mice was 14 mmHg. The cyp4a14$^{-/-}$ hypertensive phenotype was spontaneous and insensitive to dietary salt. The cyp4a14$^{-/-}$ male mice had elevated levels of plasma androgens. Castration normalized the blood pressure of the hypertensive cyp4a14$^{-/-}$ male mice, and androgen replacement restored the hypertensive phenotype to the castrated cyp4a14$^{-/-}$ male mice. In a study by Nakagawa *et al.* [11], disruption of the murine cyp4a10 gene caused salt-sensitive hypertension. When cyp4a10$^{-/-}$ mice were fed a low-salt diet, they were normotensive. When the cyp4a10$^{-/-}$ mice were fed a normal or high-salt diet, however, they became hypertensive. Compared with wild-type mice, the male and female cyp4a10$^{-/-}$ mice had a significantly increased systolic blood pressure when fed a 0.3% NaCl diet, with an average increase in systolic blood pressure of 27 and 22 mmHg for the male and female mice, respectively. The findings for the cyp4a14$^{-/-}$ mice and the cyp4a10$^{-/-}$ mice were therefore similar in that the male knockout mice had more severe hypertension than the female mice. These findings suggest that the effects of cyp4a14 and cyp4a10 mediated by androgens are involved in the pathophysiology of hypertension, and that these knockout mice are promising models of gender-specific human hypertension.

The isoforms cyp4a14 and cyp4a10 do not exist in humans. Human CYP4A11 is 72.69% identical with murine cyp4a14, and is 73.02% identical with murine cyp4a10. This suggests that CYP4A11 plays a role in humans similar to that of cyp4a14 and cyp4a10 in mice [12]. In humans, CYP4A11 acts mainly as an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE). In the kidney, CYP4A11 and CYP4F2 are involved in the regulation of blood pressure [13]. Available evidence suggests that the human CYP4A11 gene is an essential hypertension causative gene candidate.

The human CYP4A11 gene is located at chromosome 1p33, spans approximately 12.57 kilo-base-pairs, and contains 12 exons. More than 120 single-nucleotide polymorphisms (SNPs) of the human CYP4A11 gene are listed in the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/SNP).

There have been case–control studies on the association between CYP4A11 and hypertension [14,15], but none of these studies were haplotype-based. The aim of the present haplotype-based case–control study was to assess the association between essential hypertension and the human CYP4A11 gene in Japanese men and women.

Materials and methods

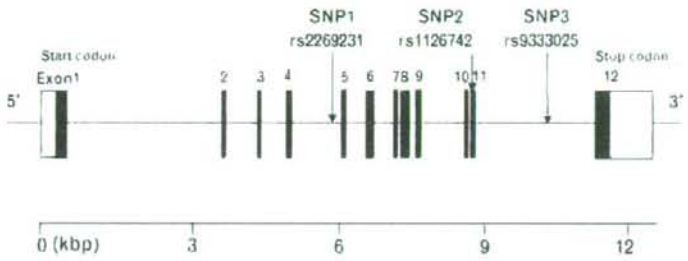
Participants

Patients diagnosed with essential hypertension were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo from 1993 to 2003. We enrolled 304 essential hypertension patients in the present study, with a men/women ratio of 2.27. Essential hypertension was diagnosed based on the following criteria: seated systolic blood pressure greater than 160 mmHg or diastolic blood pressure greater than 100 mmHg on three occasions within 2 months after the first blood pressure reading. None of the essential hypertension patients were receiving antihypertensive medication. Patients diagnosed with secondary hypertension were excluded. A total of 207 normotensive, age-matched individuals (men/women ratio = 1.92) were enrolled as control participants. None of the control individuals had a family history of hypertension, and all had a systolic blood pressure below 130 mmHg and a diastolic blood pressure less than 85 mmHg. Informed consent was obtained from each participant in accordance with the protocol approved by the Human Studies Committee of Nihon University [16].

Genotyping

There are 121 SNPs for the human CYP4A11 gene listed in the National Center for Biotechnology Information SNP database Build 127 (http://www.ncbi.nlm.nih.gov/SNP). We also screened the data for the Tag SNPs on the International HapMap Project website (http://www.hapmap.org/index.html.ja). The data for all SNPs listed for the Japanese population were classified into three groups depending upon frequency, including: having large minor allele frequencies (MAF) of 0.367–0.417, having a middle MAF of 0.179–0.200, and having a MAF less than 0.1. Five SNPs (rs 4660978, rs9333025, rs3890011, rs9333016 and rs9332982) were included in the first group and three SNPs (rs9333029, rs4660980 and rs9332998) were included in the second group. For use as a marker, the first group was thought preferable because the minor allele frequencies were high. Since high r^2 values were shown between the SNP pairs in the first group, we selected one SNP for this group (rs9333025). Genotyping for this SNP was done using a kit from Applied Biosystems Inc. (Foster City, California, USA). There were eight SNPs for the human CYP4A11 gene that were registered in the Japanese Single Nucleotide Polymorphisms database. Of these SNPs there was only one SNP (rs2269231) that was found to have an allele frequency within the Japanese population. We selected rs2269231 for our experiment because the MAF of this SNP was 0.389. This SNP could also be genotyped using the kit from Applied Biosystems Inc. The MAF for both SNPs (rs9333025, rs2269231) was greater than 30%, which indicates that they were effective genetic markers. In addition to this standard, we chose rs1126742, which is a nonsynonymous substitution at amino acid [14], as this SNP has been reported to have a loss-of-function and associated hypertension. This SNP was not registered in the genotyping kit from Applied Biosystems Inc., so we purchased a Custom TaqMan SNP Genotyping Assay (Applied Biosystems Inc.). We designated these SNPs as SNP1 (rs2269231, C_15876257_10), SNP2 (rs1126742) and SNP3

Fig. 1



Structure of the human CYP4A11 gene. The gene consists of 12 exons (boxes) separated by 11 introns (lines; intergenic regions). Filled boxes, coding regions; arrows, locations of single-nucleotide polymorphisms (SNPs). kbp, kilo-base pairs.

(rs9333025, C_29846881_10), which were in order of increasing distance from the 5′ end of the gene (Fig. 1). SNP1 and SNP3 were located in the introns. SNP2, which is located in exon 11, involves a thymidine-to-cytosine substitution (T8590C) that leads to a nonsynonymous phenylalanine-to-serine (F-to-S) substitution at amino acid residue 434 of CYP4A11 [14].

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes by phenol and chloroform extraction [17].

Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems Inc.). The TaqMan SNP Genotyping Assays were performed using the method of Taq amplification [18]. 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 that hybridizes to single-stranded targets with greater sequence-specificity than ordinary DNA probes. This reduces nonspecific probe hybridization, and results in low background fluorescence in the 5′ nuclease PCR assay (TaqMan; Applied Biosystems Inc.). Cleavage results in increased emission of a reporter dye. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe is labeled with two reporter dyes at the 5′ end. In the present study, VIC and FAM were used as the reporter dyes. The primers and probes used in the TaqMan SNP Genotyping Assays (Applied Biosystems Inc.) were chosen based on information available on the Applied Biosystems Inc. website (http://myscience.appliedbiosystems.com).

PCR amplification was performed using 6 μl TaqMan Universal Master Mix, No. AmpErase UNG (2×) (Applied Biosystems Inc.) in a 12 μl final reaction volume containing 2 ng DNA, 0.22 μl TaqMan SNP Genotyping Assay Mix (20× or 40×), primers at a concentration of 900 nmol/l each, and probes at a final concentration of 200 nmol/l each. Thermal cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s; and 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system (Applied Biosystems Inc.).

Each 96-well plate contained 80 DNA samples of an unknown genotype and four reaction mixtures containing reagents but no DNA (control). The control samples without DNA are a necessary part of the Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems Inc.). The plates were read on the SDS 7700 instrument with the end-point analysis mode of the SDS version 1.6.3 software package (Applied Biosystems Inc.). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X–Y scatter-plot of the SDS software. The genotypes were also determined automatically by the signal processing algorithms of the software. The results of each scoring method were saved in two separate output files for later comparison [19].

Biochemical analysis

We measured the plasma concentration of total cholesterol and high-density lipoprotein cholesterol, and the serum concentration of creatinine and uric acid, using standard methods employed by the Clinical Laboratory Department of Nihon University Hospital.

Statistical analysis

All continuous variables were expressed as the mean ± SD. Differences in continuous variables between essential hypertension patients and control individuals were analyzed using the Mann–Whitney U-test. Differences in categorical variables were analyzed using Fisher's exact test. Hardy–Weinberg equilibrium was assessed by chi-squared analysis. Differences in distributions of genotypes and alleles between essential hypertension patients and control individuals were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations,

we performed linkage disequilibrium analysis and haplotype-based case–control analysis, using the expectation maximization algorithm [20] and the software SNPAlyze version 3.2 (Dynacom Co., Ltd., Yokohama, Japan). The pairwise linkage disequilibrium analysis was performed using three SNP pairs. We used $|D'|$ values greater than 0.5 to assign SNP locations to one haplotype block. SNPs with an r^2 value less than 0.5 were selected as tagged. In the haplotype-based case–control analysis, haplotypes with a frequency below 0.02 were excluded. The frequency distribution of the haplotypes was calculated by chi-squared analysis. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at P values less than 0.05. Statistical analyses were performed using SPSS software for Windows (version 12; SPSS Inc., Chicago, Illinois, USA).

Results

Table 1 presents the clinical characteristics of the study participants. For men, women and total participants, the following values were significantly higher for the essential hypertension patients than the control individuals: body mass index, systolic blood pressure, diastolic blood pressure, and pulse rate. For men and total participants, serum creatinine was significantly higher for the essential hypertension patients than the control individuals. There was no significant difference in the following variables between the essential hypertension patients and control individuals: plasma concentration of total cholesterol, plasma concentration of high-density lipoprotein cholesterol, plasma concentration of uric acid, and the incidence of hyperlipidemia. The incidence of diabetes was significantly higher for essential hypertension patients than for control individuals. There was no significant difference in alcohol consumption between essential hypertension patients and control individuals. For men, smoking prevalence was significantly higher for essential hypertension patients than for control individuals.

Table 2 presents the distribution of the genotypes and alleles of the three SNPs. The genotype distribution of the each SNP did not show significant difference from the Hardy–Weinberg equilibrium values (data not shown). For total participants, the genotype distribution of rs1126742 differed significantly between the essential hypertension patients and control individuals ($P = 0.005$). For the total participants, men, and women, the distribution of the recessive model of rs1126742 (CC versus TC + TT) differed significantly between the essential hypertension patients and control individuals ($P = 0.007$, $P = 0.043$, and $P = 0.045$, respectively). Dominance and recessiveness of the models were defined by their frequency among total control individuals.

Table 3 presents the results of logistic regression analysis. Logistic regression was performed with and without

Table 1 Characteristics of study participants

	Total			Men			Women		
	Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value
Age (years)	59.1 ± 10.0	60.9 ± 11.9	0.077	57.8 ± 9.3	59.0 ± 11.6	0.293	62.1 ± 11.1	64.4 ± 11.8	0.199
Body mass index (kg/m^2)	23.7 ± 4.7	22.6 ± 3.5	0.005*	24.0 ± 4.6	23.0 ± 3.9	0.040*	23.2 ± 4.0	22.0 ± 2.5	0.036*
Systolic blood pressure (mmHg)	164.6 ± 23.2	115.5 ± 9.7	<0.001*	163.5 ± 22.6	115.4 ± 9.6	<0.001*	167.3 ± 24.6	115.6 ± 10.0	<0.001*
Diastolic blood pressure (mmHg)	97.8 ± 16.6	70.2 ± 7.8	<0.001*	98.6 ± 16.4	70.6 ± 7.9	<0.001*	96.2 ± 17.2	69.5 ± 7.7	<0.001*
Pulse (beats/min)	76.1 ± 14.4	71.9 ± 13.6	0.002*	76.3 ± 14.7	71.6 ± 15.2	0.007*	76.9 ± 13.7	71.5 ± 9.7	0.002*
Creatinine (mg/dl)	0.9 ± 0.3	0.8 ± 0.2	0.003*	1.0 ± 0.3	0.9 ± 0.2	0.006*	0.7 ± 0.3	0.7 ± 0.2	0.400
Total cholesterol (mg/dl)	205.1 ± 37.2	206.3 ± 39.6	0.727	200.1 ± 34.9	199.5 ± 36.4	0.885	216.5 ± 39.9	219.6 ± 42.3	0.651
High-density lipoprotein cholesterol (mg/dl)	58.0 ± 17.5	56.6 ± 18.9	0.073	49.8 ± 15.9	53.9 ± 17.5	0.056	60.2 ± 19.0	64.7 ± 21.0	0.293
Uric acid (mg/dl)	5.7 ± 1.5	5.5 ± 1.4	0.169	6.1 ± 1.4	5.8 ± 1.3	0.052	4.8 ± 1.2	4.7 ± 1.5	0.663
Hyperlipidemia (%)	26	19	0.067	21	15	0.123	39	28	0.156
Diabetes (%)	13	0	<0.001*	12	0	<0.001*	15	0	<0.001*
Drinking (%)	64	58	0.259	80	69	0.058	29	27	0.844
Smoking (%)	57	48	0.118	66	53	0.033*	30	32	0.869

Continuous variables expressed as the mean ± SD. Categorical variables expressed as the percentage. *P* value of continuous variables calculated by Mann–Whitney *U*-test. *P* value of categorical variables calculated by Fisher's exact test. * $P < 0.05$.

Table 2 Genotype and allele distributions in patients with essential hypertension and in control individuals

		Total			Men			Women		
		Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value
rs2269231 (SNP1)										
Genotype	AA	70 (23.0%)	52 (25.1%)	0.607	53 (25.1%)	31 (22.8%)	0.709	17 (18.3%)	21 (29.6%)	0.227
	AT	170 (55.9%)	107 (51.7%)		115 (54.5%)	72 (52.9%)		55 (59.1%)	35 (49.3%)	
	TT	64 (21.1%)	48 (23.2%)		43 (20.4%)	33 (24.3%)		21 (22.6%)	15 (21.1%)	
Dominant model	AA	70 (23.0%)	52 (25.1%)	0.586	53 (25.1%)	31 (22.8%)	0.622	17 (18.3%)	21 (29.6%)	0.089
	AT + TT	234 (77.0%)	155 (74.9%)		258 (74.9%)	105 (77.2%)		76 (81.7%)	50 (70.4%)	
Recessive model	TT	64 (21.1%)	48 (23.2%)	0.567	43 (20.4%)	33 (24.3%)	0.393	21 (22.6%)	15 (21.1%)	0.824
	AT + AA	240 (78.9%)	159 (76.8%)		168 (79.6%)	103 (75.7%)		72 (77.4%)	56 (78.9%)	
Allele	A	310 (51.0%)	211 (51.0%)	0.953	221 (52.4%)	134 (49.3%)	0.424	89 (47.8%)	77 (54.2%)	0.253
	T	298 (49.0%)	203 (49.0%)		201 (47.6%)	138 (50.7%)		97 (52.2%)	65 (45.8%)	
rs1126742 (SNP2)										
Genotype	TT	179 (58.9%)	133 (64.3%)	0.005*	123 (58.3%)	85 (62.5%)	0.060	56 (60.2%)	48 (67.6%)	0.054
	CT	122 (40.1%)	64 (30.9%)		85 (40.3%)	44 (32.4%)		37 (39.8%)	20 (28.2%)	
	CC	3 (1.0%)	10 (4.8%)		3 (1.4%)	7 (5.1%)		0 (0.0%)	3 (4.2%)	
Dominant model	TT	179 (58.9%)	133 (64.3%)	0.222	123 (58.3%)	85 (62.5%)	0.435	56 (60.2%)	48 (67.6%)	0.330
	TC + CC	125 (41.1%)	74 (35.7%)		88 (41.7%)	51 (37.5%)		37 (39.8%)	23 (32.4%)	
Recessive model	CC	3 (1.0%)	10 (4.8%)	0.007*	3 (1.4%)	7 (5.1%)	0.043*	0 (0.0%)	3 (4.2%)	0.045*
	TC + TT	301 (99.0%)	197 (95.2%)		208 (98.6%)	129 (94.9%)		93 (100.0%)	68 (95.8%)	
Allele	T	480 (78.9%)	330 (79.7%)	0.788	331 (78.4%)	214 (78.7%)	0.940	149 (80.1%)	116 (81.7%)	0.719
	C	128 (21.1%)	84 (20.3%)		91 (21.6%)	58 (21.3%)		37 (19.9%)	26 (18.3%)	
rs9333025 (SNP3)										
Genotype	GG	169 (55.6%)	107 (51.7%)	0.348	121 (57.3%)	75 (55.2%)	0.758	48 (51.6%)	32 (45.1%)	0.480
	GA	113 (37.2%)	78 (37.7%)		76 (36.0%)	49 (36.0%)		37 (39.8%)	29 (40.8%)	
	AA	22 (7.2%)	22 (10.6%)		14 (6.7%)	12 (8.8%)		8 (8.6%)	10 (14.1%)	
Dominant model	GG	169 (55.6%)	107 (51.7%)	0.385	121 (57.3%)	75 (55.2%)	0.687	48 (51.6%)	32 (45.1%)	0.406
	GA + AA	135 (44.4%)	100 (48.3%)		90 (42.7%)	61 (44.8%)		45 (48.4%)	39 (54.9%)	
Recessive model	AA	22 (7.2%)	22 (10.6%)	0.180	14 (6.7%)	12 (8.8%)	0.450	8 (8.6%)	10 (14.1%)	0.141
	GA + GG	282 (92.8%)	185 (89.4%)		197 (93.3%)	124 (91.2%)		85 (91.4%)	51 (85.9%)	
Allele	G	451 (74.2%)	292 (70.5%)	0.199	318 (75.4%)	199 (73.2%)	0.518	133 (71.5%)	93 (65.5%)	0.244
	A	157 (25.8%)	122 (29.5%)		104 (24.6%)	73 (26.8%)		53 (28.5%)	49 (34.5%)	

P value of the genotype calculated by Fisher's exact test. * $P < 0.05$.

Table 3 Odds ratios and 95% confidence intervals for each risk factor and the TC + TT genotype of rs1126742 associated with essential hypertension

	Total			Men			Women		
Risk factor	Odd ratios	95% confidence interval	*P* value	Odd ratios	95% confidence interval	*P* value	Odd ratios	95% confidence interval	*P* value
TC + TT genotype	6.697	1.312–34.175	0.022*	5.704	1.060–30.677	0.043*	0.000	0.000–0.000	1.000
Diabetes mellitus	0.000	0.000–0.000	0.998	0.000	0.000–0.000	0.998	0.000	0.000–0.000	0.999
Smoking	0.704	0.444–1.116	0.135	0.591	0.345–1.014	0.056	1.138	0.417–3.108	0.800
Hyperlipidemia	0.737	0.422–1.289	0.285	0.773	0.390–1.532	0.461	0.829	0.301–2.285	0.717

* $P < 0.05$.

diabetes mellitus, smoking and hyperlipidemia. For the total participants and for the men, the TC + TT genotype of rs1126742 still differed significantly between the essential hypertension patients and control individuals ($P = 0.022$ and $P = 0.043$, respectively), while for women there was no difference for the TC + TT genotype of rs1126742 between the essential hypertension patients and control individuals ($P = 1.000$).

Table 4 presents patterns of linkage disequilibrium in the CYP4A11 gene, with their $|D'|$ and r^2 values. All three SNPs are located in one haplotype block because all $|D'|$ values are beyond 0.5. All three SNPs were available for the performance of a haplotype-based case–control study because all of the r^2 values were below 0.5.

In the haplotype-based case–control analysis, haplotypes were established through the use of different combinations of the SNPs (Table 5). For men, the overall distribution of the haplotypes established by SNP1, SNP3 and by SNP1, SNP2, SNP3 were significantly different between the essential hypertension patients and the control individuals ($P = 0.001$ and $P = 0.003$, respectively). For women, the frequency of the T-C haplotype (established by SNP1, SNP2) was significantly lower for essential hypertension patients than for control individuals ($P = 0.021$). For the men, the frequency of the T-A haplotype (established by SNP1, SNP3) and the frequency of the T-T-A haplotype (established by SNP1, SNP2, SNP3) were significantly lower for essential hypertension patients than for control individuals (both $P = 0.001$), while the frequency of the A-T-G haplotype (established by SNP1, SNP2, SNP3) was significantly higher for essential hypertension patients than for control individuals ($P = 0.043$).

Table 4 Pairwise linkage disequilibrium for the three single nucleotide polymorphisms

	$\|D'\|$ value							
	Essential hypertension patients				Control individuals			
	SNP	SNP1	SNP2	SNP3	SNP	SNP1	SNP2	SNP3
r^2 value	SNP1		0.925	0.786	SNP21		0.751	0.748
	SNP2	0.079		0.867	SNP32	0.060		0.928
	SNP3	0.207	0.193		SNP13	0.225	0.211	

$|D'|$ above diagonal and r^2 below diagonal.

Discussion

CYP4A11 is involved not only in fatty-acid metabolism in the liver, but also in blood pressure regulation in the kidney [13,21]. CYP4A11 converts arachidonic acid to 20-HETE, which acts in either a prohypertensive or antihypertensive manner depending on whether it is expressed at renovascular or tubular sites in the kidney, respectively. In the renal tubule, 20-HETE blocks sodium transport and acts primarily as a natriuretic, antihypertensive substance. In the renal vasculature, 20-HETE has vasoconstricting and prohypertensive effects [22,23]. Both animal and human studies [22,24–26] indicate that the cyp4A11 gene is a candidate causative gene of hypertension. Gainer *et al.* [14] were the first to identify the T8590C variant of the CYP4A11 gene (registered as rs1126742 in the NCBI online database). In in-vivo experiments examining the kinetic properties of CYP4A11 and 20-HETE, they found that a thymidine-to-cytosine substitution in CYP4A11 resulted in a phenylalanine-to-serine (F-to-S) amino acid substitution, which affects the catalytic activity of 20-HETE synthase via a loss-of-function mechanism. Studies have revealed an association between rs1126742 and hypertension in large study populations (Tennessee cohort and Framingham offspring cohort) [14]. In the third MONICA Augsburg survey, Mayer *et al.* [15] also found an association between rs1126742 and hypertension, but did not find any evidence that rs1126742 had a blood-pressure-independent modulatory effect on cardiac size, cardiac structure, or left ventricular function. In the present study, we genotyped three SNPs in CYP4A11 (including rs1126742) in Japanese individuals, and assessed the association between CYP4A11 and essential hypertension using a haplotype-based case–control analysis.

In the present study that examined three groups, for the total participants, for the men, and for the women the genotypic distribution of the recessive model (CC versus TC + TT) of rs1126742 significantly differed between essential hypertension patients and control individuals, indicating that the risk of essential hypertension is increased in individuals with the T allele of rs1126742. Some studies have produced conflicting results regarding whether the recessive, dominant or both models of this genotype modulate blood pressure [14,15,27]. The present results indicate that the recessive model of rs1126742 is associated with essential hypertension.

Table 5 Haplotype analysis in patients with essential hypertension and control participants

Haplotype				Overall *P* value			Frequency in total			Frequency in men			Frequency in women		
				Total	Men	Women	Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value	Essential hypertension patients	Control individuals	*P* value
	SNP1	SNP2		0.660	0.835	0.098									
H1	**A**	**T**					0.535	0.522	0.697	0.543	0.526	0.663	0.516	0.504	0.870
H2	**T**	**T**					0.256	0.281	0.377	0.243	0.263	0.552	0.285	0.313	0.624
H3	**A**	**C**					0.210	0.197	0.628	0.214	0.211	0.921	0.199	0.151	0.304
H4	**T**	**C**											0.000	0.032	0.021*
	SNP1		SNP3	0.494	0.001*	0.489									
H1	**A**		**G**				0.279	0.251	0.314	0.295	0.260	0.338	0.243	0.236	0.841
H2	**T**		**G**				0.231	0.258	0.301	0.232	0.233	0.994	0.236	0.307	0.138
H3	**A**		**A**				0.463	0.454	0.760	0.473	0.472	0.942	0.472	0.419	0.362
H4	**T**		**A**				0.027	0.036	0.364	0.000	0.035	0.001*	0.049	0.038	0.559
		SNP2	SNP3	0.988	0.607	0.362									
H1		**T**	**G**				0.313	0.315	0.948	0.320	0.285	0.338	0.297	0.371	0.157
H2		**C**	**G**				0.204	0.200	0.876	0.209	0.211	0.951	0.192	0.179	0.754
H3		**T**	**A**				0.483	0.485	0.948	0.471	0.504	0.405	0.511	0.450	0.278
	SNP1	SNP2	SNP3	0.855	0.003*	0.556									
H1	**A**	**T**	**G**				0.081	0.064	0.338	0.095	0.054	0.043*	0.052	0.083	0.253
H2	**T**	**T**	**G**				0.231	0.250	0.502	0.228	0.229	0.908	0.245	0.292	0.350
H3	**A**	**C**	**G**				0.203	0.196	0.772	0.208	0.209	0.964	0.192	0.169	0.596
H4	**A**	**T**	**A**				0.458	0.461	0.942	0.470	0.476	0.847	0.470	0.431	0.493
H5	**T**	**T**	**A**				0.027	0.029	0.788	0.000	0.032	0.001*	0.041	0.024	0.407

Haplotypes with frequency >0.02 were estimated using software SNPAlyze (version 3.2; Dynacom Co., Ltd., Yokohama, Japan). *P* value calculated by chi-square analysis. * $P < 0.05$.

Although the studies by Gainer *et al.* [14] and by Mayer *et al.* [15] concluded that this SNP was not associated with gender-specific hypertension, in the present results logistic regression indicates that the TC + TT genotype distribution of rs1126742 for men significantly differed between the essential hypertension patients and the control individuals, which means the SNP was associated with gender-specific hypertension. The discrepant results that were noted may be due to racial differences; for example, all participants in the study by Gainer *et al.* [14] were Caucasian. These discrepancies may also be due to the differences in the designs of these studies. The criteria for essential hypertension in the present study (systolic blood pressure >160 mmHg or diastolic blood pressure >100 mmHg) were stricter than those of the study by Gainer *et al.* [14] (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg). Use of age-matched case–control analysis is generally thought to produce more accurate results in such studies.

Morris and Kaplan [28] found that, for genes with multiple susceptibilities, analysis based on haplotypes has advantages over analysis based on individual SNPs, particularly when linkage disequilibria between SNPs are weak. Consequently, in the present study we successfully established haplotypes for the CYP4A11 gene from the different combination of the three SNPs. For the present female participants, although the frequency of the T-C haplotype (established by SNP1, SNP2) was significantly lower for essential hypertension patients than for control individuals ($P = 0.021$), we believe that this difference is not indicative of the true situation, as the overall distribution of the haplotype established by SNP1, SNP2 was not significantly different between the essential hypertension patients and the control individuals within the female group ($P = 0.098$). For the present male participants, the frequency of the A-T-G haplotype (established by SNP1, SNP2, SNP3) was significantly higher for essential hypertension patients than for control individuals ($P = 0.043$). When SNP2 was not present, the A-G haplotype (established by SNP1, SNP3) did not show any differences between the essential hypertension patients and the control individuals ($P = 0.338$). We therefore speculate that haplotypes that are carrying rs1126742 are more valuable as genetic markers for essential hypertension in Japanese men. In addition, for men, we also successfully identified two resistance haplotypes for essential hypertension (T-A, established by SNP1, SNP2; and T-T-A, established by SNP1, SNP2, SNP3).

Some case–control studies have identified gene variants associated with gender-specific susceptibility to essential hypertension [29,30]. The present haplotype-based case–control study also showed gender-specific (for men only) significant differences in genetic markers between essential hypertension patients and control individuals. This is very interesting in light of the previous findings that male cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice have higher blood pressures than female cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice, respectively. The combination of these previous findings and the present results suggest that animal cyp4a loss-of-function models of essential hypertension are useful models for the human hypertensive phenotype associated with CYP4A11. The finding that the male cyp4a14$^{-/-}$ mice exhibited increases in plasma androgen, and that their hypertensive phenotype was androgen sensitive [10], suggests that the A-T-G haplotype and the T-A, T-T-A haplotype are associated with androgen-mediated activity of the CYP4A11 enzyme. Unfortunately, we could not obtain data on plasma androgen, as we were not able to obtain informed consent to collect blood samples for the purpose of measuring plasma androgen levels. It appears that functional mutations in the CYP4A11 gene and/or neighboring genes are associated with essential hypertension in men.

In conclusion, rs1126742 and the A-T-G haplotype of the human CYP4A11 gene appear to be genetic markers of essential hypertension in Japanese men. Further studies are needed to isolate functional mutations in the CYP4A11 gene that regulate blood pressure, and to evaluate the function of CYP4A11 variants that are involved in the metabolism of sex hormones.

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There are no conflicts of interest.

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Association of *TNFRSF4* gene polymorphisms with essential hypertension

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Background **Essential hypertension is a complex disorder that results from the interaction of a number of susceptibility genes and environmental factors. The *TNFRSF4* (tumor necrosis factor receptor superfamily, member 4) gene was one of the genes that showed altered renal expression in long-term salt loading in mice. Moreover, association of the *TNFRSF4* and *TNFSF4* (tumor necrosis factor (ligand) superfamily, member 4) genes with myocardial infarction was recently reported. Since essential hypertension is a well-known risk factor for myocardial infarction, we hypothesized that *TNFRSF4* could be a susceptibility gene for essential hypertension.**

Methods **We performed a case–control study of *TNFRSF4* in two independent population.**

Results **Extensive investigation of single nucleotide polymorphisms of the entire gene suggested that it resided in one linkage disequilibrium block, and four single nucleotide polymorphisms in the 5′ flanking region sufficiently represented major haplotypes. In the combined population, the frequency of the most frequent haplotype, C-C-A-A, was significantly lower ($P = 8.07 \times 10^{-5}$) and that of the second most frequent haplotype, C-T-G-A, was significantly higher ($P = 6.07 \times 10^{-4}$) in hypertensive subjects than in control subjects. This difference was observed only in female patients. The C-T-G-A haplotype showed a lower promoter activity than other haplotypes, suggesting a relationship with disease susceptibility.**

Conclusion **Our results suggest that *TNFRSF4* is a female-specific susceptible gene for essential hypertension.** *J Hypertens* **26:902–913 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: association studies, essential, haplotype, hypertension, single nucleotide polymorphisms, *TNFRSF4* gene

Abbreviations: 95% CI, 95% confidence interval; *Agt*, mouse angiotensinogen gene; ANOVA, analysis of variance; APC, antigen-presenting cell; CRP, C-reactive protein; DBP, diastolic blood pressure; EH, essential hypertension; HT, hypertensive; LD, linkage disequilibrium; MI, myocardial infarction; NT, normotensive; OR, odds ratio; PCR, polymerase chain reaction; SBP, systolic blood pressure; SD, standard deviation; SNP, single nucleotide polymorphism; TaqMan-ASA, TaqMan allele-specific amplification; TNFRSF4 (OX40), tumor necrosis factor receptor superfamily, member; 4TNFSF4 (OX40L), tumor necrosis factor (ligand) superfamily, member 4; UTR, untranslated region

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Introduction

Hypertension affects more than 25% of the adult population worldwide [1]. Essential hypertension (EH) accounts for more than 90% of hypertension cases and is a multifactorial disorder resulting from the interaction of a number of susceptibility genes and environmental factors. It is estimated that the genetic contribution to blood pressure variation ranges from 30 to 50% [2]. Identification of susceptibility genes for hypertension would provide a clue to the pathophysiology of the disease.

Several approaches exists for genetic causes of EH: candidate–gene linkage studies, genome–scanning linkage studies, candidate–gene association studies, genetic studies in animal models, and gene expression profiling in animal models [3]. Each approach has its own strengths and weaknesses, and some argue that integration of the approaches is a more efficient way forward [4]. The Millennium Genome Project for Hypertension in Japan has adopted the candidate–gene association strategy because of its relatively higher statistical power and

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convenience of collecting samples [5]. Candidate genes are selected on the basis of the accumulation of experimental evidence (expression profiling in animal models) and information in the literature. As a first step in this project, we performed DNA microarray experiments in mice to screen genes whose renal expression was changed by long-term salt loading, because genes that showed salt sensitivity were considered to be candidate genes for EH. The results showed that more than 300 genes were either downregulated or upregulated. For the genetic association study, from these 300 genes, we nominated 121 that had been reported in the literature as candidate genes. To date, 70 genes have been screened, 10 of which showed significant association with EH on haplotype-based analysis. Three of these 10 genes were positive in both the expression profiling and genetic association studies. The tumor necrosis factor receptor superfamily, member 4 (*TNFRSF4*) gene was one of the three.

TNFRSF4 (OX40) is a member of the tumor necrosis factor receptor (TNFR) superfamily, and is primarily expressed as a transmembrane protein on activated $CD4^+$ T cells after antigen recognition [6–9]. Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4, also called OX40L) [10], the ligand for TNFRSF4 on activated $CD4^+$ T cells, is expressed on antigen-presenting cells (APCs) including activated B cells, macrophages, and dendritic cells, as well as on endothelial cells and some activated T cells [11–14]. The TNFRSF4–TNFSF4 interaction between T cell and APC contributes to proinflammatory T-cell function. In particular, TNFRSF4–TNFSF4 interactions are crucial for the generation of memory $CD4^+$ T cells by promoting the survival of effector T cells [15–18]. Thus, it is suggested that the TNFRSF4–TNFSF4 pathway is involved in inflammation and immune response.

T lymphocyte activation involving several receptor–ligand pairs such as TNFRSF4–TNFSF4 is suggested to promote atherosclerosis [12,19,20], which is now considered to be an inflammatory disease [21]. Recently, *TNFSF4* was identified as a susceptibility gene for atherosclerosis and a genetic variation in *TNFSF4* was reported to be associated with myocardial infarction (MI) and severity of coronary artery disease [22]. Genetic variation in *TNFRSF4* was also shown to be associated with MI [23]. These reports suggested that the TNFRSF4–TNFSF4 pathway plays an important role in the pathogenesis of atherosclerosis and MI in humans. It is generally believed that hypertension is one of the major risk factors for atherosclerosis and MI [24]; however, MI and hypertension often coexist, as seen in the SHEEP study cohort in which MI patients were significantly associated with hypertension [25]. Thus, the association between MI and TNFRSF4/TNFSF4 in human subjects may be due to not only atherogenesis but also hypertension itself. We hypothesized that *TNFRSF4* and/or *TNFSF4* were potential candidate genes for EH.

The aim of the present study was to investigate the association between genetic variations of the *TNFRSF4* gene and EH in the Japanese population. We performed a case–control study using two independent population of Japanese patients with EH.

Methods

Study subjects

Initial screening of candidate genes involved 1035 subjects with EH (762 men and 273 women) and 1058 age-matched controls (792 men and 266 women) who were recruited through the study group of the Millennium Genome Project for Hypertension [5]. Six medical institutes took part in the collaborative study and recruited subjects in Japan. Recruitment procedures, case–control criteria, and clinical characteristics are described in detail elsewhere [5].

The clinical characteristics of the subjects included in this study for *TNFRSF4* gene analyses are shown in Tables 1 and 2. Subjects in population 1 were part of the population recruited through the study group of the Millennium Genome Project for Hypertension [5]. Subjects in population 2 were recruited from Ohasama, a cohort in a rural community of northern Japan [26].

Each subject was assigned to one of the blood pressure diagnostic categories defined by the criteria of the 1999 WHO/ISH guidelines for the management of hypertension [27]. Hypertensive (HT) subjects had systolic blood pressure (SBP) of at least 140 mmHg or diastolic blood

Table 1 Characteristics of subjects in population 1

	Total subjects			Male subjects			Female subjects		
Parameters	NT	HT	*P*	NT	HT	*P*	NT	HT	*P*
No. of subjects	562	587		301	316		261	271	
Age (years)	61.6 ± 9.2	60.1 ± 11.2	0.011*	59.9 ± 9.0	58.5 ± 11.1	0.083	63.6 ± 9.1	62.0 ± 11.1	0.056
BMI (kg/m^2)	22.2 ± 2.8	23.9 ± 3.3	< 0.001*	22.1 ± 2.9	23.8 ± 3.1	< 0.001*	22.3 ± 2.7	24.0 ± 3.6	< 0.001*
SBP (mmHg)	111.7 ± 8.9	163.7 ± 21.1	< 0.001*	111.8 ± 8.8	162.1 ± 18.4	< 0.001*	111.5 ± 9.1	166.1 ± 24.4	< 0.001*
DBP (mmHg)	68.9 ± 7.3	98.3 ± 14.8	< 0.001*	69.4 ± 7.3	98.7 ± 14.0	< 0.001*	68.0 ± 7.3	97.6 ± 16.0	< 0.001*
TC (mg/dl)	205.5 ± 38.0	207.1 ± 34.9	0.596	195.8 ± 35.7	198.7 ± 33.0	0.294	216.2 ± 37.6	216.0 ± 34.7	0.970
HDL-C (mg/dl)	57.3 ± 15.1	58.3 ± 17.2	0.314	55.3 ± 15.0	56.8 ± 17.5	0.263	59.5 ± 15.0	60.0 ± 16.7	0.697
TG (mg/dl)	123.8 ± 87.4	141.7 ± 84.7	0.003*	132.1 ± 106.2	147.0 ± 94.5	0.133	116.7 ± 67.4	135.4 ± 71.3	0.007*

Values are mean ± SD. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, HDL cholesterol; HT, hypertensive patient; NT, normotensive patient; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride. *Difference was statistically significant.

Table 2 Characteristics of subjects in population 2

Parameters	Total subjects			Male subjects			Female subjects		
	NT	HT	P	NT	HT	P	NT	HT	P
No. of subjects	925	732		317	323		608	409	
Age (years)	54.6 ± 11.5	61.6 ± 9.7	< 0.001*	55.8 ± 11.1	61.5 ± 10.2	< 0.001*	54.0 ± 11.6	61.7 ± 9.3	< 0.001*
BMI (kg/m^2)	23.4 ± 3.1	24.2 ± 3.3	< 0.001*	23.5 ± 3.0	23.6 ± 3.1	0.506	23.4 ± 3.1	24.6 ± 3.4	< 0.001*
SBP (mmHg)	123.9 ± 9.8	142.2 ± 12.1	< 0.001*	125.4 ± 8.6	143.9 ± 11.4	< 0.001*	123.1 ± 10.3	140.8 ± 12.5	< 0.001*
DBP (mmHg)	70.3 ± 7.1	80.2 ± 9.1	< 0.001*	71.6 ± 6.9	81.9 ± 9.4	< 0.001*	69.6 ± 7.2	78.8 ± 8.6	< 0.001*
TC (mg/dl)	193.4 ± 34.2	195.0 ± 33.8	0.358	186.4 ± 33.8	183.9 ± 34.0	0.352	197.1 ± 33.9	203.8 ± 31.0	0.001*
HDL-C (mg/dl)	55.3 ± 14.1	53.8 ± 14.6	0.028*	51.2 ± 14.1	52.8 ± 14.4	0.180	57.4 ± 13.7	54.4 ± 14.7	0.001*
TG (mg/dl)	128.9 ± 73.4	142.5 ± 89.8	0.001*	139.1 ± 85.1	146.3 ± 103.0	0.340	123.7 ± 66.1	139.5 ± 77.9	0.001*

Values are mean ± SD. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, HDL cholesterol; HT, hypertensive patient; NT, normotensive patient; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride. *Difference was statistically significant.

pressure (DBP) of at least 90 mmHg or were patients currently taking chronic antihypertensive medication. Normotensive (NT) subjects had SBP/DBP lower than 140/90 mmHg and had never been treated with antihypertensive medication. Informed consent was obtained from each individual as per the protocol approved by each institution's ethics committee.

DNA microarray experiments in mice

In DNA microarray experiments, we used two lines of mice having different numbers of the functional mouse angiotensinogen gene (*Agt*) [28,29], kindly donated by Professor Oliver Smithies (Department of Pathology, University of North Carolina, Chapel Hill, North Carolina, USA). To observe distinct effects by long-term salt loading, *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) were fed a high-salt diet containing 8% NaCl for 6 months, whereas *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) were fed a low-salt diet containing 0.3% NaCl. Total RNA was isolated from the kidneys of mice and differences in gene expression were examined using mouse cDNA microarray (Incyte Genomics Inc., Palo Alto, California, USA), which contains 9222 mouse cDNA clones.

Screening of candidate genes

We selected a total of 121 candidate genes (Supplemental Table S1) based on the following criteria: (1) genes reported as candidates in the literature or with functions relevant to the blood pressure regulation and (2) human homologue of genes in which renal expression was changed by long-term salt loading in mice. For an initial screening of these candidate genes, some of the available single nucleotide polymorphisms (SNPs) per gene were selected from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/) or dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and were genotyped in 1035 patients and 1058 controls using the PCR–SSP–FCS method [30]. Haplotype-based association analyses were performed using SNPAlyze v4.1 Pro software (DYNACOM, Mobara, Japan) based on an expectation/maximization (EM) algorithm. *P* values for overall distribution of haplotypes were calculated by permutation test at 1000 iterations. *P* values less than 0.05 were considered statistically significant.

Screening for polymorphisms in *TNFRSF4*

To identify genetic variants of the human *TNFRSF4* gene, we sequenced all seven exons, the adjacent intronic sequence, 4 kb of the 5′ flanking region, and 1.5 kb of the 3′ flanking region in 32 control subjects. Nineteen primer sets were designed on the basis of the *TNFRSF4* genomic and mRNA sequences from the GenBank database (accession numbers NT_004350.18 and NM_003327, respectively). All polymerase chain reaction (PCR) products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The sequences were analyzed and polymorphisms identified using the Genetyx program (Genetyx Corp., Tokyo, Japan).

Genotyping of polymorphisms in *TNFRSF4*

Genotyping of four SNPs in the *TNFRSF4* gene (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G and P12: −530A>G) was performed using either the TaqMan allele-specific amplification (TaqMan-ASA) method [31] or the Custom TaqMan Genomic Assays kit (Applied Biosystems). In the TaqMan-ASA method, specific primers were designed on the basis of the *TNFRSF4* genomic sequence from the GenBank database (accession number NT_004350.18). The primer sequences are shown in Table 3. The PCR mixture for the TaqMan-ASA method contained 5 μl of 2× TaqMan Universal Mix (Applied Biosystems), 0.4 μmol/l of each PCR primer, 0.12 μmol/l of TaqMan probe, and 5 ng of template DNA in a final volume of 10 μl. The samples were analyzed with an ABI PRIZM 7000 Sequence Detection system (Applied Biosystems). The thermoprofiles were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Luciferase assay

TNFRSF4 reporter constructs of 3970 bp (nt −3968 to +2) were created by means of PCR amplification of genomic DNA from homozygous subjects who had alternative haplotypes with the use of following primers: forward, 5′-GGGGTACCGTGCCACATGGCTGGAATTTAC-3′ (including *KpnI* site) and reverse, 5′-TCTAGCTAGC GTCTCTGCTGTCGCCAGAGTC-3′ (including *NheI*

Table 3 Primer sequence (5′ → 3′) for TaqMan-ASA genotyping

SNP	Allele-specific primer	Common primer	TaqMan probe[a]
P1	CACATGGCTGGAATTTACCATC CACATGGCTGGAATTTACCTCT	CTCAGCAGTGGGAGAAAAACAA	CCTCTGAAGCGTTTTCACTGGTATCATGTGT
P2	GTCGCCTTTCCCCCTCCG GTCGCCTTTCCCCCTCCA	GCTGCAGCCAATAGGCACCTT	AATAGCCACTTCGTGCGGCTGG
P8	GTCACAGGTCCAAGAAAGCCGT GTCACAGGTCCAAGAAAGCCGC	GCAGGCTGCCTTACAGACCTT	TGAGCTCTGGGTCAGTGTCCA
P12	GGTCAGGAGTTCAAGACCAGTGT GGTCAGGAGTTCAAGACCAGTTC	CCACGCCCGAATAATTTTTG	AGTAGAGACGGGATTTCGCCATGTTAGC

[a]TaqMan probes contained a 5′ FAM (6-carboxyfluorescein) reporter fluorophore and a 3′ TAMRA (6-carboxytetramethylrhodamine) quencher.

site). Amplicons of three haplotypes (Pr-H1, Pr-H2, and Pr-H5) were cloned into the pGL4.10[luc2] vector (Promega, Madison, Wisconsin, USA). Promoter constructs that contained one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G) were created by site-directed mutagenesis carried out in the Pr-H1 plasmid using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). All constructs were verified by sequencing. COS-7 cells (monkey kidney, SV40 T antigen transformed) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HEK293 cells (human embryonic kidney) were cultured in minimum essential medium supplemented with 2 mmol/l L-glutamine, 1% nonessential amino acids, 10% fetal bovine serum, and antibiotics. Cells in 12-well plates at 50–70% confluence were transfected with 500 ng of each construct and 10 ng of pGL4.74[hRluc/TK] *Renilla* luciferase vector (Promega) as an internal control for transfection efficiency, using 1.5 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h of transfection, the cells were harvested, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System and a TD-20/20 luminometer (Promega). Each experiment was repeated five or six times, and each sample was studied in triplicate.

Statistical analysis

Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview/index.php) was used to analyze and visualize the linkage disequilibrium (LD) and haplotypic patterns. Hardy–Weinberg equilibrium was assessed by χ^2 analysis. Overall distributions of the genotypes or alleles were analyzed by χ^2 analysis using 2×3 or 2×2 contingency tables between NT controls and HT patients. Haplotype frequencies were estimated using SNPAlyze v4.1 Pro software. The distributions of each haplotype between NT controls and HT patients were calculated both by χ^2 tests of one haplotype against the others (haplotype-wise test) and by permutation tests with 1000 iterations using SNPAlyze software. We calculated odds ratios (ORs) with 95% confidence intervals (CIs) using logistic regression analyses with or without clinical covariates (age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride). To estimate the contribution of the gene to the total variance of blood pressure, the variance component procedure with the analysis of variance (ANOVA) type III variance estimates was used. Comparisons in reporter assays were performed using Student's *t*-test or ANOVA. All statistical analyses were performed with SPSS software (SPSS Japan Inc., Tokyo, Japan) unless otherwise stated. *P* values less than 0.05 were considered statistically significant.

Results

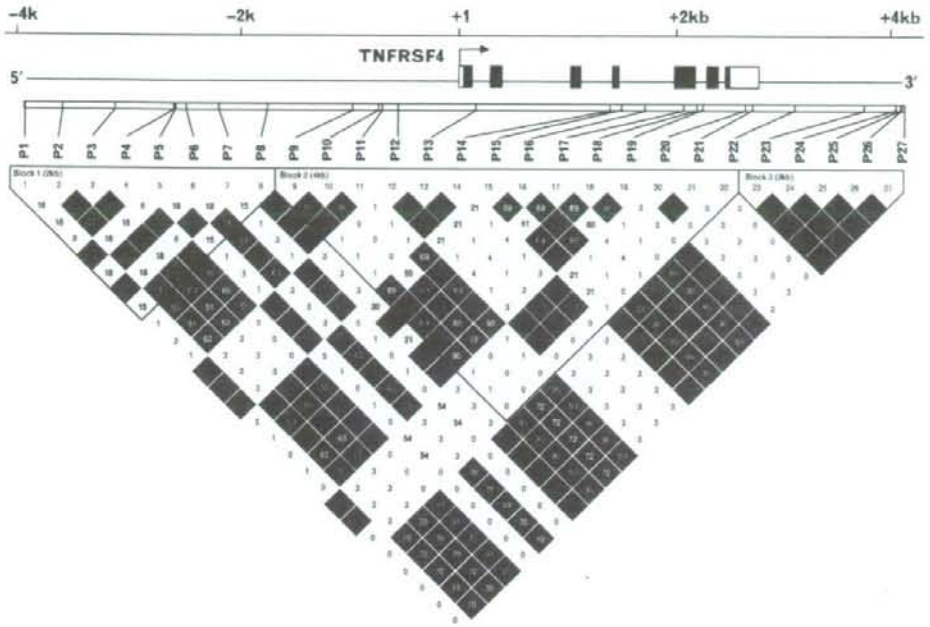
DNA microarray experiments in mice

We used cDNA microarray analyses to compare the expression profiles of 9222 genes in the kidneys of *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) with a high-salt diet versus those of *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) with a low-salt diet. Differential expression values greater than 1.3 based on internal quality control data are summarized in Supplemental Tables S2 and S3. We found that 119 genes were downregulated in the kidneys of *Agt* 2/2 mice by 1.3–3.1-fold compared with *Agt* 0/1 mice and 192 genes were upregulated by 1.3–1.9-fold. Murine *TNFRSF4* gene (*Tnfrsf4*) was the gene downregulated 1.3-fold.

Screening of candidate genes by haplotype association study

We selected a total of 121 candidate genes (Supplemental Table S1) on the basis of the following criteria: (1) genes reported as candidates in the literature or with possible involvement of blood pressure regulation and (2) human homologue of genes in which renal expression was changed by long-term salt loading in mice. We excluded genes whose genotype data were not available due to the following reasons: no SNP data was available in the databases; minor allele frequencies of SNPs in Japanese were too low (< 5%); or the genotyping of some SNPs was difficult. So far, 191 SNPs in 70 genes have been successfully genotyped for genetic association tests, and the genotyping of only a single SNP was completed in eight genes. A haplotype-based association test was performed in 62 genes and a single SNP association study in eight genes. *P* values for difference in overall distribution of the haplotype or genotype frequencies between normotension and hypertension in total (men + women), male,

Fig. 1



Haplotype block structure of the *TNFRSF4* gene. (Top) Organization of the *TNFRSF4* gene. Exons are indicated by boxes (black, coding sequences; white, untranslated sequences). (Bottom) Linkage disequilibrium structure of polymorphisms across the *TNFRSF4* gene region using data from 32 Japanese controls. Haplotype blocks were defined by the solid spine of LD method in Haploview. The number in each cell represents the LD parameter r^2 (100×), blank cells denote $r^2 = 1$. Each cell is painted with graduated color relative to the strength of LD between markers, which is defined by the r^2 value.

and female subjects are shown in Supplemental Table S4. Significant *P* values were observed for 10 genes: *Aquaporin-2* (*AQP2*), *Estrogen receptor 2* (*ESR2*), *Glycogen synthase 1* (*GYS1*), *Kallikrein 1* (*KLK1*), *Nephrin* (*NPHN*), *Solute carrier family 1 (glial high affinity glutamate transporter), member 2* (*SLC1A2*), *Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3* (*SLC9A3*), *Steroidogenic acute regulatory protein* (*STAR*), *Syntaxin binding protein 1* (*STXBP1*), and *TNFRSF4*. Three genes (*STAR*, *STXBP1*, and *TNFRSF4*) are the human homologues to the mouse genes that showed changes in renal expression in the salt-loading experiment. The *P* value for overall distribution of the haplotype of *TNFRSF4* was significant only in female subjects.

Identification of polymorphisms in *TNFRSF4*

We searched for polymorphisms in the *TNFRSF4* gene, including 4 kb of the 5′ flanking region and 1.5 kb of the 3′ flanking region. By direct sequencing in 32 Japanese individuals, a total of 44 polymorphisms were identified; 20 in the 5′ flanking region, four in exons, seven in introns, and 13 in the 3′ flanking region. Of those, 27 polymorphisms (P1–P27) with minor allele frequencies (MAF) 5% of higher (in 32 DNA samples) are presented in Table 4. A graphical overview of the structure of the human *TNFRSF4* gene showing the location of the 27 polymorphisms identified in this study is shown in Fig. 1. Pairwise LD measuring r^2 between polymorphisms and defined haplotype block structures in this region was evaluated using the solid spine of LD method in Haploview (Fig. 1). Three haplotype blocks (blocks 1, 2, and 3) were defined in the *TNFRSF4* gene region with this method. Blocks 1 and 2 appear to be separated because P8 showed low LD to other polymorphisms and blocks 2 and 3 were separated by P22 for the same reason. Strong LDs, however, were observed among certain blocks, such as between P4 and P27 ($r^2 = 0.91$). In addition, multiallelic D′ values between these blocks were high (0.86 between blocks 1 and 2; 1.0 between blocks 2 and 3). Thus, we decided to handle an entire gene region as one block, which could be analyzed by tag SNPs from the entire region. Four SNPs in the 5′ flanking region (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G, and P12: −530A>G) were employed for further analysis. The four SNP haplotypes constructed

Table 4 Polymorphisms with minor allele frequencies 5% or higher detected in the *TNFRSF4* genomic region in 32 Japanese controls

Name	Polymorphism[a]	Location	Amino acid change	MAF[b]	dbSNP ID	JSNP ID
P1	−3943C>T	5′ Flanking		0.06		
P2	−3601C>T	5′ Flanking		0.27	rs12036216	
P3	−3119G>T	5′ Flanking		0.27	rs11721	
P4	−2577delA	5′ Flanking		0.22		
P5	−2568C>G	5′ Flanking		0.06		
P6	−2461C>G	5′ Flanking		0.27		
P7	−2167C>T	5′ Flanking		0.06		
P8	−1720A>G	5′ Flanking		0.30	rs3813201	JST-IMS173304
P9	−936A>G	5′ Flanking		0.19	rs34115518	
P10	−699C>T	5′ Flanking		0.16	rs35339498	
P11	−669C>G	5′ Flanking		0.19	rs35659545	
P12	−525A>G	5′ Flanking		0.11	rs35107976	
P13	150 + 47G>C	Intron1		0.11	rs35737009	
P14	376-16C>G	Intron3		0.11	rs34108055	
P15	442 + 32ins35bp	Intron4		0.25		
P16	442 + 248C>T	Intron4		0.19	rs9661697	
P17	539G>A	Exon5	Glu178Glu	0.25	rs17568	
P18	639 + 25C>T	Intron5		0.19	rs2298212	JST-IMS053053
P19	640-31T>G	Intron5		0.20	rs2298211	JST-IMS053052
P20	921C>T	Exon7 (3′ UTR)		0.11	rs2298210	JST-IMS053051
P21	989C>G	Exon7 (3′ UTR)		0.11	rs2298209	JST-IMS053050
P22	1067 + 308G>A	3′ Flanking		0.08	rs2298208	JST-IMS053049
P23	1067 + 941G>C	3′ Flanking		0.20	rs34067070	
P24	1067 + 1224delTT	3′ Flanking		0.20		
P25	1067 + 1240G>C	3′ Flanking		0.20	rs34279802	
P26	1067 + 1266T>C	3′ Flanking		0.20	rs35916760	
P27	1067 + 1296C>T	3′ Flanking		0.20	rs36057244	

[a]Numbering according to the cDNA sequence of *TNFRSF4* (accession number NM_003327). [b]Minor allele frequency (MAF) on the basis of the sequencing of 32 DNA samples.

from these SNPs covered more than 85% of haplotype diversity of the entire *TNFRSF4* gene when P22 was not included for analysis.

Case–control study for *TNFRSF4* polymorphisms

The clinical characteristics of the NT and HT subjects in population 1 are summarized in Table 1. Difference in age between the NT and HT subjects was significant when men and women were jointly compared ($P = 0.011$), whereas it was not significant when men and women were separately compared.

In population 1, four SNPs (P1, P2, P8, and P12) were genotyped in 562 NT controls and 587 HT patients. All

Table 5 Genotype and allele frequencies among normotensive (NT) and hypertensive (HT) subjects in population 1

		Total subjects			Male subjects			Female subjects		
	Genotype	NT ($n = 562$)	HT ($n = 587$)	P[a]	NT ($n = 301$)	HT ($n = 316$)	P[a]	NT ($n = 261$)	HT ($n = 271$)	P[a]
P1	CC	448 (0.799)	455 (0.776)		238 (0.793)	245 (0.775)		210 (0.805)	210 (0.778)	
	CT	109 (0.194)	124 (0.212)		60 (0.200)	67 (0.212)		49 (0.188)	57 (0.211)	
	TT	4 (0.007)	7 (0.012)	0.524	2 (0.007)	4 (0.013)	0.691	2 (0.008)	3 (0.011)	0.722
	Allele									
	C	1005 (0.896)	1034 (0.882)		536 (0.893)	557 (0.881)		469 (0.898)	477 (0.883)	
	T	117 (0.104)	138 (0.118)	0.305	64 (0.107)	75 (0.119)	0.506	53 (0.102)	63 (0.117)	0.429
P2	CC	324 (0.578)	319 (0.544)		166 (0.553)	170 (0.538)		158 (0.605)	149 (0.552)	
	CT	208 (0.371)	220 (0.375)		121 (0.403)	117 (0.370)		87 (0.333)	103 (0.381)	
	TT	29 (0.052)	47 (0.080)	0.129	13 (0.043)	29 (0.092)	0.055	16 (0.061)	18 (0.067)	0.455
	Allele									
	C	856 (0.763)	858 (0.732)		453 (0.755)	457 (0.723)		403 (0.772)	401 (0.743)	
	T	266 (0.237)	314 (0.268)	0.089	147 (0.245)	175 (0.277)	0.203	119 (0.228)	139 (0.257)	0.263
P8	AA	284 (0.506)	280 (0.478)		144 (0.480)	154 (0.487)		140 (0.536)	126 (0.467)	
	AG	238 (0.424)	248 (0.423)		137 (0.457)	125 (0.396)		101 (0.387)	123 (0.456)	
	GG	39 (0.070)	58 (0.099)	0.182	19 (0.063)	37 (0.117)	0.044	20 (0.077)	21 (0.078)	0.250
	Allele									
	A	806 (0.718)	808 (0.689)		425 (0.708)	433 (0.685)		381 (0.730)	375 (0.694)	
	G	316 (0.282)	364 (0.311)	0.129	175 (0.292)	199 (0.315)	0.376	141 (0.270)	165 (0.306)	0.202
P12	AA	401 (0.716)	393 (0.671)		209 (0.699)	215 (0.680)		192 (0.736)	178 (0.659)	
	AG	148 (0.264)	175 (0.299)		84 (0.281)	88 (0.278)		64 (0.245)	87 (0.322)	
	GG	11 (0.020)	18 (0.031)	0.179	6 (0.020)	13 (0.041)	0.318	5 (0.019)	5 (0.019)	0.144
	Allele									
	A	950 (0.848)	961 (0.820)		502 (0.839)	518 (0.820)		448 (0.858)	443 (0.820)	
	G	170 (0.152)	211 (0.180)	0.069	96 (0.161)	114 (0.180)	0.355	74 (0.142)	97 (0.180)	0.093

[a]Significant P value after Bonferroni's correction for four loci is 0.0125 (0.05/4).

Table 6 Four SNP haplotypes (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in population 1

		Male subjects				Female subjects			
	Haplotype[a]	NT ($n=298$)	HT ($n=316$)	P^b	Permutation P	NT ($n=261$)	HT ($n=270$)	P^b	Permutation P
H1	C-C-A-A	404 (0.677)	413 (0.653)	0.371	0.363	376 (0.720)	356 (0.659)	0.031	0.021*
H2	C-T-G-A	81 (0.136)	96 (0.152)	0.419	0.420	60 (0.116)	75 (0.138)	0.267	0.259
H3	T-T-G-G	63 (0.106)	73 (0.115)	0.584	0.559	52 (0.099)	62 (0.115)	0.405	0.402
H4	C-C-G-G	16 (0.026)	21 (0.033)	0.484	0.470	17 (0.033)	14 (0.027)	0.578	0.617
H5	C-C-A-G	17 (0.029)	18 (0.029)	0.967	0.958	1 (0.002)	19 (0.036)	6.78×10^{-5}*	<0.001*
H6	C-C-G-A	12 (0.021)	5 (0.008)	0.063	0.066	9 (0.018)	12 (0.021)	0.646	0.682
	Others	3 (0.005)	6 (0.010)			7 (0.013)	2 (0.004)		
Entire distribution				0.722[c]				0.003[c,*]	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant P value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). [c] P value for the entire distribution with permutation test. * Difference was statistically significant.

of these SNPs were in Hardy–Weinberg equilibrium in the NT group. Table 5 shows the distribution of genotypic and allelic frequencies of the four SNPs in each group. The overall distribution of genotype and allele did not significantly differ between the HT and NT groups for total, male, or female subjects. The P value of χ^2 test for the difference in the genotypic frequency of P8 between male HT and NT groups was 0.044, which was not significant after Bonferroni's correction (multiplied by 4).

We next analyzed the four SNP haplotypes in population 1 (Table 6). Six common haplotypes (H1–H6) covered approximately 99% of the subjects in the HT and NT groups. The frequencies of each haplotype in men did not differ between the HT and NT groups. In women, the frequency of the major C-C-A-A haplotype (H1) of the HT subjects was significantly lower than that of the NT subjects ($P=0.031$). Multiple logistic regression in women revealed that the association of the H1/H1 diplotype with hypertension remained significant ($P=0.006$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1 diplotype against the others was 0.56 with a 95% CI of 0.37–0.85. The frequency of the minor C-C-A-G haplotype (H5) of the HT subjects was significantly higher than that of the NT subjects ($P=6.78\times10^{-5}$). H5 haplotype was significantly associated with hypertension in a dominant model ($P=0.004$) after adjustment for the above factors. The OR of the H5/H5 + H5/other diplotype against the others was 6.93 with a 95% CI of 1.88–25.5.

To confirm an association of the four SNP haplotypes in women with EH, we genotyped them using the second case–control population (population 2) comprising 925 NT controls and 732 HT patients. Table 2 presents the clinical features of the NT controls and HT patients in population 2. All genotype results of four SNPs in each group were consistent with Hardy–Weinberg equilibrium. Table 7 shows the distribution of genotypic and allelic frequencies of four SNPs in each group of population 2. The overall distribution of genotype and allele of all four SNPs did not significantly differ between the HT and NT groups for total or male subjects. Among women, however, significant differences were observed in the allelic frequencies of P2 ($P=0.005$) and the genotypic and allelic frequencies of P8 ($P=0.005$ and 0.003, respectively) between the HT and NT subjects even after Bonferroni's correction (multiplied by 4). P2 was still significantly associated with hypertension in women in both a dominant ($P=0.007$) and recessive model ($P=0.038$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of T/T + C/T against C/C (dominant model) was 1.22 with a 95% CI of 1.05–1.40, and the OR of T/T against C/T + C/C (recessive model) was 1.94 with a 95% CI of 1.04–3.62. P22 was also significantly associated with hypertension in women in both a dominant model ($P=0.011$) and recessive model ($P=0.002$) after adjustment for the above factors. The OR of G/G + A/G against A/A (dominant model) was 1.20 with a 95% CI of 1.04–1.38, and the OR of G/G against A/G + G/G (recessive model) was 1.49 with a 95% CI of 1.16–1.92.

Table 8 shows the frequency of four SNP haplotypes in population 2. Among women, the HT subjects showed a significantly lower frequency of H1 (C-C-A-A) ($P=8.48\times10^{-4}$) and a significantly higher frequency of H2 (C-T-G-A) ($P=6.46\times10^{-4}$) than the NT subjects, whereas in men no significant difference in frequencies of haplotypes between the HT and NT groups was observed. Multiple logistic regression in women revealed that the association of H1 haplotype with hypertension remained significant in both a dominant ($P=0.006$) and recessive model ($P=0.005$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1 + H1/other diplotype against the others (dominant model) was 0.52 with a 95% CI of 0.32–0.83, and the OR of the H1/H1 diplotype against the others (recessive model) was 0.67 with a 95% CI of 0.50–0.89. The H2 haplotype was also significantly associated with hypertension in women in a dominant model ($P=0.001$) after adjustment for the above factors. The OR of the H2/H2 + H2/other diplotype against the others was 1.40 with a 95% CI of 1.18–1.65. In population 2, the frequency of H5

Table 7 Genotype and allele frequencies among normotensive (NT) and hypertensive (HT) subjects in population 2

		Total subjects			Male subjects			Female subjects		
	Genotype	NT ($n=925$)	HT ($n=732$)	P^a	NT ($n=317$)	HT ($n=323$)	P^a	NT ($n=608$)	HT ($n=409$)	P^a
P1	CC	729 (0.792)	573 (0.786)		253 (0.801)	249 (0.778)		476 (0.788)	324 (0.792)	
	CT	181 (0.197)	147 (0.202)		58 (0.184)	66 (0.206)		123 (0.204)	81 (0.198)	
	TT	10 (0.011)	9 (0.012)	0.929	5 (0.016)	5 (0.016)	0.770	5 (0.008)	4 (0.010)	0.949
	Allele									
	C	1639 (0.891)	1293 (0.887)		564 (0.892)	564 (0.881)		1075 (0.890)	729 (0.891)	
	T	201 (0.109)	165 (0.113)	0.721	68 (0.108)	76 (0.119)	0.530	133 (0.110)	89 (0.109)	0.927
P2	CC	550 (0.598)	403 (0.553)		176 (0.555)	182 (0.567)		374 (0.620)	221 (0.542)	
	CT	323 (0.351)	282 (0.387)		118 (0.372)	123 (0.383)		205 (0.340)	159 (0.390)	
	TT	47 (0.051)	44 (0.060)	0.176	23 (0.073)	16 (0.050)	0.488	24 (0.040)	28 (0.069)	0.017
	Allele									
	C	1423 (0.773)	1088 (0.746)		470 (0.741)	487 (0.759)		953 (0.790)	601 (0.737)	
	T	417 (0.227)	370 (0.254)	0.069	164 (0.259)	155 (0.241)	0.477	253 (0.210)	215 (0.263)	0.005*
P8	AA	464 (0.508)	342 (0.472)		146 (0.465)	157 (0.489)		318 (0.530)	185 (0.458)	
	AG	384 (0.420)	316 (0.436)		139 (0.443)	143 (0.445)		245 (0.408)	173 (0.428)	
	GG	66 (0.072)	67 (0.092)	0.189	29 (0.092)	21 (0.065)	0.436	37 (0.062)	46 (0.114)	0.005*
	Allele									
	A	1312 (0.718)	1000 (0.690)		431 (0.686)	457 (0.712)		881 (0.734)	543 (0.672)	
	G	516 (0.282)	450 (0.310)	0.080	197 (0.314)	185 (0.288)	0.321	319 (0.266)	265 (0.328)	0.003*
P12	AA	630 (0.686)	479 (0.659)		214 (0.677)	208 (0.650)		416 (0.691)	271 (0.666)	
	AG	265 (0.289)	220 (0.303)		93 (0.294)	100 (0.313)		172 (0.286)	120 (0.295)	
	GG	23 (0.025)	28 (0.039)	0.213	9 (0.028)	12 (0.038)	0.690	14 (0.023)	16 (0.039)	0.301
	Allele									
	A	1525 (0.831)	1178 (0.810)		521 (0.824)	516 (0.806)		1004 (0.834)	662 (0.813)	
	G	311 (0.169)	276 (0.190)	0.128	111 (0.176)	124 (0.194)	0.405	200 (0.166)	152 (0.187)	0.231

[a] Significant P value after Bonferroni's correction for four loci is 0.0125 (0.05/4). * Difference was statistically significant.

did not significantly differ between the HT and NT groups for women.

Although trends of frequency changes in the H1 and H2 haplotypes among women in the two independent population were the same, the frequency of H2 showed a significant difference not in population 1 but in population 2. This discrepancy could have been caused by difference in the sample size. When we analyzed the differences in frequencies of each haplotype between the HT and NT groups in combined samples of the two population (Table 9), female HT subjects showed a significantly lower frequency of H1 ($P=8.07\times10^{-5}$) and a significantly higher frequency of H2 ($P=6.07\times10^{-4}$) than the NT subjects. The frequency of H5 of female HT subjects was still significantly higher than that of NT subjects ($P=0.003$). No significant difference in haplotype frequencies between male HT and NT groups was observed.

Variance component estimation of *TNFRSF4*

The variance estimates of the *TNFRSF4* diplotype and the residual in SBP of the control women of population 1 were 5.5 and 79.6, respectively. Therefore, the *TNFRSF4* gene explains 6.5% of the variation of SBP in this group. The values in DBP were 2.8 and 52.1, respectively, with the gene contributing 5.2% of the variation.

Transcriptional effects of polymorphisms in the promoter region

To study transcriptional effects of the polymorphisms, we transfected COS-7 cells and HEK293 cells with promoter constructs containing the haplotypes in the *TNFRSF4* gene (Pr-H1, Pr-H2, and Pr-H5). In COS-7 cells, promoter activity of the Pr-H2 construct was significantly lower than that of the Pr-H1 or Pr-H5 construct (0.89 for Pr-H2/Pr-H1, $P=0.008$ and 0.91 for Pr-H2/Pr-H5, $P=0.026$; Fig. 2a). The same results were observed in HEK293 cells (0.92

Table 8 Four SNP haplotypes (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in population 2

		Male subjects				Female subjects			
	Haplotype[a]	NT ($n=303$)	HT ($n=299$)	P^b	Permutation P	NT ($n=584$)	HT ($n=388$)	P^b	Permutation P
H1	C-C-A-A	403 (0.665)	403 (0.674)	0.743	0.714	839 (0.718)	502 (0.647)	8.48×10^{-4}*	<0.001*
H2	C-T-G-A	86 (0.142)	75 (0.125)	0.400	0.388	115 (0.098)	116 (0.149)	6.46×10^{-4}*	0.001*
H3	T-T-G-G	66 (0.109)	71 (0.119)	0.592	0.593	125 (0.107)	84 (0.108)	0.939	0.926
H4	C-C-G-G	30 (0.049)	24 (0.040)	0.429	0.443	47 (0.040)	45 (0.058)	0.067	0.074
H5	C-C-A-G	11 (0.018)	20 (0.034)	0.095	0.113	21 (0.018)	18 (0.023)	0.434	0.451
H6	C-C-G-A	8 (0.014)	4 (0.007)	0.265	0.374	15 (0.013)	8 (0.010)	0.606	0.583
	Others	2 (0.003)	1 (0.002)			6 (0.005)	3 (0.004)		
Entire distribution				0.533[c]				0.026[c]*	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant P value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). [c] P value for the entire distribution with permutation test. * Difference was statistically significant.

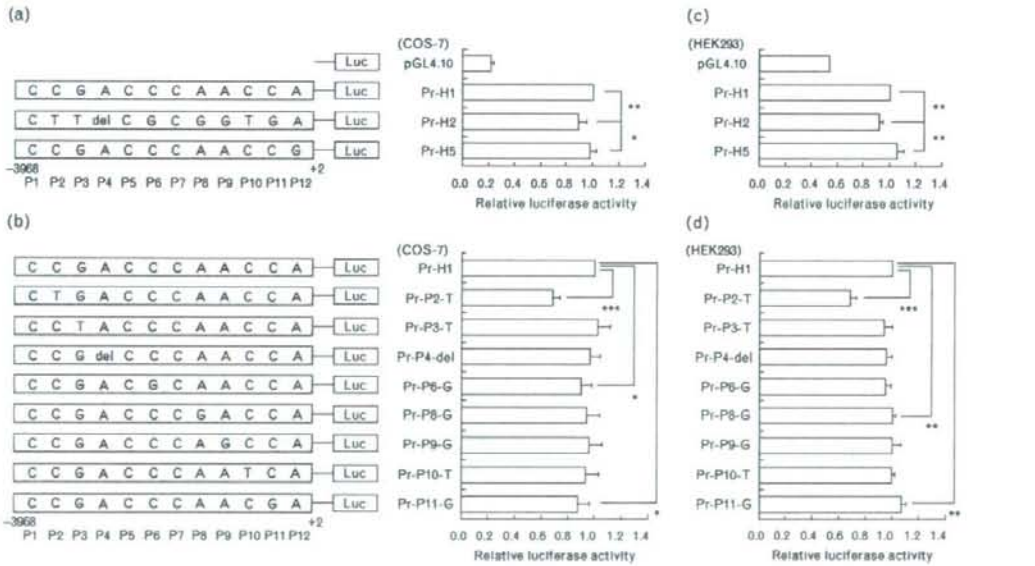
Table 9 Four SNP haplotype (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in the combined population (population 1 and 2)

		Male subjects			Female subjects		
	Haplotype*	NT ($n=602$)	HT ($n=615$)	P^b	NT ($n=845$)	HT ($n=658$)	P^b
H1	C-C-A-A	806 (0.671)	816 (0.663)	0.682	1215 (0.719)	858 (0.652)	8.07×10^{-5}*
H2	C-T-G-A	167 (0.139)	171 (0.139)	0.989	175 (0.104)	191 (0.145)	6.07×10^{-4}*
H3	T-T-G-G	129 (0.107)	144 (0.117)	0.446	177 (0.104)	146 (0.111)	0.578
H4	C-C-G-G	45 (0.038)	45 (0.036)	0.846	64 (0.038)	60 (0.045)	0.306
H5	C-C-A-G	29 (0.024)	38 (0.031)	0.263	22 (0.013)	37 (0.028)	0.003*
H6	C-C-G-A	21 (0.017)	9 (0.008)	0.033	24 (0.014)	20 (0.015)	0.906
	Others	5 (0.004)	7 (0.006)		13 (0.008)	6 (0.004)	

* Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant P value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). * Difference was statistically significant.

for Pr-H2/Pr-H1, $P=0.001$ and 0.88 for Pr-H2/Pr-H5, $P=0.001$; Fig. 2c). There was no significant difference in promoter activity between the Pr-H1 and Pr-H5 constructs in both cells. These results suggest that expression of TNFRSF4 mRNA in cells is lower in individuals who have the H2 haplotype than in cells from individuals who have other types of haplotypes. To clarify the responsible SNP(s) for the lower promoter activity of Pr-H2, we performed an additional assay using a series of promoter constructs that contained only one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G). In COS-7 cells, promoter activities of Pr-P2-T, Pr-P6-G, and Pr-P11-G were significantly lower than that of Pr-H1 (0.69 for Pr-P2-T/Pr-H1, $P<0.0001$, 0.90 for Pr-P6-G/Pr-H1, $P=0.016$, and 0.88 for Pr-P11-G/Pr-H1, $P=0.015$; Fig. 2b). In HEK293 cells, as in COS-7 cells, Pr-P2-T showed significantly lower promoter activity when compared with Pr-H1 (0.71 for Pr-P2-T/Pr-H1, $P=0.0001$; Fig. 2d). The results of other constructs, however, were different: promoter activities of Pr-P8-G and Pr-P11-G were significantly higher than that of Pr-H1 (1.04 for Pr-P8-G/Pr-H1, $P=0.002$ and 1.10 for

Fig. 2



Effect of haplotypes and each polymorphism on the transcriptional activity of the *TNFRSF4* promoter. (a and c) Effect of haplotypes on the transcriptional activity of the *TNFRSF4* promoter. (b and d) Effect of each polymorphism on the transcriptional activity of the *TNFRSF4* promoter. Relative luciferase activities after transient transfection in COS-7 (a and b) and HEK293 (c and d) cell lines are shown. Activities of the Pr-H1 constructs were considered as 100%. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for six (COS-7) or five (HEK293) independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Pr-P11-G/Pr-H1, $P=0.003$; Fig. 2d). Only Pr-P2-T showed consistent change in promoter activity in the two different cell lines. These results suggest that P2 had the largest impact on the decreased promoter activity of the H2 haplotype.

Discussion

The significance of *TNFRSF4* in the pathogenesis of female subjects with EH was indicated in two independent sets of population. Haplotype analysis using four SNPs (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G and P12: −530A>G) in the 5′ upstream region showed that the frequency of H1 (C-C-A-A) was significantly low among female HT patients when compared with female NT controls in both population 1 ($P=0.031$) and population 2 ($P=8.48\times10^{-4}$). The frequency of H2 (C-G-T-A) of female HT patients was significantly higher than that of female NT controls in population 2 ($P=6.46\times10^{-4}$), but not in population 1. In the combined population, both significantly lower frequency of H1 ($P=8.07\times10^{-5}$) and significantly higher frequency of H2 ($P=6.07\times10^{-4}$) were observed in female HT patients compared with female NT controls. No difference in haplotype frequencies between the HT and NT groups was observed in the male subjects of either the combined or separate population. These results of association of the *TNFRSF4* haplotype with hypertension suggested that the H1 haplotype is a protective allele and that the H2 haplotype is a high-risk allele for EH in women. The promoter activity of the H2 haplotype was significantly lower than that of the H1 and H5 (C-C-A-G) haplotypes. Furthermore, the Pr-P2-T construct showed lower promoter activity than other constructs. Allelic association of P2 (−3606C>T, rs12036216) with female HT patients was significant in population 2 and the combined population (data not shown), but not in population 1. These data suggested that P2 is the responsive SNP that modifies the risk for hypertension in females, although it is possible that unidentified variant(s) in LD with this haplotype have function(s) that influence disease susceptibility. We also observed a significant difference in frequency of the H5 haplotype in the combined population ($P=0.003$) and in population 1 ($P=6.78\times10^{-5}$), but not in population 2. We, however, could not find any transcriptional effect of H5 haplotype.

The TNFRSF4–TNFSF4 interactions on T lymphocytes enhance proliferation and differentiation of the cells as well as generation and survival of memory $CD4^+$ T cells in the process of inflammation and immune response [15–18]. Several inflammatory markers, such as soluble leukocyte adhesion molecules, cytokines, specific growth factors, heat shock proteins, CD40L, and C-reactive protein (CRP), were reported to increase in patients with EH [32–41]. Although the relationship between inflammation and hypertension has not been well established, a growing body of evidence indicates that vascular inflammation may be involved in both the initiation and development of hypertension [42–46]. Sesso *et al.* [46] showed that elevated plasma CRP, a well-known marker of inflammation, was associated with the future development of hypertension in a dose-dependent manner. Furthermore, hypertension has been suggested to trigger inflammation through the increased expression of several mediators, including leukocyte adhesion molecules, chemokines, specific growth factors, heat shock proteins, endothelin-1, and angiotensin [47–54]. Given our findings that variants of the *TNFRSF4* gene, which might affect the inflammatory cascade, were associated with EH among women, it is likely that inflammation may play a role in initiation and/or development of hypertension.

Inflammatory process [21] and T-lymphocyte activation [12,19,20] are implicated to be involved in the pathogenesis of atherosclerosis. Thus, alteration(s) in the TNFRSF4–TNFSF4 pathway could influence atherosclerosis formation. Indeed, Wang *et al.* [22] found that polymorphisms of *TNFSF4* are associated with MI in women. Furthermore, a polymorphism in *TNFRSF4* was also reported to be associated with MI [23]. These studies strongly suggested that genes involved in the TNFRSF4–TNFSF4 pathway play a role in the pathogenesis of atherosclerosis and MI, particularly in women.

Our findings combined with those of the studies mentioned above suggested that genetic variations in the TNFRSF4–TNFSF4 pathway may be involved in the pathogenesis of both atherosclerosis and hypertension. So, which comes first, atherosclerosis or hypertension? Hypertension is one of the principal risk factors for atherosclerosis and MI [24], but the exact mechanism underlying the association is not fully understood. Although arterial stiffness, which is a predictor of atherosclerosis [55,56], has been thought to be the result of hypertension rather than its cause, recent studies suggested that arterial stiffness is related to the development of hypertension [57,58]. These data indicated that the relationship between hypertension and arterial stiffness may be bidirectional [59]. Therefore, three different scenarios are possible to explain the results that genetic variations in the TNFRSF4–TNFSF4 pathway are associated with both hypertension and MI. First, inflammation may directly increase arterial stiffness and induce the development of an atherosclerotic lesion, which may lead to the development of hypertension. Second, inflammation may induce hypertension, which may result in increase in arterial stiffness and atherosclerosis. Third, inflammation may promote the development of hypertension and atherosclerosis by different pathways. Although it is not clear whether atherosclerosis is a cause of hypertension, our findings and that of previous studies indicate that the inflammation may be an important part of the link between hypertension and atherosclerosis and cardiovascular events, such as MI.

TNFSF4 is also a potential candidate for a susceptibility gene involved in the pathogenesis of EH in women. We, therefore, examined the putative association between polymorphisms in the *TNFSF4* gene and hypertension in population 1. The allele frequencies of four SNPs (rs1234315, rs3850641, rs1234313, and rs3861950) and its haplotype did not significantly differ between the HT group and the NT group for women (data not shown). In contrast to the case of MI in which susceptibility was affected by variations of both *TNFRSF4* and *TNFSF4*, susceptibility for hypertension may be affected only by *TNFRSF4*, though more extensive studies are required before we conclude an association of *TNFSF4* with hypertension.

In the present study, we found that variations of *TNFRSF4* affected hypertension susceptibility only in women. This is an interesting similarity to women-specific MI susceptibility exerted by *TNFSF4* and *TNFRSF4*. Some case–control studies [5,60,61] have identified gene variants associated with sex-specific susceptibility to EH. Recently, Nakayama *et al.* [5] reported that an SNP in the 5′-untranslated region of the follicle-stimulating hormone receptor (*FSHR*) gene, in which mutations were reported to cause hereditary hypergonadotropic ovarian failure [62], was associated with EH in women and affected the levels of transcriptional activity. In this study, the functional mutation of the gene was clearly identified in patients with EH in a sex-specific manner. Currently, the reason for women-specific association of *TNFRSF4* with EH is an open question. One possibility is the involvement of the female sex hormone, estrogen. After menopause, women are at increased risk of inflammatory cardiovascular diseases such as atherosclerosis and coronary heart disease, suggesting that estrogens modulate the initiation and progress of inflammation [63–65]. Recently, Xing *et al.* [66] suggested that estrogen may exert anti-inflammatory effects by inhibiting tumor necrosis factor-α-mediated chemokine production in vascular smooth muscle cells. Estrogen, however, is also known to increase CRP, which is an inflammatory marker [63]. These findings indicate that estrogen may modulate the production of several proinflammatory molecules in distinct pathways. It is possible that TNFRSF4 and estrogen cross talk in inflammation networks.

In conclusion, the present study revealed that haplotypes of the *TNFRSF4* gene were associated with EH among women in two Japanese population, suggesting an involvement of the *TNFRSF4* gene in the pathogenesis of female EH.

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There are no conflicts of interest.

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