to other populations with different lifestyles and genetic background. However, the present study provides a cross-validation of 4 of 7 SNPs (most likely representing 3 of 6 independent signals) derived from European GWAS. Replication studies in other Japanese[12] and Korean[13] populations also reported the cross-validation of European GWAS-derived SNP. Conservation of susceptible loci for hypertension was independent of ethnic background. This finding suggests an existence of unidentified common etiology of essential hypertension in relation to the susceptible genes and their physiological pathways.

Although individual common genetic variants confer a modest risk of hypertension, their combination showed a large impact on hypertension. The genetic risk score was associated with ≤2.27-times greater odds for hypertension. Similar observations have been found in other common diseases and multifactorial phenotypes, including, for example, type 2 diabetes mellitus,[20] serum lipid levels,[21] and serum uric acid levels.[22] We reported previously that the findings of the cross-sectional analysis revealed a similar association in the longitudinal analysis[23]; the fat mass and obesity-associated gene polymorphism was an independent risk factor for the future development of obesity after adjustment for possible confounding factors. The present cross-sectional study cannot address the question of whether the *ATP2B1* polymorphism and other susceptible variants predict future development of hypertension. However, recent articles investigating a prognostic significance of susceptible variants for type 2 diabetes mellitus[24] and cardiovascular disease[25] showed poor predictive performance of common variants in spite of the high OR observed in subjects carrying multiple risk alleles. A small proportion of the genetically high-risk persons attributed to independent inheritance of risk alleles may make it difficult to discriminate intermediate-risk persons. Genetic information may be most useful to identify a high-risk individual's need for early intervention.

Several definitions of hypertension were used in this study to explore susceptible SNPs with modest effects and to further validate the susceptibility. Since it was expected to be underpowered to detect the effects of common variants in a dichotomized analysis with slightly elevated BP, subjects with high normal BP were excluded from the 65 347 case-control analyses. All of the alleles associated with hypertension in a dichotomized analysis (Table S7) were also associated with BP levels (Table 2). Our methodology may, thus, be appropriate to identify susceptible variants for hypertension.

Perspectives

We have identified SNPs located in the *ATP2B1* gene region as susceptibility loci for hypertension in Japanese using a multistage association study, an association that has now been confirmed across different ethnic groups. Differences in the ex vivo *ATP2B1* mRNA expression levels further supported the disease susceptibility of SNP rs1110578. We also replicated the susceptibility of the European GWAS-derived SNPs in Japanese. Because hypertension is a trait that is preventable by dietary and exercise interventions, early detection of at-risk populations using genetic information may be useful in preventing future hypertension-related diseases.

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Disclosures

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CLINICAL STUDY

Association of *HSD3B1* and *HSD3B2* gene polymorphisms with essential hypertension, aldosterone level, and left ventricular structure

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Abstract

Background: HSD3B1 and HSD3B2 are crucial enzymes for the synthesis of hormonal steroids, including aldosterone. Therefore, *HSD3B* gene variations could possibly influence blood pressure (BP) by affecting the aldosterone level.

Methods: We performed a haplotype- and diplotype-based case–control study to investigate the association between the *HSD3B* gene variations and essential hypertension (EH), aldosterone level, and left ventricular hypertrophy (LVH). A total of 275 EH patients and 286 controls were genotyped for four SNPs of the *HSD3B1* gene (rs3765945, rs3088283, rs6203, and rs1047303) and for two SNPs of the *HSD3B2* gene (rs2854964 and rs1819698). Aldosterone and LVH were investigated in 240 and 110 subjects respectively.

Results: Significant differences were noted for the total and the male subject groups for the recessive model (CC versus TC+TT) of rs6203 between the controls and EH patients ($P=0.030$ and $P=0.008$ respectively). The frequency of the T-C haplotype established by rs3088283-rs1047303 was significantly higher for EH patients compared with the controls ($P=0.014$). Even though the polymorphism of *HSB3B1* was not associated with LVH, the diplotype established by rs3088283-rs1047303 in the total subject group, along with the systolic BP, diastolic BP, and aldosterone level were significantly higher for those subjects who had the T-C haplotype versus those who did not ($P=0.025$, $P=0.014$, and $P=0.006$ respectively).

Conclusion: rs6203 and rs1047303 in the *HSD3B1* gene are useful genetic markers for EH, while polymorphisms of *HSD3B1* are associated with the BP and aldosterone level.

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Introduction

It is likely that essential hypertension (EH) is a polygenic disorder that results from the inheritance of a number of susceptibility genes. The causal genes identified may be responsible for 30–50% of the observed inter-individual variations in blood pressure (BP) (1). Subphenotypic stratification and measurement of biochemical and physiological intermediate phenotypes provide an important way to dissect out these genetic factors.

Aldosterone is a potent mineralocorticoid that promotes sodium retention and leads to elevation of the arterial pressure. Additionally, aldosterone may also play a role in cardiac hypertrophy that is independent of its effect on BP. Therefore, genetic variations in the regulation of aldosterone synthesis might influence the structure and function of the left ventricle.

The 3β-hydroxysteroid dehydrogenases (3β-HSD)/$\Delta^{4,5}$-isomerase is the enzyme responsible for catalyzing the 3β-HSD dehydrogenation and $\Delta^{4,5}$-isomerization of the Δ^5-steroid precursors into their respective Δ^4-ketosteroids (2). This activity is crucial for the synthesis of hormonal steroids, which includes aldosterone, cortisol, and testosterone. The two 3β-HSD isoenzymes are chronologically designated as type 1 and type 2. These isoenzymes are 93.5% homologous and encoded by two different genes that are located on chromosome 1p13.1 (3). The type 1 gene (*HSD3B1*) is almost exclusively expressed as 3β-HSD in the placenta and peripheral tissues, including in the mammary gland, the prostate, and the skin. In contrast, the type 2

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gene (*HSD3B2*) is predominantly expressed as 3β-HSD in the adrenal gland, ovary, and testis (4).

A recent study in mice shows that type VI 3β-HSD (*Hsd3b6*), which is functionally similar to human *HSD3B1*, is associated with hypertension (HT) (5). In humans, a genetic variation in *HSD3B1* can lead to an elevation in plasma aldosterone with a resultant increase in intravascular volume and HT (6). In this study, it was reported that rs6203, which is the T→C silent substitution at codon 338 in exon 4 of *HSD3B1*, was associated with an elevated BP in a population of Swedish men. These previous studies indicate that the *HSD3B1* and *HSD3B2* genes may modulate BP and cardiac hypertrophy via changes in circulating aldosterone. To clarify this, we attempted to determine if the common SNPs and haplotypes/diplotypes of these genes were associated with BP and aldosterone levels. In addition, we also examined the differences in the echocardiographic left ventricular hypertrophy (LVH) between EH patients with normal BP and those who had never been treated for EH.

Materials and methods

Subjects

Subjects diagnosed with EH were recruited at Nihon University Itabashi Hospital and at other neighboring hospitals in Tokyo from 1993 to 2003. We enrolled 275 EH patients in the present study, with a male/female (m/f) ratio of 1.89. All the subjects had been previously screened for EH and had undergone physical and laboratory examinations. To be diagnosed with EH, the subjects had to have a family history of HT and a seated systolic BP (SBP) >160 mmHg or a diastolic BP (DBP) >100 mmHg on three occasions within 2 months after their first BP reading. A family history of HT was defined as prior diagnosis of HT in grandparents, uncles, aunts, parents, or siblings. None of the EH patients were receiving antihypertensive medications. Any patients diagnosed with secondary HT, including primary aldosteronism, were excluded from the study. A total of 286 normotensive (NT) age-matched healthy individuals (m/f ratio=1.80) were recruited at Nihon University Itabashi Hospital and at other neighboring hospitals from 1993 to 2003 and were enrolled as control subjects. None of the control subjects had any family history of HT, and all the controls had an SBP <130 mmHg and a DBP <85 mmHg. All EH subjects and controls were Japanese. Informed consent was obtained from each subject in accordance with the protocol approved by the Ethics Committee of Nihon University (7). This investigation was performed in line with the guidelines of the Declaration of Helsinki.

SNP selection and genotyping

A total of six SNPs were selected from the public databases available at the NCBI dbSNP and at the International HapMap Project websites (http://www.ncbi.nlm.nih.gov and http://www.hapmap.org respectively). The structures of the *HSD3B* genes are shown in Fig. 1. Since the linkage disequilibrium (LD) analysis, which was based on the JPT HapMap (release 19/phaseII October 2005, on NCBI B34 assembly, dbSNP b124), found that the SNPs tagged for each locus were on the same block ($r^2>0.8$), and this made it possible to perform a complete linkage block analysis.

Standard methods were used to extract DNA, while the genotyping was performed using a previously described Taq amplification method of the TaqMan SNP Genotyping Assay (Applied Biosystems Inc., Norwalk, CT, USA) (8). In the present study, we genotyped three SNPs that spanned the *HSD3B1* gene and which included, rs3765945 in the intron region (also known as T3216C), rs3088283 in the third exon (also known as The 54 Ile), and rs6203 and rs1047303 in the fourth exon (also known as Leu 338 Leu and as Thr 367 Asn respectively). The two SNPs genotyped in the *HSD3B2* gene included rs2854964 (A1167T) in the second intron and rs1819698 (G7747A) in the UTR-3. Genotyping was carried out blinded to the phenotypic information with controls of the known genotype included in each genotyping run. There was a 90% success rate for the genotyping with higher and minor allele frequencies all >5%. The estimated genotyping error rate was <1%.

Haplotype and diplotype construction

Based on the genotype data for the genetic variations, we performed a LD analysis along with haplotype-based case-control analysis and diplotype construction. These analyses used the expectation maximization

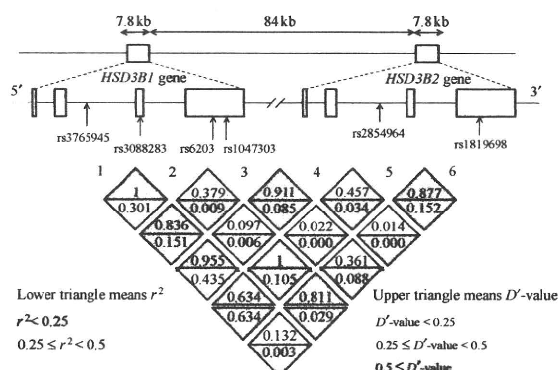

Figure 1 Top, genomic structure of *HSD3B1* and *HSD3B2*. The exons and the introns are indicated by lines and boxes respectively. The sizes of the genes are indicated by the scale at the top of the figure. Bottom, r^2 and pairwise LD (D') estimates are provided in the upper and lower triangles of each box respectively.

Table 1 Characteristics of the study participants. Continuous variables are expressed as means ± s.d. Categorical variables are expressed as percentages. The *P* value for the continuous variables were calculated using the Mann–Whitney *U* test. The *P* value for the categorical variables were calculated using Fisher's exact test.

	Total			Males			Females		
	NT (n=286)	EH (n=275)	P value	NT (n=184)	EH (n=180)	P value	NT (n=102)	EH (n=95)	P value
Age (years)	51.84±9.65	51.01±6.13	0.226	51.73±6.57	50.94±6.59	0.253	52.04±13.58	51.21±5.21	0.578
BMI (kg/m²)	22.78±3.23	27.02±5.89	<0.0001	22.94±3.18	24.64±3.31	<0.0001	22.49±3.33	24.41±4.01	0.000
SBP (mmHg)	112.97±11.70	173.27±20.46	<0.0001	113.34±10.65	171.10±19.09	<0.0001	112.33±13.41	177.39±22.28	<0.0001
DBP (mmHg)	69.67±8.66	106.44±13.83	<0.0001	70.46±8.24	107.06±13.90	<0.0001	68.25±9.23	105.44±13.75	<0.0001
Pulse (beats/min)	73.56±13.05	77.59±15.01	0.003	72.51±13.33	77.38±15.46	0.004	75.88±12.29	77.99±14.04	0.336
Creatinine (mg/dl)	0.83±0.22	0.85±0.25	0.413	0.90±0.20	0.94±0.24	0.162	0.71±0.18	0.68±0.18	0.293
Tot. chol. (mg/dl)	198.67±45.45	210.59±41.41	0.002	193.63±44.90	205.32±42.45	0.014	207.81±45.27	220.31±37.54	0.041
HDL chol. (mg/dl)	55.67±16.88	56.20±17.80	0.737	53.80±15.48	52.48±16.92	0.473	59.07±18.78	63.03±17.37	0.153
Uric acid (mg/dl)	5.37±1.48	5.65±1.60	0.041	5.82±1.42	6.20±1.47	0.016	4.55±1.20	4.60±1.28	0.777
PAC (pg/ml)	106.34±55.44	117.82±58.87	0.222	108.24±45.79	115.58±59.31	0.590	104.87±62.75	121.91±58.28	0.222
PRA (pg/ml per h)	3.24±8.01	3.20±11.09	0.979	4.60±10.86	3.28±11.04	0.612	2.19±4.75	3.05±11.26	0.702
ARR	126.81±126.73	172.23±267.69	0.254	116.94±159.29	155.14±262.63	0.520	134.49±96.84	203.54±275.89	0.209
LVDd (mm)	47.56±6.01	50.57±6.50	0.183	48.56±6.01	50.57±6.50	0.182	47.12±3.31	50.09±5.73	0.183
Hyperlipidemia (%)	26.6	40.8	<0.0001	23.4	38.9	0.001	32.4	49.5	0.015
Diabetes (%)	7.3	14.9	0.004	10.0	15.6	0.098	2.9	13.7	0.006
Drinking (%)	38.8	62.2	<0.0001	48.4	76.1	<0.0001	21.6	35.8	0.030
Smoking (%)	28.0	51.3	<0.0001	35.9	62.2	<0.0001	13.7	30.5	0.004

NT, normotension; EH, essential hypertension; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Tot. chol., total cholesterol; HDL chol., high density lipoprotein cholesterol; PAC, plasma aldosterone concentration; PRA, plasma renin activity; ARR, PAC to PRA ratio; LVDd, left ventricular diastolic dimensions.

algorithm (9) and the SNPAlyze version 3.2 software (Dynacom Co., Ltd, Yokohama, Japan). A total of six SNPs were used for the pairwise LD analysis, with $|D'|$ values >0.5 used to assign SNP locations to a single haplotype block. SNPs with a r^2 value <0.5 were selected as tagged. In the haplotype-based case–control analysis, haplotypes with a frequency <0.02 were excluded. A diplotype was defined as a pair of haplotypes in which one haplotype comes from the mother, while the other comes from the father. Diplotypes for each subject were also constructed by applying SNPAlyze version 3.2 (Dynacom Co., Ltd) (10).

Biochemical analysis

Standard methods employed by the Clinical Laboratory, Department of Nihon University Hospital were used to measure the total cholesterol, low-density lipoprotein cholesterol, aldosterone level, and renin activity in the plasma, and the creatinine and uric acid concentrations in the serum. Samples used to determine the aldosterone levels and renin activities were obtained from the subjects after they had been in a sitting position for a minimum of 30 min.

Echocardiographic examination

Echocardiography was performed in 110 individuals, (87 of the 287 EH patients and 23 of the 253 age-matched control subjects), using a standardized procedure to measure the wall thickness in 16 left ventricular wall segments. An experienced echocardiographer, who was blinded to the subject's status including genotype, examined each individual in a supine, left lateral position in accordance with the recommendations of the American Society of Echocardiography (11). Left ventricular diastolic dimensions (LVDd) were measured online from the standard 2D-guided M-mode registrations that were determined according to the leading edge convention. The end diastolic phase (R-wave peak of the ECG trace) was considered to define the interventricular septal thickness (IVST) and posterior wall thickness (PWTd). Left ventricular mass (LVM) was calculated using the American Society of Echocardiography's recommended formula for estimation of LVM from 2D-LV linear dimensions:

$$\mathrm{LVM} = 0.8 \times (1.04((\mathrm{LVDd} + \mathrm{PWTd} + \mathrm{IVST})^3 - (\mathrm{LVDd})^3)) + 0.6\ \mathrm{g}$$

To calculate the LVM index (LVMI), the LVM was divided by the body surface area.

Statistical analysis

Based on our initial estimated sample size, the number of patients enrolled was considered to be sufficient for a gene polymorphism study. In our analyses, we obtained

Table 2 Genotype and allele frequencies in normotensives and patients with EH.

Variants	Function		Total			Males			Females		
			NH (n=286)	EH (n=275)	P value	NH (n=184)	EH (n=180)	P value	NH (n=102)	EH (n=95)	P value
rs3765945	Intron	Genotype									
		TT	81.8	82.5		81.0	79.4		83.3	88.4	
		TC	18.2	17.1		19.0	20.0		16.7	11.6	
		CC	0.0	0.4	0.565	0.0	0.6	0.579	0.0	0.0	N.C.
		Dominant model									
		TT	81.8	82.5		81.0	79.4		83.3	88.4	
		TC+CC	18.2	17.5	0.822	19.0	20.6	0.713	16.7	11.6	0.307
		Recessive model									
		CC	0.0	0.4		0.0	0.6		0.0	0.0	
		TC+TT	100.0	99.6	0.307	100.0	99.4	0.311	100.0	100.0	N.C.
		Allele									
		T	90.9	91.1		90.5	89.4		91.7	94.2	
		C	9.1	8.9	0.915	9.5	10.6	0.639	8.3	5.8	0.326
rs3088283	Missense mutation (The 54 Ile)	Genotype									
		TT	94.1	97.1		92.4	96.7		97.1	97.9	
		TC	5.9	2.9		7.6	3.3		2.9	2.1	
		CC	0.0	0.0	N.C.	0.0	0.0	N.C.	0.0	0.0	N.C.
		Dominant model									
		TT	94.1	97.1		92.4	96.7		97.1	97.9	
		TC+CC	5.9	2.9	0.082	7.6	3.3	0.074	2.9	2.1	0.709
		Recessive model									
		CC	0.0	0.0		0.0	0.0		0.0	0.0	
		TC+TT	100.0	100.0	N.C.	100.0	100.0	N.C.	100.0	100.0	N.C.
		Allele									
		T	97.0	98.5		96.2	98.3		98.5	98.9	
		C	3.0	1.5	0.085	3.8	1.7	0.078	1.5	1.1	0.711
rs6203	Silent mutation (Leu 338 Leu)	Genotype									
		TT	45.1	44.4		46.2	43.9		43.1	45.3	
		TC	46.5	41.5		47.8	41.7		44.1	41.1	
		CC	8.4	14.2	0.082	6.0	14.4	0.026	12.7	13.7	0.909
		Dominant model									
		TT	45.1	44.4		46.2	43.9		43.1	45.3	
		TC+CC	54.9	55.6	0.860	53.8	56.1	0.658	56.9	54.7	0.846
		Recessive model									
		CC	8.4	14.2		6.0	14.4		12.7	13.7	
		TC+TT	91.6	85.8	0.030	94.0	85.6	0.008*	87.3	86.3	0.764
		Allele									
		T	68.4	65.1		70.1	64.7		65.2	65.8	
		C	31.6	34.9	0.246	29.9	35.3	0.121	34.8	34.2	0.902
rs1047303	Missense mutation (Thr 367 Asn)	Genotype									
		AA	90.9	85.5		91.3	86.7		90.2	83.2	
		AC	9.1	12.7		8.7	11.7		9.8	14.7	
		CC	0.0	1.8	0.025	0.0	1.7	0.141	0.0	2.1	0.221
		Dominant model									
		AA	90.9	85.5		91.3	86.7		90.2	83.2	
		AC+CC	9.1	14.5	0.022	8.7	13.3	0.079	9.8	16.8	0.141

Table 2 *Continued*

Variants	Function		Total			Males			Females		
			NH (n=286)	EH (n=275)	P value	NH (n=184)	EH (n=180)	P value	NH (n=102)	EH (n=95)	P value
		Recessive model									
		CC	0.0	1.8		0.0	1.7		0.0	2.1	
		AC+AA	100.0	98.2	0.045	100.0	98.3	0.157	100.0	97.9	0.145
		Allele									
		A	95.5	91.8		95.7	92.5		95.1	90.5	
		C	4.5	8.2	0.938	4.3	7.5	0.071	4.9	9.5	0.776
rs2854964	Intron	Genotype									
		AA	58.4	56.0		55.4	56.7		66.7	54.7	
		AT	37.1	37.5		39.7	36.1		29.4	40.0	
		TT	4.5	6.5	0.532	4.9	7.2	0.564	3.9	5.3	0.230
		Dominant model									
		AA	58.4	56.0		55.4	56.7		66.7	54.7	
		AT+TT	41.6	44.0	0.561	44.6	43.3	0.813	33.3	45.3	0.086
		Recessive model									
		TT	4.5	6.5		4.9	7.2		3.9	5.3	
		AT+AA	95.5	93.5	0.278	95.1	92.8	0.351	96.1	94.7	0.652
		Allele									
		A	77.0	74.7		75.3	74.7		81.4	74.7	
		T	23.0	25.3	0.377	24.7	25.3	0.864	18.6	25.3	0.111
rs1819698	UTR-3	Genotype									
		GG	36.0	37.5		39.1	37.2		30.4	37.9	
		GA	46.5	48.0		44.6	47.8		50.0	48.4	
		AA	17.5	14.5	0.627	16.3	15.0	0.823	19.6	13.7	0.393
		Dominant model									
		GG	36.0	37.5		39.1	37.2		30.4	37.9	
		GA+AA	64.0	62.5	0.724	60.9	62.8	0.708	69.6	62.1	0.267
		Recessive model									
		AA	17.5	14.5		16.3	15.0		19.6	13.7	
		GA+GG	82.5	85.5	0.343	83.7	85.0	0.732	80.4	86.3	0.266
		Allele									
		G	59.3	61.5		61.4	61.1		55.4	62.1	
		A	40.7	38.5	0.454	38.6	38.9	0.933	44.6	37.9	0.176

Allele frequencies are given as percentages. NT, normotension; EH, essential hypertension. N.C. indicates that the calculation of the χ^2 from the contingency table could not be performed due to inclusion of a sample that did not contain any DNA. *Means remained significant P value after Bonferroni's correction ($P<0.0083$).

90% power during the detection of the disequilibrium at the 5% level of significance. Results of a previously published study also confirmed that our sample sizes were appropriate for this type of case–control study (12).

All continuous variables were expressed as the mean ± S.D. Differences in continuous variables between the EH patients and control subjects were analyzed using the Mann–Whitney *U* test. Differences in categorical variables were analyzed using Fisher's exact test. Differences in the distributions of the genotypes and alleles between the EH patients and control subjects were analyzed using Fisher's exact test. The frequency distribution of the haplotypes was calculated by performing a permutation test using the bootstrap method. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at $P<0.05$. All *P* values were adjusted for the number of tests performed by Bonferroni's correction. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL, USA).

Results

In the present study, 275 EH patients and 286 age-matched controls were genotyped for four SNPs that are the genetic markers for the *HSD3B1* gene (rs3765945, rs3088283, rs6203, and rs1047303) and for the two SNPs that are the markers for the *HSD3B2* gene (rs2854964 and rs1819698). Table 1 presents the clinical characteristics of the study participants, while the distributions of the genotypes and alleles of the six SNPs are listed in Table 2. The six SNPs examined in our study population were consistent with the Hardy–Weinberg equilibrium ($P>0.05$), and the allele frequency for each of these SNPs was in agreement with the previously reported HapMap data. In males, there was a significant difference for the rs6203 genotype distribution between the EH patients and the control subjects ($P=0.026$). For the total and male subject groups, there were also significant differences noted for the distribution of the recessive model of rs6203 (CC versus TC+TT) between the EH patients and the control subjects ($P=0.030$ and 0.008 respectively). In male subjects, the association between the recessive model of rs6203 and EH remained significant at $P<0.05$ after Bonferroni's correction on the number of tests performed. For rs1047303 in the total subject group, the genotype distribution, the distribution of the dominant model (AA versus AC+CC), and the recessive model (CC versus AC+AA) differed significantly between the EH patients and the controls ($P=0.025$, $P=0.022$, and $P=0.045$ respectively). Dominance and recessiveness of the model were defined by the frequency found for the total control individuals. While rs6203 and rs1047303 are located in the fourth exon of *HSD3B1*, the SNPs located within the *HSD3B2* gene were not associated with EH.

To investigate the effect of the gene polymorphism on BP, we performed a logistic regression analysis that examined age, body mass index (BMI), total cholesterol, presence or absence of diabetes mellitus, and smoking. For the total and the male subject groups, the CC genotype of rs6203 differed significantly between the EH patients and the control subjects ($P=0.041$ and $P=0.031$ respectively). There was also a significant difference for the AC+CC genotype of rs1047303 between the EH patients and control subjects of the total group ($P=0.027$, Table 3).

LD patterns for the *HSD3B1* and *HSD3B2* genes are shown at the bottom of Fig. 1. As all the r^2 values were below 0.5, all the six SNPs were considered to be located in a single haplotype block and, thus, were

Table 3 Odds ratio for hypertension risk factors and genotypes.

	Total			Male			Female		
Risk factor	Odds ratios	95% CI	*P* value	Odds ratios	95% CI	*P* value	Odds ratios	95% CI	*P* value
Odds ratios and 95% CI for hypertension risk factors and the CC genotypes of rs6203									
CC genotype	1.660	0.910–3.024	0.041	2.330	1.037–5.238	0.031	0.979	0.381–2.512	0.964
Age	0.987	0.964–1.011	0.282	0.985	0.950–1.021	0.420	0.989	0.959–1.020	0.497
BMI	1.135	1.073–1.200	<0.001	1.131	1.051–1.216	0.001	1.142	1.039–1.256	0.006
Total cholesterol	1.006	1.002–1.011	0.008	1.004	0.997–1.011	0.274	1.002	0.993–1.011	0.707
Smoking	1.368	1.251–1.593	0.031	3.176	2.030–5.220	<0.001	3.759	1.730–8.120	0.013
Diabetes mellitus	1.659	0.910–3.024	0.098	0.776	0.406–1.484	0.443	0.265	0.069–1.022	0.540
Odds ratios and 95% CI for hypertension risk factors and the AC+CC genotypes of rs1047303									
AC+CC genotype	1.495	0.265–1.922	0.027	0.797	0.382–1.663	0.545	1.947	0.705–5.376	0.198
Age	0.987	0.964–1.010	0.260	0.983	0.949–1.019	0.352	0.991	0.961–1.023	0.582
BMI	1.132	1.070–1.196	<0.001	1.126	1.048–1.210	0.001	1.137	1.033–1.252	0.009
Total cholesterol	1.006	1.002–1.011	0.008	1.004	0.997–1.011	0.286	1.005	0.997–1.013	0.232
Smoking	1.027	1.253–1.544	<0.001	3.193	1.994–5.103	<0.001	2.732	0.701–4.203	0.013
Diabetes mellitus	0.687	0.381–1.237	0.211	0.797	0.382–1.663	0.545	0.270	0.070–1.042	0.570

CI, confidence intervals.

Table 4 The haplotypes with the lowest overall *P* values in each of the groups.

				Frequency of the haplotype					
			Overall *P* value	NT	EH	*P* value	Adjusted *P* value	**Odds ratio for EH (95% CI)**	***P* value**
Haplotype established by rs3088283-rs1047303 (*Haplotype analysis in total subjects*)									
rs3088283	rs1047303		0.011						
T	A			0.925	0.918	0.888			
C	A			0.030	0.022	0.112			
T	C			0.045	0.063	0.014	0.056	1.702 (1.008–2.875)	0.047
Haplotype established by rs3088283-rs6203-rs1819698 (*Haplotype analysis in male subjects*)									
rs3088283	rs6203	rs1819698	0.016						
T	T	G		0.504	0.426	0.057			
T	C	G		0.109	0.192	0.002	0.016	1.661 (0.989–2.788)	0.055
T	T	A		0.192	0.220	0.358			
T	C	A		0.165	0.162	0.876			
C	C	A		0.030	0.000	0.033	0.264	N.C.	N.C.
Haplotype established by rs1047303-rs2854964 (*Haplotype analysis in female subjects*)									
rs1047303	rs2854964		<0.001						
A	A			0.808	0.677	0.003	0.012	0.513 (0.145–1.812)	0.003
C	A			0.000	0.081	0.001	0.004	N.C.	N.C.
A	T			0.157	0.242	0.039	0.156	1.678 (0.926–3.041)	0.088
C	T			0.035	0.000	0.284			

NT, normotension; EH, essential hypertension; CI, confidence intervals. Haplotype with frequency >0.02 were estimated by using SNPAlyze software. The *P* value of haplotype frequency was calculated by permutation test using bootstrap method. Adjusted *P* values were adjusted for the number of tests by Bonferroni's correction. Odds ratios were estimated for haplotypes that occurred at significantly different frequencies between EH and controls with control haplotypes as the reference. N.C. indicates that the calculation could not be performed due to the absence of haplotype.

suitable for use in a haplotype-based case–control study. In a haplotype-based case–control analysis, haplotypes are created via the use of different SNP combinations. Combinations based on the six SNPs used in this study resulted in a total of 51 haplotype patterns. The haplotypes with the lowest overall *P* value in each of the groups are shown in Table 4. For the total subject group, the frequency of the T-C haplotype established by rs3088283-rs1047303 was significantly higher for the EH patients compared with the control subjects ($P=0.014$). The frequency of the T-C-G haplotype established by rs3088283-rs6203-rs1819698 in the male subjects, and the frequency of the C-A haplotype established by rs1047303-rs2854964 in the female subjects were also significantly higher for the EH patients compared with the control subjects ($P=0.002$ and $P=0.001$ respectively). By using haplotypes in the controls as references, the odds ratios for EH were estimated for haplotypes which were present at a significantly different frequency between the EH and control subjects, and the T-C haplotype established by rs3088283-rs1047303 showed a significantly high odds ratio of 1.702 ($P=0.047$).

Subsequently, we used the haplotypes that had the lowest overall *P* values to establish diplotypes (Table 5). The diplotype established by rs3088283-rs1047303 in the total group was associated with significantly higher SBP, DBP, and aldosterone levels in those subjects who had the T-C haplotype compared with those who did not ($P=0.025$, $P=0.014$, and $P=0.006$ respectively). Hypertensive subjects who had one or two copies of the T-C haplotype were also found to have higher SBP and aldosterone levels compared to those lacking the T-C haplotype ($P=0.031$ and $P=0.049$ respectively). In males with the diplotype established by rs3088283-rs6203-rs1819698, both the SBP and aldosterone levels were significantly higher in subjects who had double T-C-G haplotypes compared with those who only had a single or who were completely lacking the T-C-G haplotype ($P=0.012$ and $P=0.039$ respectively). In females with the diplotype established by rs1047303-rs2854964, SBP and DBP were significantly higher in subjects who had the C-A haplotype compared with those who did not ($P=0.005$ and $P=0.001$ respectively). It should be noted, however, that these diplotypes had no association with echocardiographic parameters such as LVDd, IVST, PWTd, LVM or LVMI (data of PWTd and LVM, are not shown in Table 5). Furthermore, we investigated the effect of the T-C haplotype on the aldosterone level. We performed a logistic regression analysis that examined age, BMI, and the plasma renin activity and showed that the haplotype is associated with higher aldosterone level (>120 pg/ml; Table 6).

Discussion

The current study is the first to use haplotype and diplotype analyses in the same subjects in order to investigate the association between the *HSD3B1* gene and BP, aldosterone level, and echocardiographic

Table 5 Association between diplotypes and blood pressure, aldosterone level, and echocardiographic parameters.

	Diplotype	SBP (mmHg)	*P* value	DBP (mmHg)	*P* value	PAC (pg/ml)	*P* value	LVDd (mm)	*P* value	LVMI (g/m^2)	*P* value
Diplotype established by rs3088283-rs1047303 haplotypes in total subjects											
NT+EH	One or two T-C haplotypes	151.44±40.08 (*n*=66)	0.025	93.86±23.93 (*n*=66)	0.014	137.48±70.55 (*n*=34)	0.006	51.65±6.98 (*n*=16)	0.315	157.76±79.97 (*n*=13)	0.110
	No T-C haplotype	141.35±33.45 (*n*=495)		86.91±21.30 (*n*=495)		107.24±56.47 (*n*=206)		49.90±6.33 (*n*=94)		130.00±53.79 (*n*=86)	
EH	One or two T-C haplotypes	179.70±22.66 (*n*=40)	0.031	109.73±15.93 (*n*=40)	0.112	136.42±71.27 (*n*=32)	0.049	51.65±6.98 (*n*=16)	0.466	157.76±79.97 (*n*=13)	0.307
	No T-C haplotype	172.18±19.87 (*n*=235)		105.96±13.42 (*n*=235)		114.10±55.59 (*n*=160)		50.33±6.41 (*n*=71)		139.12±55.95 (*n*=66)	
Diplotype established by rs3088283-rs6203-rs1819698 haplotypes in male subjects											
NT+EH	Two T-C-G haplotypes	167.60±28.98 (*n*=10)	0.012	101.10±20.33 (*n*=10)	0.062	159.50±93.60 (*n*=7)	0.039	57.75±11.04 (*n*=4)	0.052	184.66±103.26 (*n*=4)	0.140
	No or one T-C-G haplotype	141.18±32.60 (*n*=354)		88.21±21.53 (*n*=354)		109.56±55.82 (*n*=139)		50.18±7.18 (*n*=61)		139.48±54.35 (*n*=55)	
Diplotype established by rs1047303-rs2854964 haplotypes in female subjects											
NT+EH	One or two C-A haplotypes	170.29±34.66 (*n*=14)	0.005	104.64±21.15 (*n*=14)	0.001	144.70±63.11 (*n*=10)	0.070	47.82±2.66 (*n*=5)	0.369	129.87±37.51 (*n*=4)	0.747
	No C-A haplotype	141.67±36.82 (*n*=183)		84.77±21.42 (*n*=183)		107.46±60.57 (*n*=85)		49.651±4.36 (*n*=40)		120.27±57.41 (*n*=37)	

NT, normotension; EH, essential hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; PAC, plasma aldosterone concentration; LVDd, left ventricular diastolic dimension; LVMI, left ventricular mess index.

findings. The use of haplotypes and/or diplotypes is a more recent approach that may very well be used to elucidate relationships between candidate genes and specific traits. To avoid the possible confounding effect of ethnicity, we restricted our analysis to Japanese subjects. Since we selected our experimental population on the basis of a strong family history of HT, there is an increased likelihood that our findings of a genetic contribution to HT were associated with the inherent biological power of our patient cohort. Therefore, the positive results of this study might potentially be able to explain why conflicting results have been reported in the past when HT was used as the phenotype.

HSD3B is a key rate-limiting enzyme in the steroid biosynthesis pathways that produce aldosterone, estradiol, testosterone, and cortisol. It is expressed as two tissue-specific isoforms (HSD3B1 and HSD3B2) that have different substrate affinities (13). A genome-wide linkage analysis of 420 markers (353 microsatellites and 67 restriction fragment length polymorphisms) has shown that the locus for BP variations is within the region of the *HSD3B1* gene on chromosome 1p13.1 (14). Most recently, Doi *et al.* (5) have shown that Cry-null mice show salt-sensitive HT due to abnormally high synthesis of the mineralocorticoid aldosterone by the adrenal gland. In Cry-null mice, *Hsd3b6* mRNA and protein level are constitutively high, leading to a marked increase in Hsd3b activity and, as a consequence, enhanced aldosterone production. They also found that human *HSD3B1*, but not *HSB3B2*, is a functional counterpart of mouse *Hsd3b6*. In clinical examination of the human *HSD3B1* gene of Swedish NTs, a positive association between BP and rs6203 (the T→C Leu 338 variant of *HSD3B1*) was found with higher SBP and DBP occurring in carriers of the C allele (6). Conversely, a later study indicated that this SNP was not associated with HT in a Caucasian–Australian HT cohort, even though the study revealed that there was a tendency for higher DBP in those with the C allele (15). Our analysis, however, did confirm that the CC genotype of rs6203 was associated with higher BP in male but not in female subjects. As in a recent study (6), we also found that the aldosterone level tended to increase in accordance with the number of C alleles of rs6203 present. In addition, extremely high aldosterone levels were observed for the CC genotype of rs1047303 compared with the AA and the AC genotypes (data not shown). Although the reason for the gender difference was not clear, the potential mechanisms underlying the gender-related differences observed in our study merit comment. Both estrogen and aldosterone receptors are present in cardiac fibroblasts, myocyte, endothelial, and vascular smooth muscle cells. In addition, in vascular tissue, the rapid responses to aldosterone may be important in the acute control of BP (16). Aldosterone and estrogen also elicit similar rapid nongenomic responses that use common signaling pathways (protein kinase C), and some of the responses are shown to have gender

Table 6 Odds ratios and 95% CI for higher aldosterone level (>120 pg/dl).

	NT+EH (*n*=240)			**EH** (*n*=192)		
Risk factor	Odds ratios	95% CI	*P* value	Odds ratios	95% CI	*P* value
T-C haplotype	2.302	1.040–5.096	0.040	5.408	1.580–18.508	0.007
Age	1.064	1.021–1.109	0.003	1.166	1.060–1.282	0.002
BMI	0.956	0.915–0.999	0.056	0.948	0.860–1.044	0.383
PRA	0.990	0.965–1.015	0.412	0.980	0.938–1.025	0.276

NT, normotension; EH, essential hypertension; CI, confidence intervals; BMI, body mass index; PRA; plasma renin activity. Haplotype was constructed with rs3088283 and rs1047303.

specificity (16). Because our present sample size was not sufficiently large to define gender differences, subsequent study is warranted to investigate whether an interaction between the signaling effect of estrogen and aldosterone receptors contributes to the observed gender-related differences in BP.

In animal models, both cardiac load and high circulating aldosterone levels can stimulate fibrosis within the myocardium, leading to LVH (17). Pathological patterns of the LV geometry have also been shown to be associated with an elevation of plasma aldosterone concentrations in the EH patients (18, 19). It has been estimated that the role of genetic factors in causing cardiac mass variance may be as high as 60% (20), and that these different gene variants are associated with LVH and diastolic dysfunction in EH. Heller *et al.* (21) assessed the influence of C344T aldosterone synthase polymorphism (*CYP11B2*) in left ventricular structure and humoral parameters in young NT males. They found that the TT genotype of the C344T polymorphism of the *CYP11B2* gene was associated with significantly higher plasma levels of aldosterone and higher values of the LVMI. On the other hand, a systematic review (22) of the association of the *CYP11B2* gene with echocardiographic parameters found that there was no significant association between the C344T polymorphism and LVM. Similarly, no significant association was found for interventricular septal wall thickness. However, the previous study did not examine the LVMI, which is an important parameter of LVH. In the present study, we evaluated the association between the *HSD3B1* gene and the LVMI, in addition to other echocardiographic parameters. However, we were also unable to reach a definitive conclusion with regard to the association between the diplotypes of *HSD3B1* and LVMI. One of the potentially limiting factors in the present study was the relatively small number of patients who underwent the echocardiographic examinations. Thus, the small sample size of our cohort may have limited the statistical power to detect weak associations between the diplotype and the echocardiographic parameters. We also recognize that our analysis was limited in that we only examined six of the SNPs for the *HSD3B* genes, and that we did not investigate the *CYP11B1* and *CYP11B2* genes, which are key to the control of the production of hormone during the late stages of aldosterone synthesis.

In concordance with a recent report (23), the findings for the tag SNPs and haplotype analysis in our study population further support the involvement of *HSD3B1* in BP regulation with rs6203 and rs1047303 both showing a definite association with BP. In contrast, our study indicates that the polymorphism within the *HSD3B2* gene, which is only 8.4 kb away from the *HSD3B1* gene, was not associated with BP. Thus, the observation that the *HSD3B1* gene is associated with increased BP and aldosterone levels suggests that a gain of function through a mutation in the *HSD3B1* gene is actually responsible for augmenting the biosynthesis of this hormone. In addition, recent studies have indicated that mutations of the enzymes involved in aldosterone biosynthesis can result in elevated or suppressed aldosterone levels (23, 24). Therefore, genetic polymorphisms such as rs1047303, which is a missense mutation of Thr 367 Asn in the exon of *HSD3B1*, might potentially modulate the exchange of the enzyme, thereby contributing to the inherited variability of the BP and aldosterone levels.

In conclusion, we employed a candidate-gene approach that uses a small set of highly informative SNPs to probe for significant associations with the BP and aldosterone levels. We observed a significant association between the *HSD3B1* variants and BP. Furthermore, the results of this study provide the first evidence that *HSD3B1* diplotype variations not only have a significant association with BP but also with aldosterone level in the present subjects. Overall, our results reinforce the view that inherited variations in the steroid biosynthetic pathway could have effects on both the circulating aldosterone level and BP in the Japanese population. However, our present study only included Japanese subjects, and the sample size was too small to clearly confirm the associations between SNPs at the *HSD3B1* gene and EH and plasma aldosterone. A replication study with a larger and/or another population is indispensable to confirm our present results.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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ORIGINAL CONTRIBUTIONS

Association Study of the Elastin Microfibril Interfacer 1 (*EMILIN1*) Gene in Essential Hypertension

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BACKGROUND
Elastin microfibril interfacer 1 (EMILIN-1) is a negative regulator of the transforming growth factor-β (TGF-β) signaling, which is involved in blood pressure (BP) homeostasis. *Emilin1* knockout mice display elevated BP. The aim of the present study was to assess the association between the human *EMILIN1* gene and essential hypertension (EH) using a haplotype-based case–control study.

METHODS
A total of 287 EH patients and 253 age-matched controls were genotyped for the five single-nucleotide polymorphisms (SNPs) used as genetic markers for the human *EMILIN1* gene (rs2289408, rs2289360, rs2011616, rs2304682, and rs4665947). Data were analyzed for three separate groups: the total subjects, men, and women.

RESULTS
For the total, the genotypic distribution of rs2289360, rs2011616, and rs2304682 differed significantly between control and EH ($P = 0.010$, $P = 0.009$, and $P = 0.008$, respectively). For the total and men, there were significant differences noted between the controls and the EH patients for both the dominant model (GG vs. AA+AG) ($P = 0.006$, $P = 0.021$, respectively), and the recessive model (AA vs. AG+GG) ($P = 0.028$, $P = 0.038$, respectively) of rs2011616. For the total and the men, logistic regression analysis indicated that the AG+GG genotype of rs2011616 was significantly higher in EH patients ($P = 0.033$, $P = 0.043$, respectively). The frequency of the G-G-T haplotype (established by rs2536512, rs2016116, rs17881426) was significantly higher in EH men ($P = 0.007$), and the G-A-T haplotype (established by rs2536512, rs2016116, rs17881426) was significantly higher in control men ($P < 0.001$).

CONCLUSIONS
We confirmed that rs2289360, rs2011616, and rs2304682 in the human *EMILIN1* gene, as well as the haplotype constructed using rs2536512, rs2011616, and rs17881426 are useful genetic markers of EH in Japanese men.

***Keywords:** androgen; blood pressure; case–control study; EMILIN1; essential hypertension; haplotype; hypertension; single-nucleotide polymorphism*

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Essential hypertension (EH), which in general increases the arterial blood pressure (BP), is a major health concern and risk factor for other diseases such as myocardial infarction and kidney failure.[1–3] Despite its prevalence, the pathogenesis of hypertension is poorly understood. However, it is known that the etiology of EH is multifactorial, with both genetic determinants, such as allelic variation in genes involved in renal salt absorption, along with environmental factors associated with the diet playing roles in the development of the disease.[4]

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Arterial BP is a function of both the cardiac output, which is the amount of blood pumped out by the heart, and the vasculature resistance. Changes in the vasculature that result in a narrowing of the arteries can increase peripheral resistance and cause hypertension. The extracellular matrix (ECM) in the vascular wall has recently been found to be a critical determinant. Elastin haploinsufficiency mice have been shown to have changes of the arterial wall structure. These mice have been found to be stably hypertensive from birth, with a mean arterial pressure 25–30 mm Hg higher than their wild-type counterparts.[5] Also, individuals with mutations in the ECM component elastin develop supravalvular aortic stenosis, which is characterized by a narrowing of the ascending aorta.[6] Thus, vessel compliance dictated by the ECM is also involved in modulating the BP.

Recently, studies examining BP homeostasis have discovered a new link between ECM and the transforming growth factor β (TGF-β). TGF-β directly stimulates the synthesis of matrix molecules and blocks matrix degradation.[7] In addition, TGF-β may regulate the BP level via the endothelin and/or renin–angiotensin system.[8] In 2006, Zacchigna *et al.* found that the elastin microfibril interface-located protein (Emilin-1) inhibits

TGF-β signaling by specifically binding to the proTGF-β precursor, thereby preventing its maturation by furin convertases in the extracellular space.[9] The study additionally revealed that *Emilin1* deficient mice became hypertensive (systolic BP (SBP): 120 ± 2 vs. 101 ± 1, $n = 46$ per group, $P < 0.01$), and had an increased peripheral vascular resistance and a reduced vessel size, all of which are independent of cardiac output. In support of these findings, genetic inactivation of Emilin-1 has been shown to increase the TGF-β signaling within the vascular wall. Strikingly, the high BP in *Emilin1* deficient mice returned to normal levels upon inactivation of a single *TGF-β* allele. This study highlighted the importance of modulating the relationship between Emilin-1 and TGF-β availability in the pathogenesis of hypertension.

In humans, an increase in the circulating TGF-β has been found in individuals with EH.[10] Li *et al.* have shown that a positive correlation exists between the circulating levels of TGF-β protein and BP in humans.[11] In addition, it has also been reported there is an association between EH and single-nucleotide polymorphisms (SNPs) of *TGF-β*.[10,11] These findings suggest there is a possible association between EH and the human *EMILIN1* gene, which is a negative regulator of TGF-β. EMILIN-1 is an ECM glycoprotein that is abundantly expressed in elastin-rich tissues such as the blood vessels, skin, heart, and lung. Elastic fibers are present at the interface between the amorphous elastin and the microfibrils. The human *EMILIN1* gene, which encodes EMILIN-1, consists of 955 amino acids and is located on chromosome 2p23.3–p23.2. There are 45 SNPs of the human *EMILIN1* gene listed in the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/SNP). This gene is quite small and consists of approximately 7.3 kilobase pairs (kbp) that contain eight exons, which are interrupted by seven introns.[12]

METHODS

Subjects. Subjects diagnosed with hypertension were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo from 1993 to 2003. A total of 1,123 patients who consistently had BPs of at least 140 mm Hg systolic and/or 90 mm Hg diastolic were diagnosed as being hypertensive (stage I of World Health Organization criteria).[13] Genomic DNA was extracted from each of these patients and stored until analyses were performed. For the strict case–control study EH patients, subjects were diagnosed based on the following criteria: seated SBP >160 mm Hg and/or diastolic BP (DBP) >100 mm Hg (stage II or stage III of World Health Organization criteria) on three occasions within 2 months after the first BP reading. All EH subjects investigated in the present study had a familial history of EH. A family history of hypertension was defined as prior diagnosis of hypertension in grandparents, uncles, aunts, parents, or siblings. None of the EH patients were receiving any antihypertensive medications. After initial patient enrollment, plasma aldosterone levels and renin activity were determined in all subjects. Patients determined to have secondary hypertension, such as primary aldosteronism, were excluded from the present study. After completion of the screening of the original group, a total of 287 EH patients met the inclusion criteria and were enrolled in the study.

For the control group, a total of 253 normotensive (NT) age-matched healthy individuals (male/female ratio = 1.63) were enrolled. None of the controls had any family history of hypertension, and all subjects had a SBP of <130 mm Hg and a DBP of <85 mm Hg. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.[14]

Genotyping. There are 45 SNPs for the human *EMILIN1* gene listed in the National Center for Biotechnology Information SNP database Build 129 (http://www.ncbi.nlm.nih.gov/SNP). In this study, we also screened the data for the Tag SNPs on the International HapMap Project website (http://hapmap.ncbi.nlm.nih.gov/index.html.en). The data for all SNPs listed for the Japanese population were classified into three groups according to their minor allele frequency (MAF). The frequencies for the large MAF group ranged from 0.367 to 0.417, whereas the middle MAF group ranged from 0.179 to 0.200. In the final group, the MAF was <0.1. SNPs with relatively high minor allele frequencies have been shown to be useful as genetic markers in genetic association studies. Unfortunately, because there were no SNPs of the *EMILIN1* gene included in the first and the second groups, we selected three SNPs (rs2289360, rs2011616, and rs2304682) that had a MAF of >0.15 as the markers for the genetic association experiment (**Figure 1**). This selection was based on the SNP allelic frequency information that had been previously registered with National Center for Biotechnology Information, Celera Discovery System–Applied Biosystems, and the International HapMap project. These three SNPs were located in the intron regions. Because only one out of the three SNPs (rs2011616) were shown on the International HapMap Project website, we were unaware of the |D′| and r^2 between the three SNPs prior to starting the present study. In order to perform the haplotype-based case–control analysis, we added two SNPs upstream and downstream of the gene. rs2289408 is located 54 kbp upstream from the start codon in exon 1, whereas rs4665947 is located 39 kbp downstream from the stop codon in exon eight (**Figure 1**). We designated

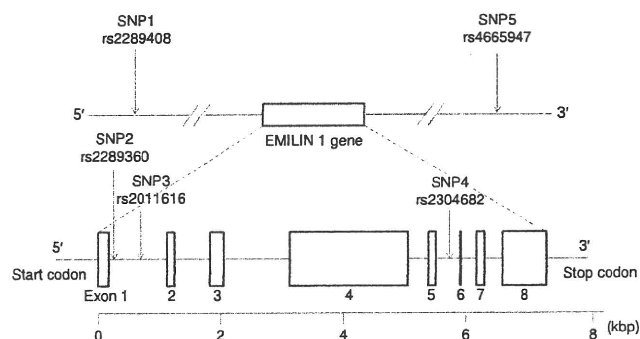

Figure 1 | Structure of the human *EMILIN1* gene. The gene consists of eight exons (boxes) separated by seven introns (lines; intergenic regions). Filled boxes indicate the coding regions, while arrows indicate the locations of single-nucleotide polymorphism (SNPs). kbp, kilobase pairs.

the five SNPs as SNP1 (rs2289408, C_15881576_10), SNP2 (rs2289360, C_3240194_1), SNP3 (rs2011616, C_3240196_10), SNP4 (rs2304682, C_3240197_1_), and SNP5 (rs4665947, C_11789131_10), which were in order of increasing distance from the 5′ end of the gene (**Figure 1**). Genotyping for this SNP was done using a kit from Applied Biosystems (Foster City, CA).

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes using phenol and chloroform extraction.[15]

Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems). The TaqMan SNP Genotyping Assays were performed using the Taq amplification method.[16] In the first step of the 5′ nuclease assay, allele-specific fluorogenic probes are hybridized to the template. Subsequently, the 5′ nuclease activity of the Taq polymerase makes it possible for discrimination to occur during the PCR. The probes contain a 3′ minor groove-binding group that hybridizes to single-stranded targets that have greater sequence specificity than the ordinary DNA probes. This reduces nonspecific probe hybridization, which results in a low background fluorescence in the 5′ nuclease PCR assay (TaqMan, Applied Biosystems). 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) were chosen based on information available at the ABI website (http://www3.appliedbiosystems.com/AB_Home/index.htm).

PCR amplification was performed using 2.5 µl of TaqMan Universal Master Mix, No AmpErase UNG (2×) (Applied Biosystems) in a 5 µl final reaction volume, along with 2 ng DNA, 2.375 µl ultrapure water, 0.079 µl Tris-EDTA (TE) buffer (1×), 0.046 µl TaqMan SNP Genotyping Assay Mix (40×) containing a 331.2 nmol/l final concentration of primers, along with a 73.6 nmol/l final concentration of the probes. The thermal cycling conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 50 cycles of 95 °C for 15 s; and 60 °C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system (Applied Biosystems).

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 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems). The plates were read on the sequence detection system 7700 instrument with the end-point analysis mode of the sequence detection system version 1.6.3 software package (Applied Biosystems). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X-Y scatter-plot of the sequence detection system 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.[17]

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.

Based on our initial estimated sample size, the number of patients enrolled was considered to be sufficient for a gene polymorphism study. During our analyses, we obtained 90% power during the detection of the disequilibrium at the 5% level of significance. Results of a previously published study also confirmed that our sample sizes were appropriate for this type of case–control study.[18]

Statistical analysis. All continuous variables were expressed as mean ± s.d. Differences in continuous variables between the EH patients and control subjects were analyzed using the Mann–Whitney *U*-test. Differences in categorical variables were analyzed using Fisher's exact test. Differences in distributions of genotypes and alleles between EH patients and control subjects were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations, we performed linkage disequilibrium and haplotype-based case–control analyses using the expectation maximization algorithm[19] and the software SNPAlyze version 3.2 (Dynacom, Yokohama, Japan). The pairwise linkage disequilibrium analysis was performed using three SNP pairs. We used $|D'|$ values of >0.5 to assign SNP locations to one haplotype block. SNPs with an r^2 value of <0.5 were selected as tagged. In the haplotype-based case–control analysis, haplotypes with a frequency of <0.02 were excluded. The frequency distribution of the haplotypes was calculated by performing a permutation test using the bootstrap method. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at $P < 0.05$. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS, Chicago, IL).

RESULTS

Table 1 shows the clinical characteristics of the study participants. For total subjects, men, and women, the following values were significantly higher for the EH patients as compared to the control subjects: body mass index, SBP, DBP, and the incidence of drinking and smoking. For total subjects and men, the following values were significantly higher for the EH patients as compared to the control subjects: pulse rate, plasma concentration of uric acid, incidence of hyperlipidemia, and diabetes. There were no significant differences for the following variables between the EH patients and the control subjects: age, plasma concentration of creatinine, and the plasma concentration of high-density lipoprotein cholesterol.

Table 2 shows the distribution of the genotypes and alleles of the five SNPs. Because the genotype distribution of rs2289360 was different from the Hardy–Weinberg equilibrium values

Table 1 | Characteristics of study participants

	NT	Total EH	*P* value	NT	Men EH	*P* value	NT	Women EH	*P* value
Number of subjects	253	287		157	193		96	94	
Age (years)	51.07 ± 9.89	49.80 ± 6.84	0.080	50.45 ± 5.75	49.08 ± 7.34	0.056	52.10 ± 14.29	51.29 ± 5.27	0.608
BMI (kg/m²)	22.56 ± 3.20	24.67 ± 3.65	<0.0001*	22.77 ± 3.14	24.83 ± 3.48	<0.0001*	22.21 ± 3.27	24.36 ± 3.96	<0.0001*
SBP (mm Hg)	112.06 ± 10.76	173.15 ± 20.04	<0.0001*	112.51 ± 10.56	170.93 ± 18.91	<0.0001*	111.32 ± 11.09	177.69 ± 21.59	<0.0001*
DBP (mm Hg)	69.06 ± 8.59	106.57 ± 13.31	<0.0001*	69.06 ± 8.59	106.57 ± 13.31	<0.0001*	67.74 ± 9.17	105.46 ± 13.38	<0.0001*
Pulse (beats/min)	73.03 ± 12.55	77.78 ± 14.80	0.0008*	72.40 ± 13.52	77.68 ± 15.14	0.004*	74.01 ± 10.88	77.99 ± 14.13	0.067
Creatinine (mg/dl)	0.85 ± 0.25	0.827 ± 0.21	0.200	0.92 ± 0.20	0.94 ± 0.4	0.385	0.69 ± 0.14	0.68 ± 0.18	0.803
Total cholesterol (mg/dl)	200.70 ± 43.85	209.21 ± 40.02	0.022*	195.99 ± 42.73	203.78 ± 40.14	0.896	208.36 ± 44.79	219.01 ± 37.78	0.610
HDL cholesterol (mg/dl)	57.20 ± 17.58	55.77 ± 17.31	0.379	55.13 ± 16.51	52.26 ± 16.10	0.132	60.59 ± 18.81	62.62 ± 17.65	0.473
Uric acid (mg/dl)	5.38 ± 1.76	5.72 ± 1.59	0.0264*	5.86 ± 1.34	6.25 ± 1.48	0.0143*	4.59 ± 2.07	4.63 ± 1.22	0.870
Hyperlipidemia (%)	18.7	26.8	0.0243*	15.3	24.4	0.0447*	24.2	31.9	0.260
Diabetes (%)	3.174603175	9.4	0.0034*	3.2	10.4	0.0095*	3.2	7.4	0.188
Drinking (%)	38.5	62.4	<0.0001*	48.4	74.6	<0.0001*	22.1	37.2	0.0262*
Smoking (%)	27.8	53.3	<0.0001*	35.0	63.7	<0.0001*	15.8	31.9	0.0106*

Continuous variables are expressed as mean ± s.d. Categorical variables are expressed as percentages.
BMI, body mass index; DBP, diastolic blood pressure; EH, essential hypertension; HDL, high-density lipoprotein; NT, normotension; SBP, systolic blood pressure.
The *P* value of the continuous variables was calculated by the Mann–Whitney *U*-test.
The *P* value of the categorical variables was calculated by Fisher's exact test. *$P < 0.05$.

($P = 0.038$), we omitted this SNP from all of the statistical analyses in the simple case–control and haplotype-based case–control studies. The genotype distribution of the other four SNPs were in good agreement with the Hardy–Weinberg equilibrium (data not shown). For total subjects, the genotype distribution of rs2011616 and rs2304682 differed significantly between the EH patients and the control subjects ($P = 0.009$ and $P = 0.008$, respectively). For men, the genotype distribution of these two SNPs differed significantly between the EH patients and the control subjects ($P = 0.028$ and $P = 0.046$, respectively). For the total subjects and men, the distribution of the dominant model of rs2011616 (GG vs. AA+AG) differed significantly between the two groups ($P = 0.006$ and $P = 0.021$, respectively). There was also a difference noted for the distribution of the recessive model of rs2011616 (AA vs. AG+GG) between the two groups ($P = 0.028$ and $P = 0.038$, respectively). However, for all of the SNPs selected for present study, there were no differences for the genotype distributions, or the dominant and recessive model distributions noted for the women. Dominance and recessiveness of the models were defined by their frequency among the total subjects.

Logistic regression was performed with and without diabetes mellitus, smoking, and hyperlipidemia. As shown in Table 3, there were significant differences noted for the total and the men for the AG+GG genotype of rs2011616 between the EH patients and the control subjects ($P = 0.033$ and $P = 0.043$, respectively). There was also a difference noted for the logistic regression analysis of the GG genotype of rs2011616 ($P = 0.033$ and $P = 0.049$, respectively, data not shown). In contrast, there was no difference noted for the GG+AG genotype of rs2011616 between the women EH patients and control subjects ($P = 0.412$).

The $|D'|$ and r^2 values for the linkage disequilibrium patterns are presented in Table 4. All five SNPs are located in one haplotype block, as all of the $|D'|$ are beyond 0.5. Three SNPs (rs2289360, rs2011616, and rs2304682) were not simultaneously available for the performance of a haplotype-based case–control study, as all of the r^2 values were beyond 0.5. rs2289360 was omitted because there was no agreement with the Hardy–Weinberg equilibrium. We selected rs2011616 because the MAF of the SNP was larger than that of rs2304682. Therefore, the haplotype-based case–control study was performed using only three SNP (rs2289408, rs2011616, and rs4665947).

In the haplotype-based case–control analysis, haplotypes were established through the use of different combinations of the SNPs (Table 5). For the total subjects and men, the overall distribution of the haplotypes established by SNP1-SNP3, SNP3-SNP5, and SNP1-SNP2-SNP3 were significantly different between the EH patients and the control subjects. For the total subjects and men, the frequencies of the G-G haplotype established by SNP1-SNP3 were significantly higher for the EH patients compared to the control subjects ($P = 0.002$ and $P = 0.006$, respectively) while the frequencies for the G-A haplotype were significantly lower for EH patients compared to the control subjects ($P = 0.002$ and $P = 0.011$, respectively). For the total subjects and men, there was also a significant difference

Table 2 | Genotype and allele distributions in normotensives and patients with EH

	Total			Men			Women		
Variants	NT (*N* = 253)	EH (*N* = 287)	*P* value	NT (*N* = 157)	EH (*N* = 193)	*P* value	NT (*N* = 96)	EH (*N* = 94)	*P* value
rs2289408 (SNP1)									
Genotype									
AA	0 (0)	2 (0.007)		0 (0)	2 (0.010)		0 (0)	0 (0)	
AG	39 (0.154)	35 (0.122)		27 (0.172)	25 (0.130)		12 (0.125)	10 (0.106)	
GG	214 (0.846)	250 (0.871)	0.141	130 (0.828)	166 (0.860)	0.150	84 (0.875)	84 (0.894)	0.688
Dominant model									
GG	214 (0.846)	250 (0.871)		130 (0.828)	166 (0.860)		84 (0.875)	84 (0.894)	
AA+AG	39 (0.154)	37 (0.129)	0.400	27 (0.172)	27 (0.140)	0.409	12 (0.125)	10 (0.106)	0.161
Recessive model									
AA	0 (0)	2 (0.007)		0 (0)	2 (0.010)		0 (0)	0 (0)	
GG+AG	253 (1)	285 (0.993)	0.183	157 (1)	191 (0.990)	0.201	96 (1)	94 (1)	1.000
Allele									
A	39 (0.077)	39 (0.068)		27 (0.086)	29 (0.075)		12 (0.063)	10 (0.053)	
G	467 (0.923)	535 (0.932)	0.563	287 (0.914)	357 (0.925)	0.277	180 (0.938)	178 (0.947)	0.698
rs2289360 (SNP2)									
Genotype									
AA	23 (0.091)	12 (0.042)		16 (0.102)	7 (0.036)		7 (0.073)	5 (0.053)	
AG	84 (0.332)	78 (0.272)		46 (0.293)	48 (0.249)		38 (0.396)	30 (0.319)	
GG	146 (0.577)	197 (0.686)	0.010	95 (0.605)	138 (0.715)	0.013	51 (0.531)	59 (0.628)	0.399
Dominant model									
GG	146 (0.577)	197 (0.686)		95 (0.605)	138 (0.715)		51 (0.531)	59 (0.628)	
AA+AG	107 (0.423)	90 (0.314)	0.008	62 (0.395)	55 (0.285)	0.030	45 (0.469)	35 (0.373)	0.178
Recessive model									
AA	23 (0.091)	12 (0.042)		16 (0.102)	7 (0.036)		7 (0.073)	5 (0.053)	
GG+AG	230 (0.909)	275 (0.958)	0.021	141 (0.898)	186 (0.964)	0.014	89 (0.927)	89 (0.947)	0.576
Allele									
A	130 (0.257)	102 (0.178)		78 (0.248)	62 (0.161)		52 (0.271)	40 (0.212)	
G	376 (0.743)	472 (0.822)	0.002	236 (0.752)	324 (0.839)	0.004	140 (0.729)	148 (0.787)	0.186
rs2011616 (SNP3)									
Genotype									
AA	21 (0.083)	11 (0.038)		14 (0.089)	7 (0.036)		7 (0.073)	4 (0.043)	
AG	88 (0.348)	80 (0.279)		50 (0.318)	49 (0.254)		38 (0.396)	31 (0.330)	
GG	144 (0.569)	196 (0.683)	0.009	93 (0.592)	137 (0.710)	0.028	51 (0.531)	59 (0.628)	0.352
Dominant model									
GG	144 (0.569)	196 (0.683)		93 (0.592)	137 (0.710)		51 (0.531)	59 (0.628)	
AA+AG	109 (0.431)	91 (0.317)	0.006	64 (0.408)	56 (0.290)	0.021	45 (0.469)	35 (0.372)	0.178
Recessive model									
AA	21 (0.083)	11 (0.038)		14 (0.089)	7 (0.036)		7 (0.073)	4 (0.043)	
GG+AG	232 (0.917)	276 (0.962)	0.028	143 (0.911)	186 (0.964)	0.038	89 (0.927)	90 (0.957)	0.370
Allele									
A	130 (0.257)	102 (0.178)		78 (0.248)	63 (0.163)		52 (0.271)	39 (0.207)	
G	376 (0.743)	472 (0.822)	0.002	236 (0.752)	323 (0.837)	0.005	140 (0.729)	149 (0.793)	0.148

Table 2 | Continued on next page

Table 2 | Continued

Variants	Total NT (*N* = 253)	Total EH (*N* = 287)	Total *P* value	Men NT (*N* = 157)	Men EH (*N* = 193)	Men *P* value	Women NT (*N* = 96)	Women EH (*N* = 94)	Women *P* value
rs2304682 (SNP4)									
Genotype									
CC	20 (0.079)	10 (0.035)		13 (0.083)	7 (0.036)		7 (0.073)	3 (0.032)	
CG	88 (0.348)	80 (0.279)		50 (0.318)	49 (0.254)		38 (0.396)	31 (0.330)	
GG	145 (0.573)	197 (0.686)	0.008	94 (0.599)	137 (0.710)	0.046	51 (0.531)	60 (0.638)	0.221
Dominant model									
GG	145 (0.573)	197 (0.686)		94 (0.599)	137 (0.710)		51 (0.531)	60 (0.638)	
CC+CG	108 (0.427)	90 (0.314)	0.006	63 (0.401)	56 (0.290)	0.029	45 (0.469)	34 (0.362)	0.134
Recessive model									
CC	20 (0.079)	10 (0.035)		13 (0.083)	7 (0.036)		7 (0.073)	3 (0.032)	
GG+CG	233 (0.921)	277 (0.965)	0.025	144 (0.917)	186 (0.964)	0.062	89 (0.927)	91 (0.968)	0.206
Allele									
C	128 (0.253)	100 (0.174)		76 (0.242)	63 (0.163)		52 (0.271)	37 (0.197)	
G	378 (0.747)	474 (0.826)	0.002	238 (0.758)	323 (0.837)	0.009	140 (0.729)	151 (0.803)	0.089
rs4665947 (SNP5)									
Genotype									
CC	29 (0.114)	26 (0.091)		19 (0.120)	17 (0.088)		10 (0.104)	9 (0.096)	
CT	119 (0.469)	136 (0.474)		70 (0.443)	87 (0.451)		49 (0.510)	49 (0.521)	
TT	106 (0.417)	125 (0.436)	0.654	69 (0.437)	89 (0.461)	0.606	37 (0.385)	36 (0.383)	0.978
Dominant model									
TT	106 (0.417)	125 (0.436)		69 (0.437)	89 (0.461)		37 (0.385)	36 (0.383)	
CC+CT	148 (0.583)	162 (0.564)	0.669	89 (0.563)	104 (0.539)	0.647	59 (0.615)	58 (0.617)	0.972
Recessive model									
CC	29 (0.114)	26 (0.091)		19 (0.120)	17 (0.088)		10 (0.104)	9 (0.096)	
CT+TT	225 (0.886)	261 (0.909)	0.641	139 (0.880)	176 (0.912)	0.323	86 (0.896)	85 (0.904)	0.847
Allele									
C	177 (0.348)	188 (0.328)		108 (0.342)	121 (0.313)		69 (0.359)	67 (0.356)	
T	331 (0.652)	386 (0.672)	0.468	208 (0.658)	265 (0.687)	0.426	123 (0.641)	121 (0.643)	0.952

EH, essential hypertensives; *N*, number of participants; NT, normotensives; SNP, single-nucleotide polymorphism.

for the frequencies of the G-T haplotype and A-T haplotype established by SNP3-SNP5 between the ET patients and the control subjects. For the total subjects and men, the frequencies of the G-G-T haplotype established by SNP1-SNP3-SNP5 were significantly higher for the EH patients as compared to the control subjects (P = 0.002 and P = 0.007, respectively). The frequency of the G-A-T haplotype established by SNP1-SNP3-SNP5 was significantly lower for the EH patients vs. that for the control subjects (P = 0.019 and $P < 0.001$, respectively). For the women, there were no significant differences noted for the distribution of the haplotypes established by SNP1-SNP3, SNP3-SNP5, and SNP1-SNP2-SNP3 between the EH patients and the control subjects.

DISCUSSION

TGF-β has an essential role in various processes ranging from embryonic development to tumorgenesis. For example, mutations in the TGF-β-related signaling pathway lead to primary pulmonary hypertension, which is an autosomal dominant disease.[20] Elevated BP has been attributed to a dual action of TGF-β in which vasoactive molecules are increased and there is remodeling of the blood vessel architecture. In humans, TGF-β_1 levels are higher in hypertensives as compared to NTs. In a previous study, the mean±s.e.m. TGF-β_1 protein concentration in hypertensive subjects (n = 61) was found to be 261 ± 9 ng/ml whereas it was 188±7 ng/ml in NT controls (n = 90) ($P < 0.0001$).[21] Recently, the relationship between aldosterone and TGF-β has been examined. Chun *et al.* found that aldosterone increased the gene expression of profibrotic factors, such as PAI-1, osteopontin, and TGF-β, in the kidney.[22] Because these changes could very well cause important differences between the EH patients and subjects with hypertension due to hyperaldosteronism, we measured the plasma aldosterone levels in all of the patients at the start of the study.

Table 3 | Odds ratios and 95% confidence intervals for each of the risk factors and the AG+GG genotypes of rs2011616 associated with essential hypertension

	Total			Men			Women		
Risk factor	Odds ratios	95% Confidence interval	*P* value	Odds ratios	95% Confidence interval	*P* value	Odds ratios	95% Confidence interval	*P* value
AG+GG genotype	2.391	1.071–5.339	0.033*	2.801	1.040–7.584	0.043*	1.773	0.451–0.967	0.412
Diabetes mellitus	0.476	0.203–1.113	0.087	0.435	0.152–1.239	0.119	0.554	0.124–2.486	0.441
Smoking	0.101	0.056–0.181	<0.001*	0.181	0.084–0.387	<0.001*	0.104	0.037–0.292	<0.001*
Hyperlipidemia	0.711	0.455–1.112	0.135	1.378	0.764–2.484	0.287	0.699	0.348–1.403	0.313

*$P < 0.05$.
SNP, single-nucleotide polymorphism.

All those subjects found to have hyperaldosteronism were excluded from the study.

Emilin-1 is a negative regulator of TGF-β signaling within the vascular wall.[9] In Emilin-1 deficient mice, there are no defects in the vascular contractility or mechanical properties, even though an increase in the peripheral resistance due to vessel size is observed. The hypertensive mice exhibited significantly narrowed arteries. Although these mice are fertile and have no obvious abnormalities, histological and ultrastructural examinations show there are alterations of the elastic fibers in the aorta and skin. It has also been shown that there is an abnormal formation of the elastic fibers by these mutant embryonic fibroblasts in cultures.[23] Thus, these animal models indicate that Emilin-1 plays an important role in BP and histological vascular changes.

In the present study, the genotype distribution along with the dominant model and recessive model distributions of the three SNPs of the human *EMILIN1* gene were significantly different between the male EH patients and control subjects. In contrast, no associations between the *EMILIN1* gene and EH were found in women. The present study showed significant gender-specific differences (for men only) in genetic markers between EH patients and control subjects. The reason for these gender-dependent differences remains unclear. In previous reports on Japanese men, a strong association between the T29→C polymorphism of the *TGF-β* gene and myocardial infarction has been noted. However, this association has not been found in women.[24] Other case–control studies have also identified gene variants that are associated with gender-specific susceptibility,[25,26] including the *TGF-β* gene variants in Japanese EH subjects.[27] In a male Russian study population, an association was also found between the 25Pro allele and 25ArgPro genotype of the *TGF-β* gene, with these individuals exhibiting a lower risk of EH.[28]

Our study is the first to investigate the association between the human *EMILIN1* gene and EH using the haplotype-based case–control study in a Japanese population. Very recently, Shen et al. have investigated two SNPs (rs3754734 and rs2011616) of the *EMILIN1* gene in the northern Han Chinese population.[29] They showed that subjects with the G allele (TG/GG genotype) of rs3754734 and those with the A allele (AG/AA genotype) of rs2011616 had an increased risk for EH. On the other hand, there was a significantly higher number of subjects with the G allele (AG/GG genotype) of rs2011616 in the EH patients as compared to the controls in our study. Shen et al. defined the hypertensive subjects as having a SBP ≥160 mm Hg and/or DBP ≥100 mm Hg, while the NT subjects had a SBP <140 mm Hg and DBP <90 mm Hg. These criteria are not the same as those used in the present study. In addition, they did not analyze their data for the three separate groups, the total subjects, men and women, which were examined in the present study. The genotypic distribution of the rs2011616 in the control subjects of the northern Han Chinese (AA, 8.0%; AG, 36.9%; GG, 55.1%)[29] did not differ ($P = 0.845$, χ^2 test) from that found for our controls (AA, 8.3%; AG, 34.8%; GG, 56.9%). Therefore, the different results of these two studies could in fact be due to the different definitions used to define the hypertensive and NT subjects rather than due to the differences in the genetic background between the two races. Because a familial history is one of the most important factors that can be used to distinguish between essential and secondary hypertension, we selected EH patients that had a familial history of hypertension while all of the control subjects were without any familial history. The EH subjects investigated in the present study were all diagnosed in accordance with the World Health Organization criteria[13] and classified as being either stage II or stage III. Thus, the severity of the criteria used to diagnose these hypertension stages may also provide further support for the results we determined in the present study.

Table 4 | Pairwise linkage disequilibrium for the five SNPs

	|D'| values				
r^2 values	SNP1	SNP2	SNP3	SNP4	SNP5
SNP1		0.806	0.774	0.765	0.827
SNP2	0.019		0.937	0.935	0.656
SNP3	0.017	0.859		1.000	0.660
SNP4	0.017	0.840	0.979		0.668
SNP5	0.031	0.282	0.280	0.281	

|D'| above the diagonal and r^2 below the diagonal. The shadowed portion indicates |D'| > 0.5 and r^2 >0.5.

Table 5 | Haplotype analysis in patients with EH and in control subjects

				overall *P* value			Frequency in total			Frequency in men			Frequency in women		
Haplotype	No1	No3	No5	Total	Man	Women	EH patients	Control subjects	*P* value	EH patients	Control subjects	*P* value	EH patients	Control subjects	*P* value
	SNP1	SNP3		0.003*	0.016*	0.288									
H1	G	G					0.759	0.671	0.002*	0.768	0.673	0.006*	0.739	0.667	0.123
H3	G	A					0.177	0.254	0.002*	0.161	0.244	0.011*	0.207	0.271	0.135
H2	A	G					0.065	0.075	0.480	0.070	0.083	0.479	0.053	0.063	0.638
		SNP3	SNP5	0.006*	0.021*	0.494									
H1		G	T				0.667	0.593	0.019*	0.684	0.608	0.066*	0.604	0.567	0.489
H2		A	T				0.000	0.057	0.015*	0.000	0.048	0.007*	0.040	0.073	0.315
H3		G	C				0.171	0.150	0.386	0.161	0.144	0.595	0.189	0.162	0.487
H4		A	C				0.162	0.200	0.118	0.155	0.200	0.148	0.168	0.198	0.466
	SNP1		SNP5	0.230	0.620	0.787									
H1	G		T				0.628	0.577	0.071	0.641	0.577	0.099	0.602	0.578	0.551
H2	A		T				0.057	0.075	0.214	0.061	0.083	0.313	0.048	0.063	0.589
H3	G		C				0.315	0.347	0.248	0.298	0.340	0.265	0.349	0.359	0.817
	SNP1	SNP3	SNP5	0.006*	0.007*	0.583									
H1	G	G	T				0.621	0.525	0.002*	0.638	0.537	0.007*	0.562	0.504	0.282
H2	G	A	T				0.000	0.052	0.019*	0.000	0.041	<0.001*	0.040	0.074	0.397
H3	A	G	T				0.059	0.074	0.324	0.062	0.081	0.349	0.048	0.063	0.557
H4	G	G	C				0.160	0.148	0.577	0.146	0.141	0.882	0.185	0.163	0.577
H5	G	A	C				0.160	0.201	0.103	0.154	0.201	0.116	0.164	0.197	0.359

EH, essential hypertension; Haplotype with frequencies >0.02 were estimated using SNPAlyze software.
P value was calculated by permutation test using the bootstrap method. *$P < 0.05$.
SNP, single-nucleotide polymorphism.

The present findings indicate that these three SNPs in the human *EMILIN1* gene are associated with EH in men. However, finding just one of these three SNPs can serve as a genetic marker of EH, as pairwise linkage disequilibrium for five SNPs (**Table 3**) has shown there is linkage between these three SNPs in the human *EMILIN1* gene. In the present study, none of the SNPs were thought to be of any functional consequence. However, it might be possible that there are functional mutations in the human *EMILIN1* gene with quantitative effects on genomic transcription, posttranslational processing or amino acid sequences that have a strong linkage with genetic markers such as rs2389360, rs2011616, or rs2304682. If so, these could subsequently reduce or increase the activity of EMILIN-1 that is associated with EH in men.

There were no differences noted in the plasma and urine levels of TGF-β between healthy men and women.[30] However, there have been no previous studies that have compared these parameters in hypertensive subjects. It is also unknown whether gender differences play a role in the serum EMILIN-1 levels. Because we were unable to obtain written informed consent to examine the blood from those subjects who were not receiving medications, we unfortunately could not further examine any potential association between the plasma TGF-β levels and the EMILIN-1 activity.

In conclusion, this is first time that correlations between the human *EMILIN1* gene and EH have been examined in the Japanese population. The present data indicate that the human *EMILIN1* gene is a gender-specific genetic marker candidate for EH. Additional studies will need to be undertaken in order to isolate functional mutations in the *EMILIN1* gene that are responsible for regulating the BP, and to evaluate the relationship between the mutation of *EMILIN1* and EMILIN-1 activity.

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