genotype had higher blood pressure than women with the TT genotype ($p=0.044$) (Table 4). However, in men, the opposite trend was seen. The mean DBP of the 842 men with the CC+CT genotype was 4.52 mmHg lower than that of the 24 men with the TT genotype ($p=0.030$). Haplotype H5 containing the rs212528-T allele was significantly more prevalent in the normotensive group. This association also suggested that the T-allele of rs212528 was involved in blood pressure in women (Tables 3–5). Thus, the significance of rs212526 on blood pressure change should be evaluated using other population.

The association of SNP with hypertension and blood pressure change is at best marginally significant given the number of tests performed. All the p-values were more than 0.007. However, rs212528 is present in the *ECE1* gene, which encodes the endothelin-converting enzyme. In addition, this SNP showed a positive association with both hypertension and blood pressure change. Thus, we regarded this SNP as a hypertension candidate. SNP and blood pressure/hypertension described in the present study needs to be confirmed by another set of studies.

In the hypertensive population, we sequenced the coding region of the EDN1 polypeptide and its flanking region in 942 Japanese hypertensives and identified one novel missense mutation, G36R, that was not present in the EDN1 polypeptide but was present in the preproEDN-1 region (Fig. 1). At present, the effect of G36R mutation on the EDN1 function is not clear, because it was located far from the scissile site, the R52–C53 bond, by the furin-like enzyme. From the evolutionary point of view, G36 was conserved in humans, chimpanzees, cows, and dogs, but mice and rats have Val and chickens have Ala. The arginine residue at position 36 was not found in preproEDN1 in any species. To reveal the functional effect of this missense mutation on the processing of preproEDN1, an expression study of the mutant preproEDN1 is needed.

We have hypothesized that rare nonsynomymous mutations in candidate genes could collectively contribute to complex traits. In this model, the extensive sequence-based approaches focusing on identification of these mutations is necessary. So far, we have sequenced several hypertension candidate genes to evaluate whether rare variants could contribute to the etiology of hypertension. At present, however, whether rare variants contribute to hypertension is not clear due to the lack of *in vitro* or *in vivo* expression studies of the mutant protein (*14*, *15*, *17*). The exception was the nonsense mutation identified in the *RGS2* gene, which has been clearly shown to produce the defective protein (*16*). In this study, we identified one missense mutation, G36R, in preproEDN1. The further collection of such missense mutations in hypertension candidate genes could lead to an enhanced understanding of the etiology of essential hypertension.

In summary, we revealed that the rs212528 polymorphism in *ECE1* was associated with hypertension and blood pressure change. In earlier reports, the Lys198Asn polymorphism in *EDN1* showed a positive association with blood pressure elevation in overweight people (*3*–*5*). Thus, endothelin family gene polymorphisms might play an important role in the etiology of essential hypertension.

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High-density association study and nomination of susceptibility genes for hypertension in the Japanese National Project

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Essential hypertension is one of the most common, complex diseases, of which considerable efforts have been made to unravel the pathophysiological mechanisms. Over the last decade, multiple genome-wide linkage analyses have been conducted using 300–900 microsatellite markers but no single study has yielded definitive evidence for 'principal' hypertension susceptibility gene(s). Here, we performed a three-tiered, high-density association study of hypertension, which has been recently made possible. For tier 1, we genotyped 80 795 SNPs distributed throughout the genome in 188 male hypertensive subjects and two general population control groups (752 subjects per group). For tier 2 (752 hypertensive and 752 normotensive subjects), we genotyped a panel of 2676 SNPs selected (odds ratio $\geq$ 1.4 and $P \leq 0.015$ in tier 1) and identified 75 SNPs that showed similar tendency of association in tier 1 and tier 2 samples ($P \leq 0.05$ for allele frequency and $P \leq 0.01$ for genotype distribution tests). For tier 3 (619 hypertensive and 1406 normotensive subjects), we genotyped the 75 SNPs and found nine SNPs from seven genomic loci to be associated with hypertension

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($P \leq 0.05$). In three of these loci, the lowest *P*-values were observed for rs3755351 ($P = 1.7 \times 10^{-5}$) in *ADD2*, rs3794260 ($P = 0.0001$) in *KIAA0789* and rs1805762 ($P = 0.0003$) in *M6PR* when case–control comparison was made in the combined data. An SNP (rs3755351) within *ADD2* had the lowest *P*-value and its experiment-wide significance level is 0.13. Thus, these results have nominated several susceptibility genes for hypertension, and independent replication will clarify their etiological relevance.

INTRODUCTION

Essential hypertension (MIM 145500) is a multifactorial trait, in which interactions among genetic, environmental and demographic factors are involved. Substantial contribution of genetic factors to the overall disease etiology has been documented by a number of epidemiological studies. For example, family studies controlling for a common environment indicate that blood pressure heritability is in the range of 15–35% (1–3). Accordingly, considerable efforts have been made in the study of molecular genetics of hypertension, but the inherently complex nature has hampered progress in the elucidation of the genes involved (4). Over the last decade, multiple genome-wide linkage analyses have been conducted by using microsatellite markers to localize genes influencing hypertension status and/or blood pressure levels in a number of populations derived from various ethnic groups. Although no single study has so far yielded definitive evidence for 'principal' hypertension susceptibility gene(s), some of these studies provide consistency of linkage results in a few chromosomal regions (5–7). It is therefore assumed that multiple genes contribute to the etiology of hypertension independently or synergistically, with each gene exerting small effects under a certain environmental condition.

In parallel with family-based linkage analyses across the entire genome, population-based association studies have been performed, particularly focusing on individual candidate genes to search for genetic influences on hypertension. Association studies for mapping disease-related genes have recently gained popularity over traditional family-based linkage analyses mainly because of their far greater statistical power to detect the presence of genes with relatively 'minor' effects (8,9). Some researchers criticize the liability to false-positive or non-replicable claims. Nevertheless, population-based association studies have become an alternative and complementary approach to family-based linkage analyses in practice.

Given the limitation of statistical power that can be achieved by family-based linkage analyses with sample size practically collectable, population-based association studies are now underway in a genome-wide scale for a number of multifactorial diseases (10). Here, we performed a high-density association study of hypertension with a three-tiered genotyping approach in the Japanese population (Fig. 1).

RESULTS

Multi-tiered case–control study

We performed a large-scale case–control association study of hypertension using SNP markers selected from the Japanese SNP (JSNP) database (11,12). These SNP markers were distributed throughout the genome (Table 1). Only male hypertensive individuals were tested in tier 1, and a total of 80 795 SNPs distributed on 22 autosomes were used for the association study. Details of the high-throughput genotyping were same as previously described (13,14), and technical evaluation of our genotyping assay (e.g. overall success rate and accuracy of the genotyping assay) is shown in the supplementary material (Supplementary Explanation). JSNP had been developed as a database for the SNP discovery project with particular focus on common gene variations in the Japanese population. Although SNP marker resources used in the current study showed a certain degree of diversity in terms of the number of typed SNPs per gene locus, this partially reflected the variable size of re-sequenced fragments depending on the individual gene structure (12).

The gene-centered genome-wide exploratory test in tier 1 identified 2676 SNPs with odds ratio (OR) ≥ 1.4 and $P \leq 0.015$ in at least one test comparing allele frequency and/or genotype distribution (dominant or recessive models) between 188 hypertensive patients and 752 population control subjects in either of two panels (see Materials and Methods). In this exploratory test, the SNPs showing inverted tendency of OR between two pairs of case–control comparisons and significant deviations from Hardy–Weinberg equilibrium (HWE) in any panel ($P \leq 0.01$) were excluded. Subsequently, we performed a screening of these 2676 SNPs with 752 hypertensive patients and 752 normotensive controls in tier 2, which constituted the first 'case versus unaffected control' study panel, i.e. comparison between 940 cases and 752 controls, together with the 188 cases in tier 1. On the basis of relatively stringent criteria, we identified 75 SNPs that showed *P*-values of ≤ 0.01 for genotype distribution and *P*-values of ≤ 0.05 for allele frequency in the χ^2-test statistic. To further examine the association signals, we performed a replication study of these 75 SNPs with another panel of 619 hypertensive subjects and 1406 normotensive controls in tier 3. Cases and unaffected controls collected in tiers 2 and 3 were enrolled according to the identical criteria and their baseline characteristics are shown in Table 2. There were some trait differences in cases between tiers 2 and 3, such as blood pressure measurements and percentages of the subjects taking anti-hypertensive medication. This could be largely attributed to differences in sample enrollment settings between tiers 2 and 3; that is, cases in tier 3 were enrolled from either the annual medical checkup of a medical institution or the clinic practices of general practitioners, whereas a major part of cases in tier 2 were from the clinic practices of university hospitals. Among the 75 SNPs showing *P*-values between 0.05 and 4.4×10^{-5} in the first 'case versus unaffected control' study, only nine SNPs showed borderline association (at the level of $P \leq 0.05$) in

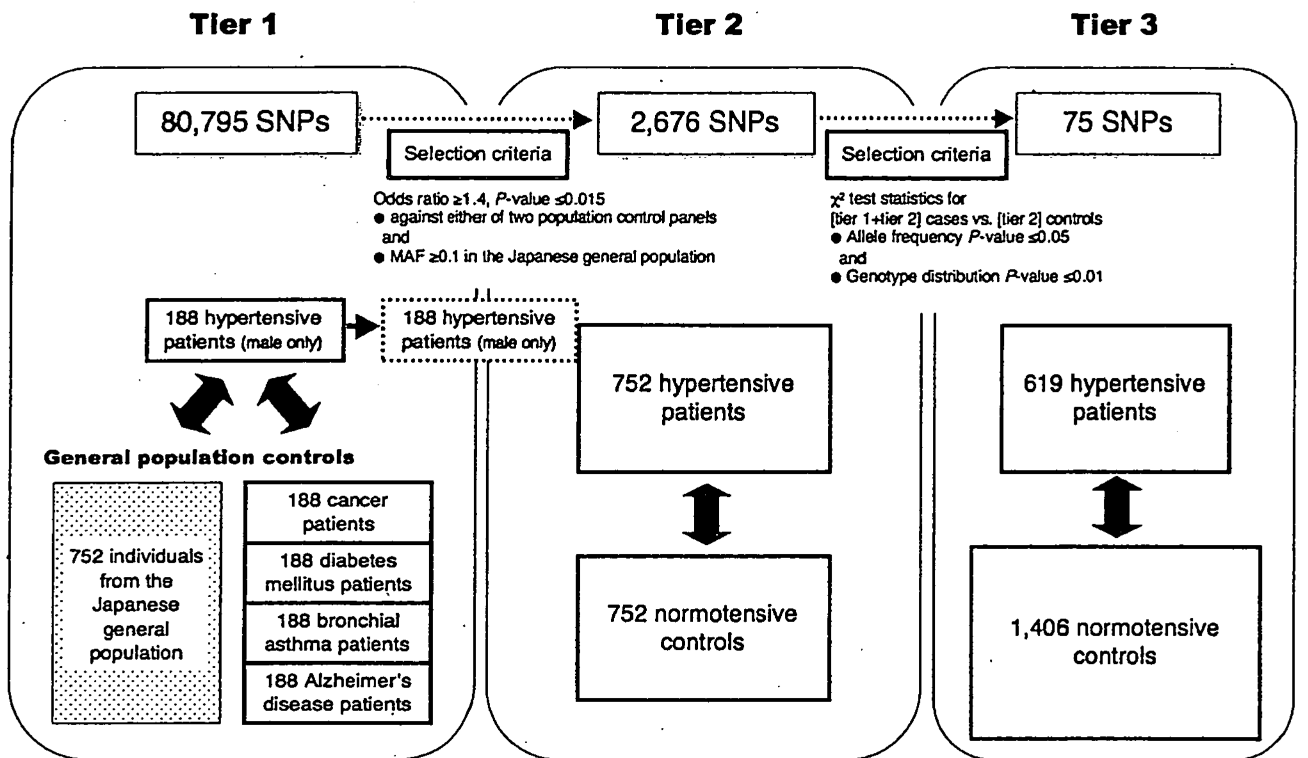

Figure 1. Schematic presentation of a three-tiered screening strategy in the present study. Gene-centered genome-wide exploratory test was performed in tier 1, followed by case–control study of disease-associated SNPs in tier 2 and tier 3 samples. A panel of 188 male hypertensive patients were compared with each of two population control panels in tier 1. Subsequently, 'case versus unaffected control' study was repeated twice to identify the best candidate SNPs. In transitions from tier 1 to tier 2, and from tier 2 to tier 3, the number of SNPs was reduced according to the selection criteria that we arbitrarily defined. See details in the Materials and Methods section.

the second 'case versus unaffected control' study (Fig. 2). Of these, we found six SNPs that showed *P*-values of $\leq$0.05 for both genotype distribution and allele frequency in the χ^2-test statistic (Table 3). rs3755351 and rs3771426 were located within the assumed intron 1 of *ADD2*, rs3787240 and rs3787241 were located in the same intron of *EYA2*, and the remaining two SNPs—rs3794260 and rs1805762—each located in *KIAA0789* and *M6PR*. To adjust for three covariates—age, gender and body mass index (BMI), we also performed logistic regression analysis for the significant SNPs (Supplementary Material, Table S1). With consideration of genetic model consistency, an SNP (rs3755351) showed the strongest association in the identical model (an additive model by logistic regression analysis) among three tiers. Further details of the association results are described in the Discussion.

SNP discovery and further test of association in three selected genes

Because a group of SNPs from three genes, *ADD2*, *KIAA0789* and *M6PR*, were particularly noted for their significant association with hypertension (Table 3), we searched for potentially functional SNPs by re-sequencing the 5′- and 3′-untranslated regions, all exons and exon–intron borders of the individual loci, on the basis of the gene structure deposited in the human genome database (http://www.ncbi.nlm.nih.gov/). We detected a total of 74 SNPs—25 SNPs in *ADD2*, 40 SNPs in *KIAA0789* and 9 SNPs in *M6PR*—and thereby selected 25 tag SNPs for genotyping 2025 subjects in tier 3 (see Supplementary Material, Table S2A, B and C). Apart from four SNPs which had been already included in the JSNP screening marker set, we found four additional SNPs, two in *ADD2* (rs2024453 and rs10084293) and one each in *KIAA0789* (rs9739493) and *M6PR* (rs1805725), to be significantly associated with hypertension (Table 3). Thus, in each gene, we identified at least two SNPs showing modest evidence of association with hypertension ($P \leq 0.05$ level in tier 3) but these SNPs did not necessarily belong to the same linkage disequilibrium (LD) block (Fig. 3 and LD group in Supplementary Material, Table S2A, B and C). The analysis of haplotypes inferable from tag SNPs did not show more significant disease association than the analysis of individual SNPs in any of three genes tested (data not shown).

Consideration of study power and multiple testing

We first estimated a type I error probability for the three-tiered screening to be 6.8×10^{-5}: 0.036 for tier 1, 0.0009 for tiers 1

Table 1. Summary of SNPs genotyped in tier-1 screening and genome coverage estimated by HapMap data

Chromosome	From JSNP screening markers		JSNP overlapped with HapMap (overlap)	From HapMap data (Release 21, JPT)			
	Total SNPs in JSNP	Proportion of SNPs unique to JSNP		SNPs in close LD ($r^2 \geq 0.8$) with overlap	(HapMap total−NA) SNPs	Coverage estimate: SNPs in LD ($r^2 \geq 0.8$)/(HapMap total−NA) SNPs	Total SNPs in HapMap
1	8378	0.370	5281	26 236	113 362	0.231	139 002
2	7336	0.293	5189	28 763	123 447	0.233	160 546
3	5128	0.358	3290	17 494	91 985	0.190	125 160
4	3172	0.366	2010	11 998	74 080	0.162	114 809
5	4973	0.311	3427	18 432	91 206	0.202	122 243
6	6220	0.272	4527	26 182	110 532	0.237	134 177
7	5813	0.358	3731	18 241	81 727	0.223	99 808
8	2388	0.246	1800	12 644	80 400	0.157	111 953
9	2818	0.218	2203	12 358	70 091	0.176	91 908
10	3159	0.322	2141	13 552	81 103	0.167	100 771
11	3636	0.248	2735	14 953	75 147	0.199	95 905
12	3816	0.223	2964	15 188	73 983	0.205	89 436
13	1291	0.290	917	6717	48 622	0.138	75 956
14	2913	0.219	2275	11 583	50 769	0.228	62 203
15	2311	0.194	1863	9903	46 599	0.213	54 210
16	2677	0.268	1959	8227	43 415	0.189	51 865
17	3246	0.258	2408	10 050	38 550	0.261	41 725
18	1243	0.207	986	6370	41 494	0.154	56 203
19	3392	0.308	2346	7626	25 524	0.299	26 949
20	2588	0.402	1548	8784	41 725	0.211	45 582
21	1761	0.275	1276	5777	23 465	0.246	26 892
22	2536	0.280	1825	7569	24 402	0.310	25 077
Total	80 795	0.298	56 701	298 647	1 451 628	0.206	1 852 380

The numbers of SNPs genotyped in tier-1 screening are demonstrated for each chromosome. Genome coverage was assessed with the HapMap data from JPT ($n = 45$); that is, the proportion of HapMap SNPs showing high r^2 (≥ 0.8) to one of the SNPs genotyped in this study (which are all derived from JSNP) is calculated. Because substantial part of the SNPs have turned out to be unique to JSNP, those overlapping with the HapMap SNPs, in the 'overlap' column, are used to estimate genome coverage. Here, NA represents a category of SNPs which have been mapped to the genome (NCBI B35) but do not have LD information against the HapMap SNPs. In this context, it is appropriate to reduce this NA SNPs from total SNPs deposited in the HapMap data when estimating genome coverage and we therefore use the number of SNPs (HapMap total−NA) as a denominator.

and 2 combined and 0.076 for tier 3 screening. Then, we estimated overall sensitivities (which could represent the statistical power) to be 0.10–0.45, 0.04–0.23 and 0.01–0.08 for a disease-associated SNP of OR = 1.4, 1.3 and 1.2, respectively, assuming the disease allele frequency within 0.1–0.9, the disease prevalence of 0.25 and the multiplicative genotype model. Since we had adopted relatively generous criteria for screening association signals, we evaluated the false discovery rate (FDR) to account for multiple testing (15). FDR for the nine SNPs found as significant was 0.69. A multi-staged screening in the current study could be largely categorized into two steps: tiers 1 and 2 (which constitute the first 'case versus unaffected control' study) and tier 3 (which constitutes the second 'case versus unaffected control' study). We therefore assessed experiment-wise type I errors with particular focus on the last-stage screening in tier 3. By permutation, the chance of observing a *P*-value of 0.0019 (for allele frequency test at rs3755351 in *ADD2*) in tier 3 was estimated to be 0.13.

DISCUSSION

With the recent advent of high-throughput genotyping technologies and high-resolution maps of SNP markers, it is expected that genome-wide association studies allow us to identify systematically the contributions of common genetic variations to human multifactorial diseases (16–18). In this line, our study has attempted to discover common hypertension susceptibility gene variants via a gene-centered genome-wide association design for the first time. Despite the modest genetic impacts assumed for hypertension, e.g. the λ-values (the relative risk for siblings of the affected probands) have been reported to be approximately 4 (19), we have nominated several susceptibility genes for hypertension (Table 3). Among these genes, findings for *ADD2* and *KIAA0789* are particularly noteworthy, because the former has been known to be a physiological candidate gene for hypertension and the latter is a novel gene with as-yet unknown physiological function.

Through a multi-tiered screening, nine SNPs derived from seven distinct gene loci have remained to show some evidence of association out of the 80 795 SNPs initially screened. Although the selection criteria were arbitrarily defined in the present study, a small percentage of the SNPs have passed the criteria in transitions from tier 1 to tier 2 (3.3%) and from tier 2 to tier 3 (2.8%). In the *ADD2* gene, for example, the minor allele frequency (MAF) of rs3755351 is lower in case groups (0.14–0.19) than that in control groups (0.21–0.22) throughout three tiers. A *P*-value of 1.7×10^{-5} and an OR of 1.30 (95% CI 1.15–1.46) are attained for allele frequency comparison of rs3755351 when the subjects studied in different tiers are combined and finally categorized into

Table 2. Clinical characteristics of participants

Variables	Case group Tier 2 panel	Tier 3 panel	Control group Tier 2 panel	Tier 3 panel
Number of subjects (female/male)	752 (353/399)	619 (280/339)	752 (366/386)	1406 (650/756)
Present age, year	62.4 ± 10.3	54.1 ± 8.4[a]	62.0 ± 8.7	58.4 ± 6.6
Age of onset, year	47.3 ± 10.2	43.2 ± 9.9	—	—
Current BMI, kg/m^2	23.9 ± 3.2	25.1 ± 3.6[a]	22.5 ± 2.8	22.4 ± 2.7
Smoking[b]				
None, %	48.6	61.6	66.0	58.6
Previous smoker, %	—	17.0	—	10.2
Current smoker, %	51.4	21.4	34.0	31.2
Blood pressure				
Systolic blood pressure, mmHg	146.4 ± 19.5[a]	150.9 ± 19.3[a]	113.8 ± 9.8	114.4 ± 10.1
Diastolic blood pressure, mmHg	86.4 ± 13.0[a]	91.4 ± 12.2[a]	69.8 ± 7.7	70.3 ± 7.2
Treatment of hypertension, %	92.6	75.4	—	—
Blood chemistry				
Serum creatinine, mg/dl	0.87 ± 0.69[a]	0.75 ± 0.50[c]	0.73 ± 0.18	0.70 ± 0.23
Fasting plasma glucose, mg/dl	105.3 ± 28.7	109.0 ± 31.0[a]	104.0 ± 41.9	99.2 ± 22.7
Serum total cholesterol, mg/dl	204.4 ± 31.1[c]	213.4 ± 33.8	209.2 ± 33.7	215.6 ± 34.1
Serum triglyceride, mg/dl	129.8 ± 82.2[a]	141.3 ± 124.6[a]	108.1 ± 67.1	110.1 ± 71.6
Serum HDL cholesterol, mg/dl	56.2 ± 16.7[a]	61.9 ± 19.5	60.6 ± 16.0	63.5 ± 17.5

Values are means ± SD.
For some variables, subjects with insufficient information are not included in the calculation.
[a]$P < 0.001$, case group versus control group by the unpaired t-test in each tier.
[b]Because of differences in the questionnaire, smoking status is categorized into two groups (non-smoker or smoker) in the tier 2 panel.
[c]$P < 0.01$, case group versus control group by the unpaired t-test in each tier.

the case (tiers 1–3) and unaffected control (tiers 2 and 3) groups. None of our results appears to be significant with the use of a strict Bonferroni correction, a very conservative evaluation of significance, and further replication in an independent population is indispensable.

The candidacy of *ADD2* as a hypertension susceptibility gene has been supported by several physiological and biochemical findings (20–22), together with some evidence from the studies of molecular genetics (23–27). Adducin is a ubiquitously expressed membrane-skeleton heteromeric protein composed of different subunits, α-, β- and γ-subunits. It is known to play a substantial role in the regulation of membrane ion transport. Point mutations of the α- and β-adducins account for up to 50% of the blood pressure difference between Milan hypertensive and normotensive rat strains, probably via the modulation of the Na^{+}–K^{+} ATPase activity (one of major Na^{+}-channels) in the kidney (23,24). In this line, of note is the fact that β-adducin-deficient mice show significant increases in systolic and diastolic blood pressures and pulse pressure (21). The human homolog of β-adducin spans over 100 kb on chromosome 2p13 and comprises 17 exons. It has been reported that a common SNP (rs4984) identified at position 1797 in exon15 is associated with an increased risk of hypertension under certain pathological conditions in European populations (25–27), whereas this SNP itself is not polymorphic in Asian populations (http://www.ncbi.nlm.nih.gov/SNP/). Also, it has to be noted that one previous study (28) showed significant evidence for hypertension linkage in the 2p13 region (a peak of 2.84 LOD at 93 cM), where the *ADD2* locus is exactly located among several positional candidate genes. Despite our investigation in the *ADD2* locus, we could not find either a clear LD block-like structure or potentially functional SNPs in the vicinity of three disease-associated SNPs (rs2024453, rs3755351 and rs3771426), which are located in the putative promoter region and intron 1, apart from rs10084293 located within an LD block of *ADD2* (Fig. 3). We have assessed the independence of multiple associated SNPs in *ADD2* by logistic regression analysis and have found that the observed association in this gene could be explained principally by the most significant SNP (rs3755351) (see Supplementary Explanation). Once these associations are validated in an independent study panel, further extensive searches of functional SNPs in the *ADD2* locus are warranted.

Our high-density association study has also highlighted the *KIAA0789* gene located on chromosome 12q23.3. This gene encodes a hypothetical protein, LOC9671, which is expressed principally in the central nervous system and modestly in the pancreas (unpublished data). The predicted gene structure of *KIAA0789* involves 9 exons, spanning ~120 kb. There is a clear LD block in the 5′ region of the putative exon 1 (~3.8 kb in size), whereas we have found two other LD block-like structures within the *KIAA0789* gene (Fig. 3). Two disease-associated SNPs (rs3794260 and rs9739493) have turned out to reside in different LD blocks, and the construction of their haplotypes does not seem to provide much additional information on disease association. Although the precise gene structure and gene function remain unknown, *KIAA0789* appears to contain a carboxy-binding WSC domain, and its homologs are likely to exist in mice and rats according to the database information (http://www.ncbi.nlm.nih.gov/). Again, detailed investigation including independent

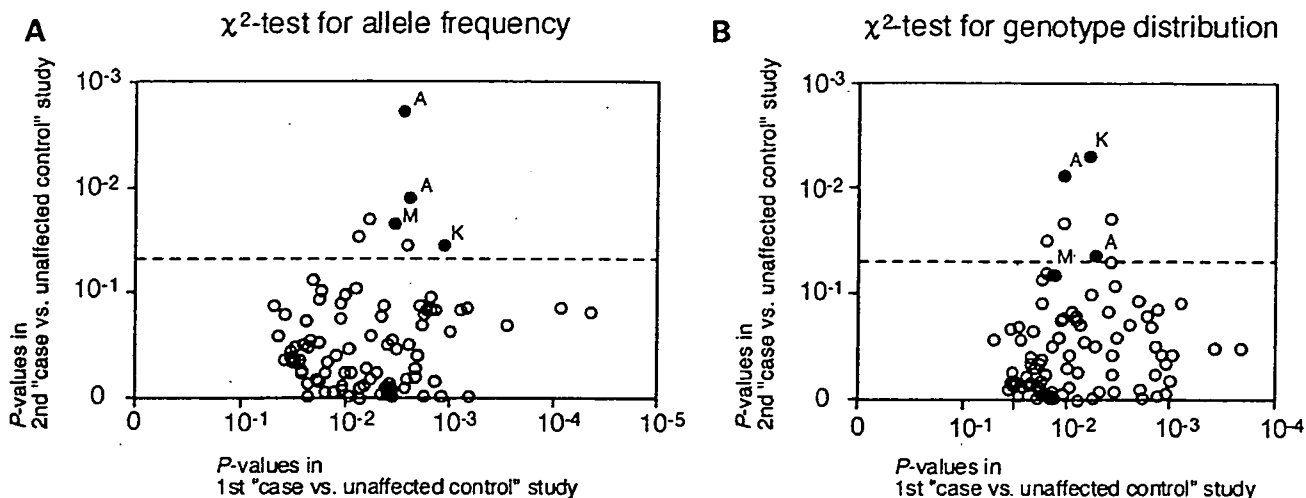

Figure 2. Statistical significance of χ^2-test of disease association in the two-staged 'case versus unaffected control' study. $-\text{Log}_{10}$ P-values were used instead of raw P-values in each scatter plot. The dashed lines indicate $P = 0.05$. (A) As for SNPs genotyped in the second 'case versus unaffected control' panel, P-values for allele frequency in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles to which the following symbols are attached: A, *ADD2*; K, *KIAA0789*; M, *M6PR*. (B) As for SNPs genotyped in the second 'case versus unaffected control' panel, P-values for genotype distribution ([2 × 3] contingency table) in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles as mentioned earlier.

replication of disease association will lead us to clarify the etiological relevance of *KIAA0789* to hypertension.

Another, potential disease association, though modest statistical significance, has been found for *M6PR*. The *M6PR* gene encodes a cation-dependent receptor for mannose-6-phosphate groups on lysosomal enzymes and plays a critical role in the segregation and targeting of lysosomal enzymes to lysosomes. Thus far, no functional relation between *M6PR* and hypertension has been reported. Similar to *KIAA0789*, this gene could also allow us to identify a novel, as-yet unnoticed blood pressure regulatory mechanism.

We should bear in mind several limitations inherent in the present study. First, the level of genome coverage is an issue of heated debate (10,18). Some people may argue that our *a priori* marker selection strategy is gene-centric without utilizing LD information and hence it is not sufficient to pick up as many modest associations as possible in genome-wide searches of hypertension susceptibility genes. A comprehensive framework of common variations throughout the human genome has been made available by the recent completion of the International HapMap Project (29). On the basis of our assessment, the JSNP screening markers in this study cover 20.6% of the HapMap SNPs, whereas a substantial proportion (~30%) of SNPs appear to be unique to JSNP (Table 1 and Supplementary Material, Fig. S1). Under these circumstances, an ideal set of SNPs for our study would encompass deliberately selected tag SNPs (principally common genetic variants) and additional 'singleton' SNPs (sometimes rare genetic variants). Besides this argument of tag SNPs, there are two points of weakness regarding genome coverage as follows: (i) sex chromosome markers have been excluded from the analysis because of the pre-determined policy of multi-disease collaborative study in the Japanese Millennium Genome Project, and (ii) a substantial part of the expressed human genes is not covered by the JSNP database (11), in which the fundamental SNP data were almost fixed in the middle of 2003. Second, the statistical power attainable by our study panel needs to be taken into consideration. For the last few years, genotype costs have fallen dramatically, yet present economic and experimental conditions make it necessary, in practice, to reduce the number of genotyped samples down to a moderately sized case group (188 subjects in our study) at the initial screening with approximately 80 000 SNPs. We arbitrarily set the selection criteria of $\text{OR} \geq 1.4$ and $P \leq 0.015$ in transition from tier 1 to tier 2, where the overall statistical power is estimated to be 10–45% for a disease-associated SNP of $\text{OR} = 1.4$ and 1–8% for that of $\text{OR} = 1.2$, assuming the disease allele frequency within 0.1–0.9 and the disease prevalence of 0.25. Thus, it is likely that our study design allows for capturing less than half of the true disease associations particularly with regard to modest genetic susceptibility. Third, ethnic diversity has not been tested within the scope of the present study. Instead of using commercially available SNP sets aimed at full genomic coverage, we have attempted to focus on potentially functional variants and also relatively common SNPs ($\text{MAF} \geq 0.1$) in the Japanese population. Accordingly, some of disease-associated SNPs listed in Table 3 may be rare or not polymorphic in the other ethnic groups. To clarify allele frequency representation of individual loci and etiological impacts attributable to them, further examination is required in the context of ethnic diversity.

During our preparation of this report, two genome-wide association studies for hypertension and/or blood pressure have been performed in Caucasians (30,31). When our results are compared with public data sets for these association statistics, a few SNPs in the regions of interest appear to show a tendency of association with hypertension or blood pressure;

Table 3. Summary of genomic SNPs associated with hypertension status in two-staged 'case versus unaffected control' study

dbSNP number	Gene symbol	Major/ minor allele	Orientation	Minor allele frequency						Association analysis												
				Case			Control			*P*-value in the first 'case (tiers 1 and 2) versus unaffected control (tier 2)' study				*P*-value in the second 'case (tier 3) versus unaffected control (tier 3)' study				Total subjects: case (tiers 1–3) versus unaffected control (tiers 2 and 3)				
				Tier 1	Tier 2	Tier 3	Tier 1[a] (Ref. 1/ Ref. 2)	Tier 2	Tier 3	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency *P*-value	Dominant *P*-value	Recessive *P*-value	Allele frequency model *P*-value	OR (95% CI)[b]
rs3755351	ADD2	C/A	fwd	0.14	0.19	0.17	(0.22/0.21)	0.22	0.21	0.011	0.041	0.007	0.003	0.007	0.071	0.003	0.002	0.00009	0.009	0.00006	0.00002	1.30 (1.15–1.46)
rs3771426	ADD2	T/C	fwd	0.13	0.16	0.16	(0.19/NA)	0.2	0.19	0.005	0.006	0.016	0.003	0.043	0.093	0.025	0.012	0.0003	0.003	0.0007	0.00008	1.28 (1.13–1.45)
rs2024453	ADD2	T/C	rev	—	—	0.29	—	—	0.33	—	—	—	—	0.034	0.013	0.132	0.021	—	—	—	—	—
rs10084293	ADD2	G/A	rev	—	—	0.44	—	—	0.48	—	—	—	—	0.089	0.083	0.063	0.027	—	—	—	—	—
rs3794260	KIAA0789	G/A	fwd	0.14	0.17	0.17	(0.21/0.18)	0.21	0.20	0.006	0.161	0.002	0.001	0.005	0.001	0.256	0.035	0.0008	0.006	0.001	0.0001	1.26 (1.12–1.42)
rs9739493	KIAA0789	T/C	fwd	—	—	0.41	—	—	0.44	—	—	—	—	0.026	0.008	0.641	0.041	—	—	—	—	—
rs1805762	M6PR	C/G	rev	0.21	0.22	0.22	(0.25/0.24)	0.26	0.25	0.015	0.045	0.009	0.003	0.061	0.332	0.019	0.022	0.001	0.615	0.0003	0.0003	1.23 (1.10–1.37)
rs1805725	M6PR	T/G	fwd	—	—	0.54	—	—	0.49	—	—	—	—	0.007	0.002	0.123	0.005	—	—	—	—	—
rs3787240	EYA2	C/T	fwd	0.25	0.21	0.22	(0.19/0.18)	0.18	0.19	0.003	0.001	0.059	0.008	0.083	0.227	0.034	0.028	0.001	0.002	0.007	0.0010	0.82 (0.73–0.92)
rs3787241	EYA2	G/A	fwd	0.24	0.21	0.22	(8.19/0.19)	0.18	0.19	0.008	0.003	0.080	0.014	0.104	0.267	0.042	0.037	0.002	0.003	0.01	0.0017	0.83 (0.74–0.93)
rs3761987	—	T/A	fwd	0.43	0.37	0.37	(0.36/0.36)	0.33	0.34	0.004	0.001	0.101	0.004	0.019	0.566	0.015	0.134	0.0007	0.031	0.004	0.0016	0.86 (0.78–0.94)
rs3741691	THAP2	A/C	fwd	0.26	0.22	0.22	(0.21/0.20)	0.19	0.21	0.011	0.043	0.007	0.002	0.021	0.139	0.066	0.317	0.007	0.727	0.002	0.0064	0.85 (0.76–0.96)
rs1298463	CCDC131	A/G	fwd	0.26	0.22	0.22	(0.21/0.19)	0.19	0.21	0.016	0.052	0.010	0.004	0.031	0.182	0.073	0.312	0.012	0.736	0.0033	0.0096	0.86 (0.77–0.96)

Two SNPs of EYA2, rs3787240 and rs3787241, are located closely (only 296 bp apart) and in complete LD ($r^2 = 1.00$) to each other.

Also, rs3741691 and rs1298463 are located closely (44 kb apart) and have turned out to be in strong LD ($r^2 = 0.99–1.00$) to each other.

[a]In tier 1 control subjects, the figures in parentheses are minor allele frequencies (MAFs) calculated separately in the Ref. 1 panel—the other disease patients who can be regarded as arbitrary general controls; Ref. 2 panel—752 individuals from the Japanese general population (see Materials and Methods).

[b]The OR was calculated as the ratio of the odds of disease in chromosomes with major alleles relative to those without them.

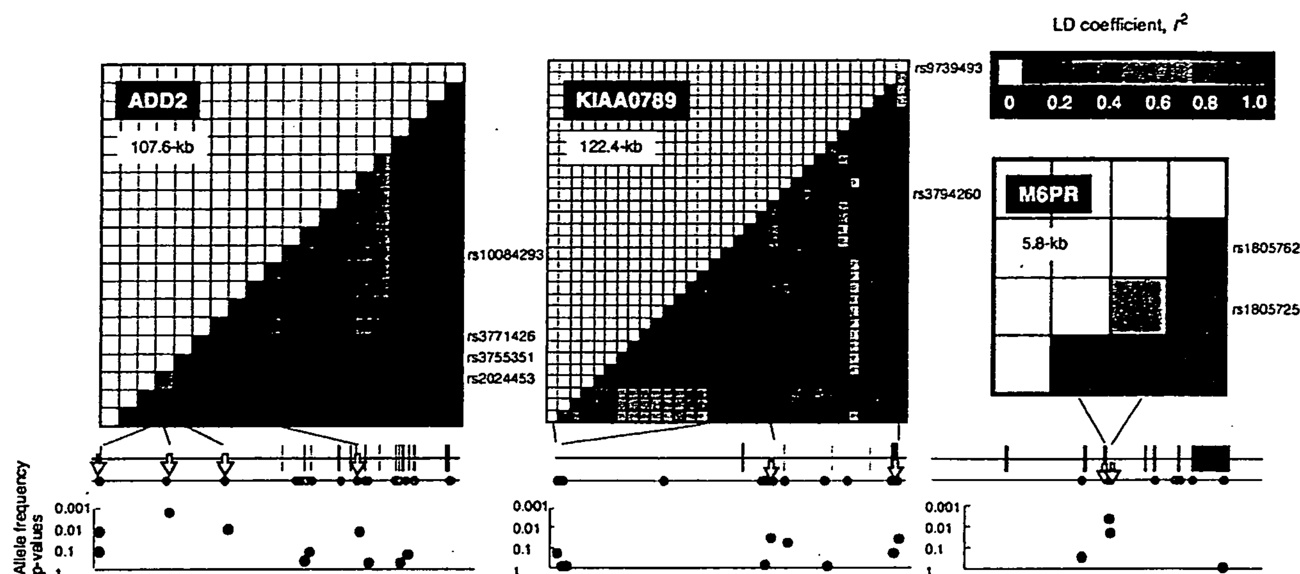

Figure 3. LD relations between SNPs in the *ADD2*, *KIAA0789* and *M6PR* genes (top) and disease association of markers from the corresponding genomic regions (bottom). In the top, the LD between a pair of markers is indicated by the color of the block above and to the left of the intersection of the markers. For the sake of readability, only the names of SNPs showing significant association are shown to the right of the vertical axis of the LD plot. The rest of the SNP information is described in Supplementary Material, Table S2A, B and C. In the upper bottom, the location of genetic markers studied in the corresponding genomic region is shown with relation to gene structure. Here, green and red circles indicate the SNPs with low (MAF < 0.05) and high (MAF ≥ 0.05) allele frequencies in the Japanese, respectively. In the lower bottom, $-\log_{10}$ *P*-values of the differences in allele frequencies between case and control subjects in tier 3 (i.e. the second 'case versus unaffected control' study) were plotted against the location of individual genetic markers genotyped.

for example, an SNP (rs17006246) in *ADD2*, which is in strong LD ($r^2 = 0.806$ and $D' = 1$ in the HapMap JPT population) with rs3755351, the most significant SNP in our study, is modestly associated with hypertension status ($P = 0.029$) in the Diabetes Genetics Initiative (DGI) study but the direction of effect is opposite between rs3755351 typed in this study and rs17006246 typed in the DGI study. On the other hand, rs1805740, in strong LD with an SNP (rs1805762) in *M6PR*, is modestly associated with hypertension status ($P = 0.036$) in the Wellcome Trust Case Control Consortium study with the same direction of effect as in this study (see Supplementary Material, Table S3).

In summary, our high-density association study provides a list of gene loci potentially predisposing people to hypertension, which awaits replication across populations. With the available samples, we have observed an association of SNPs including three SNPs clusters (or gene loci) in the Japanese populations. In face of the complex nature of disease etiology, it seems to be a formidable task but worth challenging that we eventually apply the SNPs information to improved prevention, diagnosis and treatment of hypertension.

MATERIALS AND METHODS

Study design

We performed a large-scale association study for genes susceptible to hypertension by using a three-tiered genotyping approach (tiers 1, 2 and 3) as depicted in Figure 1. All methods of the study were approved by the review committees of the individual institutions involved in the present study. All subjects provided written informed consent for participation.

In the gene-centered genome-wide exploratory test in tier 1, we carried out genotyping of 83 802 SNPs (3007 of which were excluded from the analysis because they are on sex chromosomes or in the unknown locations) using genomic DNAs from 188 Japanese male hypertensive patients and 752 unrelated Japanese individuals (referred to as general population controls) and another panel of 752 Japanese subjects (referred to as arbitrarily defined controls) who were affected with any of the other four common diseases including gastric cancer, diabetes mellitus, bronchial asthma and Alzheimer's disease; each of these was investigated as the 'Japanese Millennium Genome Project' (Fig. 1). The theoretical basis of adopting this exploratory test scheme was previously reported elsewhere (32). Cases were enrolled from the clinical practice or the annual medical checkup of university hospitals and medical institutions according to the uniformly defined criteria. These included (i) systolic blood pressure ≥160 mmHg, diastolic blood pressure ≥95 mmHg, or both on two consecutive visits for untreated subjects; (ii) patients receiving long-term antihypertensive treatments; (iii) no secondary form of hypertension as evaluated by an extensive workup; (iv) family history of hypertension, i.e. at least one hypertensive subjects detectable among parents and siblings of the participants; (v) an age of onset known to be between 30 and 59 years. Moreover, only male subjects with BMI < 25 kg/m^2 were selected in tier 1. We compared allele frequencies and/or genotype distributions in hypertensive patients and two population control panels and evaluated deviation from HWE at each of the genotyped loci. For the subsequent screening

in tier 2, we selected SNPs (i) with OR $\geq$ 1.4 and $P \leq 0.015$ against either of two population control panels and with concordant OR tendency against two control panels; (ii) with MAF $\geq$ 0.1 and (iii) not showing significant deviations ($P =$ 0.01 level) from Hardy–Weinberg expectations in the patient or control panels.

In tier 2 (which comprised 752 hypertensive patients and 752 normotensive controls), we further tested the SNPs thus screened in tier 1, which effectively constituted the first 'case (tiers 1 and 2) versus unaffected control (tier 2)' study. Here, cases in tier 2 were selected according to the criteria (i)–(v) mentioned earlier for tier 1. Normotensive controls, on the other hand, were defined as follows: (i) systolic blood pressure $\leq$130 mmHg and diastolic blood pressure $\leq$85 mmHg without receiving antihypertensive treatments; (ii) age $\geq$50 years and (iii) no family history of hypertension. Both males and females were included in tier 2 without reference to BMI. We selected SNPs (i) with P-value $\leq$0.05 when comparing allele frequency; and (ii) with P-value $\leq$0.01 when comparing genotype distribution between (tiers 1 and 2) cases and (tier 2) controls by χ^2 test statistics.

In tier 3 (which comprised 619 hypertensive patients and 1406 normotensive controls), we performed the second 'case versus unaffected control' study to examine significant associations observed in tiers 1 and 2. The diagnostic criteria in tier 3 were identical to those in tier 2. For the assessment of assumptions when using statistical models in the present study, quantile–quantile plots of P-values were depicted for each stage of association test described in Supplementary Explanation.

No significant population stratification was observed for samples in tier 1 when it was assessed with the methods reported by Patterson *et al.* (33). However, the presence of population stratification was indicated for samples in the first stage 'case (tiers 1 and 2) versus unaffected control' study. We observed moderate bias in genotype frequency of some SNPs between the two tiers, which may have resulted from technical/experimental artifacts between genotyping of cases in tiers 1 and 2. Therefore, the trend test statistic at this analytical stage was corrected according to the significant eigenvector (see Supplementary Explanation). Stratification in tier 3 was not detected but could not be ruled out because of the relatively small number of SNPs ($n = 75$) genotyped in tier 3. As for the nine SNPs that showed significant disease association after multi-stage screening, they were not correlated with the significant eigenvector detected in tiers 1 and 2 cases and tier 2 controls. The P-values for nine SNPs were similar between the nominal and the EIGENSTRAT-corrected ones; for example, the nominal P-value was 0.0029 and the EIGENSTRAT-corrected P-value was 0.0069 at rs3755351 in *ADD2*.

SNP marker resource and genotyping

Most of the SNP markers used in the present study were same as the markers used in the previous reports (14) and derived from the JSNP database. The samples in tiers 1 and 2 were genotyped by PCR amplification of multiple genomic fragments with 20 ng of genomic DNA followed by characterization with the invader assay. Genotyping of the samples in tier 3 was undertaken using the TaqMan® SNP Genotyping Assays (Applied Biosystems). To secure the accuracy and completeness of genotyping, which is critical for large-scale studies (34), we attached a set of 'flags' to individual SNP data mainly dependent on the data completeness, after two independent investigators had checked the raw data robustness by looking at the scatter plot of the assay.

SNP discovery in the selected genes

Approximately 38 kb of genomic sequence spanning the exons and the 5'- and 3'-untranslated regions of three genes, *ADD2*, *KIAA0789* and *M6PR*, was re-sequenced in 48 Japanese control individuals to identify potentially functional SNPs. Since *KIAA0789* had not been fully annotated, the arbitrary positions of translation initiation sites were estimated according to the human genome database. From the SNPs thus identified, tag SNPs were selected for the three genes with the algorithm that we previously reported (35). These tag SNPs were then used for the case–control analysis in tier 3 to further examine association signals seen throughout the multi-staged screening. We deposited the identified SNP information in the NCBI's SNP database and also in our own database, JMDBase (Japan Metabolic Disease Database).

Statistical analysis

The SNPs were tested individually for the statistical significance of disease association with the χ^2-test statistic, which evaluated three inheritance models—[2 x 3] contingency table, dominant and recessive models—for genotype distributions and independence on [2 x 2] contingency table for allele frequencies. Here, the most significant P-values among three inheritance models were adopted for genotype distributions when we selected SNPs for screening in tier 3. The criteria for declaring suggestive evidence of disease association were arbitrarily set at each analytical stage as summarized in Figure 1, and they are described in the Results section. SNPs' genotype departures from HWE were tested using the χ^2-test with 1 degree of freedom.

In the three genes showing significant association signals, the extent of LD was measured in terms of an LD coefficient r^2 before the analysis of haplotype structure. Within each LD block, haplotypes were inferred from genotype data by the SNPHAP software for the case and control groups, respectively.

We randomly permutated the genotype of individuals across different panels, 100 times per SNP, and counted the ratio of permutations that fulfill the screening criteria. This ratio indicates the specificity of the study. According to the P-value distribution of the permutations, we evaluated the probability of observing an SNP with P-value no larger than the actual minimum. This probability indicates the experiment-wise P-value. For the specific prevalence and penetrance, we calculated genotype frequency and randomly generated genotypes according to their frequency. We generated genotypes for 1000 simulations of each panel and computed the ratio of simulations that could pass the screening. This ratio is considered the sensitivity of the study.