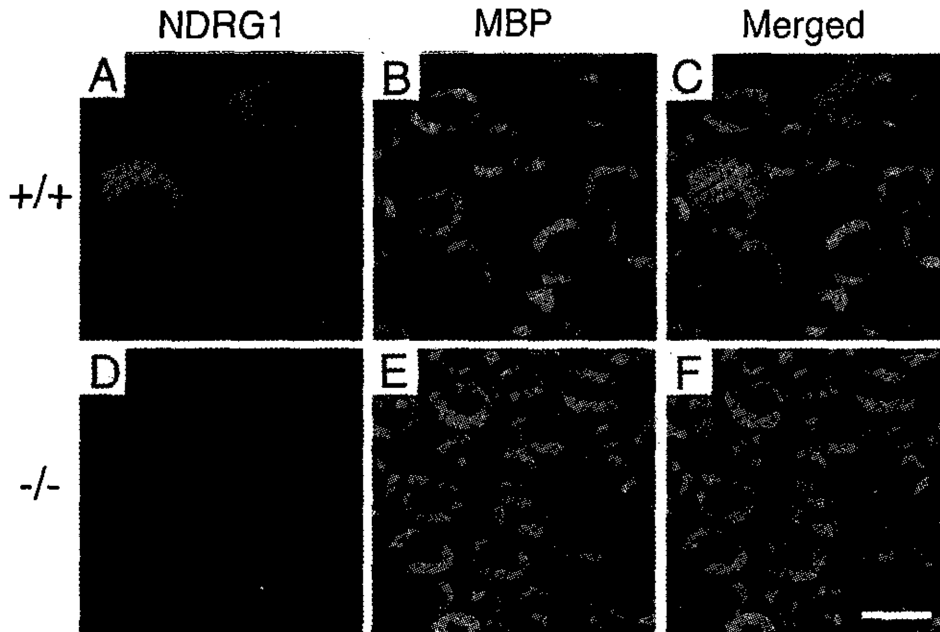

FIG. 6. Immunofluorescence analysis of sciatic nerves. Transverse sections of the sciatic nerves from mice at 3 weeks of age were double stained with anti-NDRG1 antiserum (green; A and D) and anti-MBP antibody (red; B and E). Sections from $Ndrg1^{+/+}$ (A to C) and $Ndrg1^{-/-}$ (D to F) mice were compared. The merged images are shown in panels C and F. Bar, 10 μm.

DISCUSSION

In this study, we successfully generated $Ndrg1^{-/-}$ mice. The $Ndrg1^{-/-}$ mice exhibited a progressive demyelinating disorder of the peripheral nerves. Histological and quantitative analyses revealed that Schwann cell proliferation and the initial myelination of $Ndrg1^{-/-}$ mice were normal after birth (Fig. 5 and Table 1). However, sporadic degeneration began by 5 weeks of age (Fig. 5). These results strongly suggest that the ability to form myelin sheaths is retained but some defect in the maintenance of the myelin sheath is present in the Schwann cells of $Ndrg1^{-/-}$ mice. Therefore, NDRG1 is essential for maintenance of the myelin sheath.

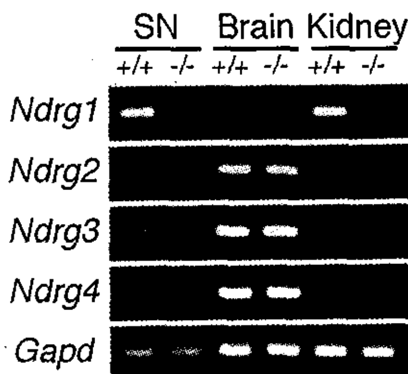

FIG. 7. mRNA expression of *Ndrg* family members. RT-PCR analysis was performed on total RNA samples from the sciatic nerves, brains, and kidneys of $Ndrg1^{+/+}$ and $Ndrg1^{-/-}$ mice at 5 weeks of age. In $Ndrg1^{+/+}$ mice, *Ndrg1* was expressed in the sciatic nerve as much as in the kidney. In contrast, *Ndrg2*, *Ndrg3*, and *Ndrg4* were abundantly expressed in the brain but less in the sciatic nerve. Expression of *Gapd* was examined as an internal control. SN, sciatic nerve.

It has been reported that NDRG1 expression is induced by differentiation or stress stimuli (21, 27, 29). NDRG1 has also been proposed to shuttle between the cytoplasm and the nucleus in cells (14). Furthermore, phosphorylation of NDRG1 depends on extracellular stimuli (2). These observations imply that NDRG1 may have a role in signal transduction. Recently, it was reported that rat NDRG1 is expressed in astrocytes only in the regions where neurons existed (28). This observation suggests that NDRG1 may also play a similar role in neuronal survival in the brain. We demonstrated that NDRG1 was abundantly expressed in the Schwann cell cytoplasm rather than in myelin sheaths (Fig. 6). This expression pattern is unique compared to that of other Charcot-Marie-Tooth disease-responsible proteins, such as peripheral myelin protein 22, myelin protein zero, connexin 32, and L-periaxin (4). These proteins are localized to the plasma membrane of Schwann cells and are thought to have a role in the formation and/or stabilization of the myelin sheaths. Cytoplasmic expression and phosphorylation of NDRG1 implies its association with intracellular signal transduction in Schwann cells. The NDRG1-mediated signals in Schwann cells related to axonal cross talk could be important for the maintenance of myelin sheaths and axonal survival.

$Ndrg1^{-/-}$ mice exhibited muscle weakness, whereas the complicated motor abilities were relatively retained (Fig. 8). These results indicate that NDRG1 deficiency causes peripheral nerve degeneration leading to muscle weakness. This suggests that peripheral nerves may be quite vulnerable to NDRG1 deficiency but that some degree of functional redundancy for NDRG1 may exist within the central nervous system. NDRG1 is one of four NDRG family members exhibiting different expression patterns (20, 22, 32). We previously demonstrated that NDRG4 is abundantly expressed in neurons in the brain but not in the peripheral nerves (32). NDRG4 ex-

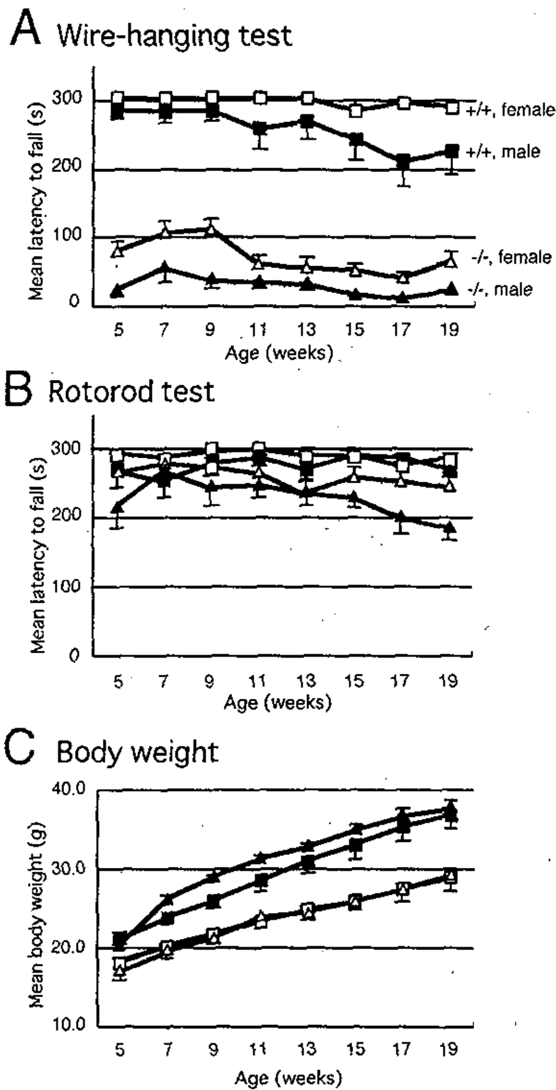

FIG. 8. Assessment of motor activity of $Ndrgl^{-/-}$ mice. (A) The wire-hanging test was carried out to measure the grip strength of $Ndrgl^{+/+}$ (seven males and seven females) and $Ndrgl^{-/-}$ (seven males and six females) mice. The time that each mouse held onto the wire netting was recorded up to a maximum of 300 s. The mean (± standard error of the mean) time before the mouse fell off is shown. (B) The rotorod test was carried out to measure more complicated motor activities in the same mice. The time that each mouse held onto the accelerating cylinder was recorded up to a maximum of 300 s. Mean (±standard error of the mean) time before the mouse fell off the cylinder is shown. Although $Ndrgl^{-/-}$ mice tended to fall sooner, the differences observed between $Ndrgl^{-/-}$ and $Ndrgl^{+/+}$ mice were less than those seen in the wire-hanging test. (C) Plot of body weights of the same mice. Mean (± standard error of the mean) body weights are shown. Each test was carried out on mice from 5 to 19 weeks of age.

pression is induced by homocysteine and reduced both the proliferation and migration rates of cultured cells (19), suggesting that NDRG4 could play a role similar to that of NDRG1 in the brain. NDRG2 and NDRG3 were expressed less in the sciatic nerve than in the brain (Fig. 7). Indeed, no apparent morphological abnormality of the brain was detected in $Ndrgl^{-/-}$ mice (data not shown). NDRG1 deficiency may be compensated for by other NDRG members in the brain.

Although the $Ndrgl^{-/-}$ mice exhibited reductive depletion of NDRG1, a nonsense mutation of human *NDRG1* (R148X) is responsible for Charcot-Marie-Tooth disease type 4D (9). The phenotypes of patients with this disease (10, 11) and of $Ndrgl^{-/-}$ mice in peripheral nerves were similar. This suggests that the C-terminal region of NDRG1 may be essential for NDRG1 function.

In conclusion, we found that NDRG1 deficiency leads to a peripheral neuropathy characterized by demyelination, though the initial formation of the myelin sheaths was normal. NDRG1 is abundantly expressed in the cytoplasm of Schwann cells and plays an essential role in maintenance of myelin sheaths. Although the exact molecular functions of NDRG1 are still under investigation, the $Ndrgl^{-/-}$ mouse will be a good model for Charcot-Marie-Tooth disease type 4D and may be used for future analysis of human peripheral nerve neuropathy as well as provide insight into potential therapies.

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Pyridoxine 5′-phosphate oxidase is a candidate gene responsible for hypertension in Dahl-S rats[☆,☆☆]

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Abstract

To identify candidate genes responsible for hypertension in Dahl salt-sensitive rats (Dahl-S), an oligonucleotide microarray analysis was performed to find differentially expressed genes in kidneys of Dahl-S and Lewis rats. We obtained 101 F2 male rats from Dahl-S and Lewis rats and performed precise measurements of blood pressure (BP) and heart rate by telemetric monitoring at 14 weeks of age after 9 weeks of salt-loading. The correlation analysis between genotypes of differentially expressed genes and BP in F2 rats indicated that pyridoxine 5′-phosphate oxidase (*Pnpo*) and catecholamine-*O*-methyltransferease (*Comt*) showed a highly significant association with BP. However, in the case of *Comt*, the Dahl-S genotype correlated with low BP. Short/branched chain acyl-CoA dehydrogenase and *Sah* also showed a significant association with systolic blood pressure. The present study provided evidence that *Pnpo* is a candidate gene responsible for hypertension in Dahl-S rats.
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Keywords: Hypertension; Dahl salt-sensitive rats; Blood pressure; Telemetry; Mammalian genetics; Pyridoxine 5′-phosphate oxidase

Hypertension is a major risk factor for cardiovascular morbidity and mortality. Blood pressure (BP) is known to be affected by genetic factors and is heritable. The identification of genes contributing to essential hypertension in humans is difficult because hypertension is a multifactorial disease resulting from environmental and genetic factors. To overcome this difficulty and facilitate genetic analyses, genetically hypertensive rat strains have been utilized [1]. Among them, the Dahl salt-sensitive rat (Dahl-S) and the spontaneously hypertensive rat (SHR) have been intensively investigated. Through analyses with these animals, many quantitative trait loci (QTLs) for BP were identified [2]. Usually, a QTL distributes in a large area on a chromosome and contains many genes, thus specifying that the genes responsible for hypertension are quite difficult. Furthermore, problems accompanied by errors of BP measurement should be considered. Conventionally, parameters such as BP or heart rate (HR) are collected by the tail-cuff method for QTL analysis. With this method, the rats are conscious and restrained, hence an error of measurement is inevitable. A precise BP measurement of the rats under freely moving condition using telemetry is desirable.

Recently, the identification of causative genes for phenotypes such as insulin-resistance of the spontaneously hypertensive rat has been successful [3]. This approach consisted of a combination of two independent methods, a microarray analysis for gene expression and mapping using a congenic strain. It can be speculated that some causative genes will be differentially expressed, thus candidates can be sought among the genes found to be differentially expressed [4,5]. We previously adopted an expression analysis using oligonucleotide microarrays to identify genes expressed differently between the SHR and normotensive Wistar–Kyoto rats [6,7].

The Dahl-S rat is a well-investigated model of salt-sensitive hypertension [2]. Using this model, many QTLs associated with BP have been identified [8,9].

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2003.11.149.

☆☆ *Abbreviations:* BP, blood pressure; Dahl-S, Dahl salt-sensitive rat; SHR, spontaneously hypertensive rat; QTL, quantitative trait locus; HR, heart rate; PNPO, pyridoxine 5′-phosphate oxidase; SBP, systolic blood pressure; DBP, diastolic blood pressure; COMT, catecholamine-*O*-methyltransferease; ACADSB, short/branched chain acyl-CoA dehydrogenase; SNP, single nucleotide polymorphism.

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Detailed analyses using congenic strains have also been performed [9–15]. However, no definite genes responsible for hypertension have been reported to date. In the present study, to identify candidate genes responsible for hypertension, we combined an expression analysis of differentially expressed genes in the kidneys of Dahl-S and Lewis rats with a cosegregation analysis of F2 rats. The kidney is thought to be important not only as a target organ of hypertension but also as an organ that may cause hypertension [16]. Furthermore, to obtain precise data for 24-h BPs and HRs of rats, we adopted telemetric measurements. Using this strategy, pyridoxine 5′-phosphate oxidase (*Pnpo*) was identified as a candidate gene responsible for hypertension in Dahl-S rats. Three other genes were also implicated.

Materials and methods

Animals. Male Dahl–Iwai salt-sensitive rats (Sunplanet, Tokyo, Japan) were mated with female Lewis rats (Charles River Japan, Yokohama, Japan). The F1 offspring were intercrossed to produce 101 F2 male rats. These rats were bred under constant temperature (22 °C) and lighting (lights on at 7 a.m. and off at 7 p.m.) throughout the experiments. The rats were fed a normal rat chow (0.5% NaCl) (Clea Japan, Tokyo, Japan) and tap water ad libitum.

Measurement of blood pressure by telemetry. The F2 rats were fed an 8% NaCl-containing diet (Oriental Yeast, Tokyo, Japan) starting at 5 weeks of age. Indwelling radiotelemetric transmitters (TA11PA-C20, Data Sciences, St. Paul, MN) were implanted into the peritoneal cavities of 9-week-old rats and connected to the lower abdominal aortas. The transmitter signals were coded in a pulse position modulated serial bit stream, which is received and monitored by the receiver (RPC-1, Data Sciences) placed underneath the animal's cage. The data were collected for 10 s every 5 min continuously day and night and stored on a hard disk. At 14 weeks of age, the systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR of F2 rats were measured continuously for 24 h, and the data were analyzed using Fluclet TM software (Dainippon Pharmaceutical, Osaka, Japan). The 12-h averages of SBP and DBP during day-time or night-time were determined. At 15 weeks of age, the rats were sacrificed and their body and heart weights were measured. The livers were also excised and the genomic DNA was extracted by the standard phenol/chloroform method. The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center in Japan.

Expression analysis of kidney of Dahl-S and Lewis rats. Male Dahl-S and Lewis rats were fed an 8% NaCl diet starting at 10 weeks of age. At 15 weeks of age, rats were sacrificed and their kidneys were excised.

Table 1
Differentially expressed genes in kidneys of Dahl-S and Lewis rats

Gene description (*Symbol*)	Chromosome location	Expression change (S–L)	Fold change (S/L)	Diff. call	F2 analysis	Accession number
SA (*Sah*)	1q35	14,576	6.0	I	Done	S62516
Isovaleryl Coenzyme A dehydrogenase (*Ivd*)	3q35	8659	2.4	I	Done	AI102838
β-Galactoside-binding lectin (*Lgals1*)	7q34	7461	2.6	I		AI172064
Early growth response 1 (*Egr1*)	18q12.1	6449	2.1	I	Done	AF023087
P2x4 ATP receptor (*P2rx4*)	12q16	3369	2.8	I		U47031
Transgelin (*Tagln*)	8q24	2818	2.5	I		M83107
Membrane metallo endopeptidase (*Mme*)	2q31	2546	2.0	I	Done	AA894298
Vascular smooth muscle α-actin (*Acta2*)	1	2057	2.7	I		X06801
Renin (*Ren1*)	13q13	−2104	−7.9	D		S60054
2-Hydroxyphytanoyl-CoA lyase (*Hpcl2*)	16p16	−2286	−2.1	D		AA893239
UDP-glucuronosyltransferase (*Udpgt*)	14p21	−2359	−18.1	D	Done	M31109
Glutathione peroxidase 2 (*Gpx2*)	6q24	−3429	−4.3	D		AA800587
Cytosolic epoxide hydrolase (*Ephx2*)	15p12	−3509	−2.1	D		X60328
Insulin-like growth factor I (*Igf1*)	7q12-q13	−3621	−2.6	D		M15481
Pyridoxine 5 -phosphate oxidase (*Pnpo*)	10	−3745	−2.2	D	Done	U91561
Betaine homocysteine methyltransferase (*Bhmt*)	2q12	−3782	−2.3	D		AF038870
Cytochrome P-452 (*Cyp4a10*)	5q36	−4484	−2.0	D		X07259
Retinol dehydrogenase type 2 (*Rdh2*)	6q33	−4735	−4.2	D		U33500
Glutathione *S*-transferase 1 θ (*Gstt1*)	20p12	−5395	−2.5	D	Done	X67654
Short/branched chain acyl-CoA dehydrogenase (*Acadsb*)	1q34	−5493	−3.3	D	Done	U64451
Lipoprotein lipase (*Lpl*)	16p14	−6790	−3.0	D	Done	L03294
3-Hydroxy-3-methylglutaryl-CoA synthase (*Hmgcs2*)	2q34	−9581	−4.6	D	Done	M33648
Cytochrome P-450 IID5 (*Cyp2d5*)	7q34	−11,431	−2.1	D		J02869
Cytochrome P-450 M-1 (*Cyp2c*)	1q54	−11,713	−3.0	D		J02657
Retinol-binding protein (*Rbp4*)	1q54	−12,508	−5.1	D	Done	M10934
Cathechol-*O*-methyltransferase (*Comt*)	11q23	−13,576	−2.6	D	Done	M93257

Three independent experiments each for Dahl-S and Lewis rats were carried out and the average value is given. Genes with more than 2-fold and 2000 change in expression are listed.

Expression change (S–L), mean of the difference in intensity obtained by subtraction of the expression intensity in Lewis from that in Dahl-S ($n = 3$).

Fold change (S/L), mean of fold changes obtained by dividing the expression intensities in Dahl-S by those in Lewis ($n = 3$).

Diff. call, Difference call judged by the GeneChip Suite software; I, Increased; D, Decreased.

Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA) from excised whole kidney. Nine batches of total RNA from the Dahl-S and Lewis rats' kidneys were prepared, and three of each were pooled for poly(A)$^+$ RNA preparation. Procedures for the oligonucleotide microarray analysis were essentially identical as described [7]. For this analysis, the rat genome U34A arrays (Affymetrix, Santa Clara, CA; containing 8799 probe sets) were used. The resulting data were analyzed with GeneChip Suite Ver. 4.0 software (Affymetrix). A comparison analysis determined the differentially expressed genes as "increased (I)" or "decreased (D)". Three independent experiments for Dahl-S and Lewis were carried out, and those genes that met the following three criteria were considered to be candidate genes; the genes showed "I" or "D" two times or more in three comparisons, the average of difference change in three comparisons was greater than 2000, and there was a greater than 2-fold difference in expression of a gene.

Identification of specific genetic polymorphisms. Specific genetic polymorphisms of the candidate genes between Dahl-S and Lewis rats were identified by sequencing. For searching the polymorphisms, the genomic PCR was performed using a HotStar Taq Master Mix kit (QIAGEN, Hilden, Germany). The PCR products were used as templates for direct single-pass sequencing using a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The reaction products were purified with a DyeEX 96 kit (QIAGEN) and analyzed on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). The obtained sequences were examined for the presence of a polymorphism using the Sequencher software (Gene Codes, Ann Arbor, MI) followed by visual inspection.

Northern blot analysis. The total RNA used for Northern blot analysis was the same as for the expression analysis. In addition, total RNA from Dahl-S rats fed a normal diet was also prepared for Northern blot analysis. Total RNA was electrophoresed in a 1% agarose gel containing 2% formaldehyde (10 μg/lane) and transferred onto a nylon membrane. To synthesize specific probes for each gene, partial cDNA fragments were synthesized by RT-PCR using total RNA derived from Lewis kidneys as templates. The PCR products were subcloned using a TOPO-TA cloning kit (Invitrogen). Fluorescein-labeled specific probes were generated by PCR as described previously [17], with the partial cDNA fragment as a template. The sense and antisense primers were 5′-CCTAAGTTGCTTGTCTGGGC-3′ and 5′-AGGCCTCGACGGAAGACAAT-3′ for *Pnpo*, 5′-ATGCCGTTGGCTGCAGTCTC-3′ and 5′-GCTGCTCCCTCTCACATACG-3′ for catecholamine-*O*-methyltransferease (*Comt*), 5′-AGGTTCTGGCTGGATTTGATAG-3′ and 5′-CCCACTGACAGTCTTTGGCAGC-3′ for *Sah*, and 5′-AAACCAGGATGGCGGTGTCT-3′ and 5′-TAGTCAAAACATCCTTGGGC-3′ for short/branched chain acyl-CoA dehydrogenase (*Acadsb*). Hybridization and detection procedures using a DNA Thunder Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) were performed according to the manufacturer's instructions. Detection was performed using a LAS-1000plus image analyzer (Fuji Film, Tokyo, Japan).

Statistical analysis. Cosegregation analysis for genotype–phenotype correlation was performed by one-way ANOVA using StatView software (SAS Institute, Berkeley, CA). Differences with a probability of $p < 0.05$ were considered to be significant.

Table 2
Blood pressure by genotype for differentially expressed genes of F2 rats

Gene	Blood pressure by genotype (mean ± SE)			P		
	S/S	S/L	L/L	Additive	Dominant	Recessive
Pnpo						
n	24	58	19			
SBP (light), mmHg	133.80 ± 11.12	132.19 ± 11.72	123.97 ± 7.98	0.0085**	0.0024**	0.1745
DBP (light), mmHg	91.19 ± 8.43	89.71 ± 8.69	83.79 ± 6.72	0.0104*	0.0033**	0.1437
SBP (dark), mmHg	142.98 ± 10.37	140.26 ± 11.26	131.45 ± 8.55	0.0016**	0.0006**	0.0615
DBP (dark), mmHg	98.47 ± 8.29	96.52 ± 8.28	89.94 ± 6.60	0.0019**	0.0007**	0.0694
Comt						
n	28	54	19			
SBP (light), mmHg	132.28 ± 10.55	127.66 ± 11.23	138.76 ± 9.02	0.0007**	0.0008**	0.4960
DBP (light), mmHg	89.76 ± 8.77	86.99 ± 8.80	93.31 ± 5.99	0.0176*	0.0136*	0.5569
SBP (dark), mmHg	140.11 ± 9.94	136.04 ± 11.58	147.19 ± 7.60	0.0006**	0.0005*	0.6379
DBP (dark), mmHg	116.96 ± 9.25	113.61 ± 10.31	121.62 ± 6.53	0.0344*	0.0223*	0.6316
Sah						
n	22	46	33			
SBP (light), mmHg	137.33 ± 11.28	129.83 ± 11.41	128.49 ± 10.23	0.0108*	0.1208	0.0030**
DBP (light), mmHg	92.51 ± 8.88	88.54 ± 8.50	87.12 ± 8.11	0.0670	0.1385	0.0271*
SBP (dark), mmHg	144.48 ± 10.17	138.00 ± 11.48	137.51 ± 10.74	0.0445*	0.2793	0.0126*
DBP (dark), mmHg	120.27 ± 9.11	115.08 ± 10.00	114.57 ± 9.50	0.1944	0.4165	0.0708
Acadsb						
n	22	45	34			
SBP (light), mmHg	137.16 ± 11.33	129.01 ± 10.93	129.73 ± 11.02	0.0151*	0.4168	0.0039**
DBP (light), mmHg	92.35 ± 8.79	88.05 ± 7.98	87.94 ± 8.98	0.1107	0.4033	0.0355*
SBP (dark), mmHg	139.37 ± 11.45	136.74 ± 10.79	139.37 ± 10.46	0.0362*	0.9390	0.0185*
DBP (dark), mmHg	98.22 ± 8.01	94.65 ± 7.99	95.59 ± 9.17	0.2653	0.8989	0.1197

The values for blood pressure are expressed as the 12-h mean in day time and night time.

SBP, systolic blood pressure; DBP, diastolic blood pressure; S, allele for Dahl-S rats; L, allele for Lewis rats; P value was obtained from one-way analysis of variance.

* $P < 0.05$.

** $P < 0.01$.

Results

Differentially expressed genes in Dahl-S and Lewis rats

We performed a gene expression analysis of kidneys from Dahl-S and Lewis rats with oligonucleotide microarrrays. As a result, 26 differentially expressed genes were identified as candidates for further analysis (Table 1). For the cosegregation analysis, exons or introns of these candidate genes of Dahl-S and Lewis rats were sequenced. Genetic polymorphisms to distinguish the genotypes between the two strains were identified in 12 genes (Supplementary Table 1). Most of the polymorphisms we identified were single nucleotide polymorphisms (SNPs). Unfortunately, we could not identify the polymorphisms in the remaining 14 genes.

F2 cosegregation analysis

We performed a cosegregation analysis of the genetic polymorphisms with various phenotypic parameters including BPs, HR, and body weight and heart weight in an F2 population (Tables 2 and 3). The PCR primers used for genotyping of the 12 candidate genes are listed in Supplementary Table 1. *Pnpo* exhibited a significant association with SBP and DBP of both day time and night time in the additive and dominant models (Table 2). In *Pnpo*, F2 rats with the Dahl-S (S) genotype showed a significantly increased BP compared to those with the Lewis (L) genotype. The BP of night time, when the rats are active, was more strongly correlated with the genotype than in the day time. *Comt* showed a significant association with SBP and DBP in the additive and dominant models. However, the heterozygotes showed a lower BP than either type of homozygote. Furthermore, F2 rats with the Lewis (L) genotype showed a significantly higher BP than those with the Dahl-S (S) genotype. Both *Sah* and *Acadsb* showed a significant association with SBP in the additive and recessive models. In both genes, F2 rats with the Dahl-S (S) genotype showed a significantly increased BP than those with the Lewis (L) genotype. None of them showed an association with HR (Table 3). *Comt* showed a significant association with body weight and heart weight in the additive and dominant models. *Sah* and *Acadsb* showed a significant association with heart weight in all statistical models.

Table 3
Heart rate, body weight, and heart weight by genotype for differentially expressed genes of F2 rats

Gene	Physiological parameters by genotype (mean ± SE)			*P*		
	S/S	S/L	L/L	Additive	Dominant	Recessive
Pnpo						
n	24	58	19			
HR (light), beats/min	295.80 ± 10.42	290.74 ± 11.21	292.94 ± 12.90	0.1855	0.8052	0.0916
HR (dark), beats/min	351.67 ± 10.91	350.73 ± 14.85	347.93 ± 12.44	0.6462	0.3730	0.6049
Body weight, g	371.75 ± 18.20	364.72 ± 23.66	370.00 ± 21.32	0.3623	0.5693	0.2694
Heart weight, g	1.255 ± 0.11	1.238 ± 0.12	1.212 ± 0.11	0.5026	0.3071	0.4046
Comt						
n	28	54	19			
HR (light), beats/min	291.08 ± 9.12	292.63 ± 13.19	293.46 ± 9.29	0.7598	0.6420	0.4892
HR (dark), beats/min	348.32 ± 13.34	352.00 ± 14.25	349.09 ± 11.54	0.4529	0.6329	0.3322
Body weight, g	365.07 ± 21.61	363.48 ± 22.53	381.90 ± 15.34	0.0051**	0.0012**	0.5166
Heart weight, g	1.25 ± 0.12	1.21 ± 0.11	1.31 ± 0.12	0.0039**	0.0031**	0.5946
Sah						
n	22	46	33			
HR (light), beats/min	292.75 ± 12.94	293.31 ± 11.76	290.77 ± 10.04	0.6169	0.3334	0.8570
HR (dark), beats/min	347.68 ± 14.43	350.22 ± 14.11	352.57 ± 12.00	0.4212	0.2710	0.2819
Body weight, g	362.73 ± 17.45	371.31 ± 23.86	364.91 ± 21.85	0.2352	0.4348	0.2650
Heart weight, g	1.28 ± 0.11	1.24 ± 0.12	1.20 ± 0.11	0.0268*	0.0189*	0.0385*
Acadsb						
n	22	45	34			
HR (light), beats/min	291.39 ± 12.83	293.30 ± 11.84	291.73 ± 10.13	0.7575	0.6980	0.6562
HR (dark), beats/min	345.41 ± 14.71	350.62 ± 13.93	353.43 ± 11.43	0.0938	0.1131	0.0483*
Body weight, g	368.45 ± 20.60	369.69 ± 22.34	363.76 ± 22.76	0.4728	0.2270	0.7988
Heart weight, g	1.30 ± 0.13	1.24 ± 0.12	1.19 ± 0.09	0.0038**	0.0062**	0.0056**

The values for heart rate (HR) are expressed as the 12-h mean in day time and night time.
S, allele for Dahl-S rats; L, allele for Lewis rats; *P* value was obtained from one-way analysis of variance.
* $P < 0.05$.
** $P < 0.01$.

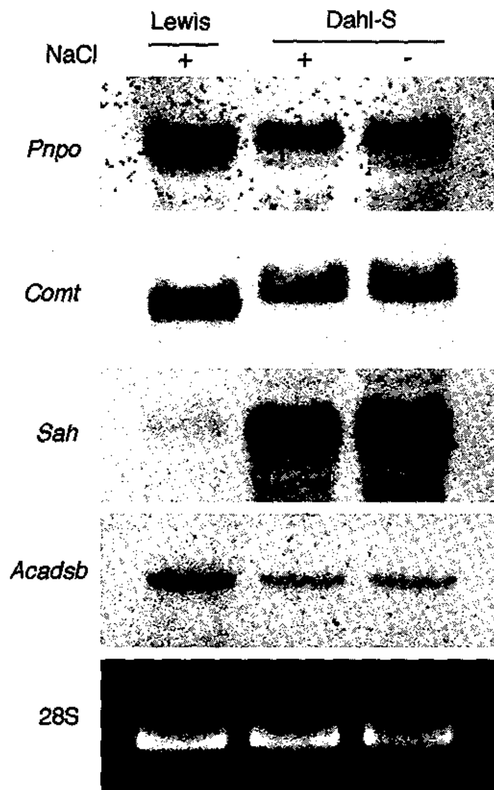

Fig. 1. Northern blot analysis of *Sah*, *Pnpo*, *Comt*, and *Acadsb*. Total RNA (10 μg per lane) obtained from kidneys of Lewis rats fed a high sodium diet, Dahl-S rats fed a high sodium diet, and Dahl-S rats fed a normal diet was used. The mRNAs of *Pnpo* and *Comt* genes from Dahl-S rats were apparently longer.

Northern blot analysis of candidate genes responsible for hypertension

Northern blot analysis for *Pnpo*, *Comt*, *Sah*, and *Acadsb* was performed using total RNA obtained from kidneys of Lewis rats fed a high sodium diet, Dahl-S rats fed a high sodium diet, and Dahl-S rats fed a normal diet (Fig. 1). The differential expression of these genes observed in the microarray analysis was confirmed by Northern blot analysis. It was also revealed that the transcript sizes of *Pnpo* and *Comt* were different between Dahl-S and Lewis rats. These results indicate that some insertion/deletion polymorphisms are present in these strains. To identify the insertion/deletion, we sequenced the PCR products from the genomic DNA of two rat strains. In the 3′-UTR region of *Pnpo*, we identified a 117-bp insertion in Dahl-S rats (data not shown). In *Comt*, an insertion (about 200 bp) was also detected in the 3′-UTR region of Dahl-S rats (data not shown).

Discussion

In the present study, we examined changes in gene expression in the kidneys of Dahl-S and Lewis rats with a microarray analysis and identified 26 differentially expressed genes. We generated 101 F2 rats whose BPs were monitored by telemetry. A cosegregation analysis of 12 differentially expressed genes using the F2 rats revealed that four genes, *Pnpo*, *Comt*, *Sah*, and *Acadsb*, showed a significant correlation with BP.

Among these four genes, *Pnpo* showed a highly significant association with BP (Table 2). PNPO (EC 1.4.3.5) catalyzes the oxidation of either the C4′ alcohol group or amino group of the two substrates pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate to an aldehyde, forming pyridoxal 5′-phosphate (PLP; vitamin B_6) [18]. PNPO is a rate-limiting enzyme in the biosynthesis of PLP, which is a critical vitamin for normal cellular function [19]. The chromosomal location of the rat *Pnpo* gene was not clear in the current version of the database (rat build 2) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The human *PNPO* gene is located between glial fibrillary acidic protein (*GFAP*) and nerve growth factor receptor (*NGFR*) on chromosome 17q21.32. The genes neighboring human *PNPO* are located on rat chromosome 10 [20]. Thus, the rat *Pnpo* gene is likely to be on chromosome 10. Rat chromosome 10 has been reported to contain two QTLs for BP [12,21,22] and one is syntenic to a human QTL for BP on chromosome 17 [23,24]. The *Pnpo*-neighboring genes, such as *Ngfr*, were located in a QTL for BP [23,24]. Therefore, the rat *Pnpo* gene was considered to be located within the QTL for BP. Considering these results, *Pnpo* is a candidate gene responsible for hypertension.

COMT (EC 2.1.1.6) is a ubiquitous enzyme that catalyzes the transfer of a methyl group from *S*-adenosylmethionine to catecholamines, including the neurotransmitters dopamine, epinephrine, and norepinephrine. This O-methylation is one of the major degradative pathways for the catecholamine transmitters. COMT plays an important role in the pathophysiology of disorders such as estrogen-induced cancers, Parkinson's disease, depression, and hypertension [25]. *Comt*-deficient mice exhibited changes in catecholamine levels and behavior [26]. Homozygous *Comt*-deficient female mice exhibited an impairment of emotional reactivity and heterozygous *Comt*-deficient mice displayed increased aggressive behavior [26]. Thus, *Comt* gene dosage may affect BP through individual behavior. We found a significant reduction of *Comt* expression in the kidney of Dahl-S rats and an insertion in the 3′-UTR region of *Comt* of Dahl-S rats that may affect the mRNA stability, leading to a reduction of COMT activity. The *Comt* gene would affect BP through catecholamine metabolism.

Sah was originally identified as a differentially expressed gene in the kidneys of SHR and Wistar–Kyoto rats [4]. *Sah* is located on rat chromosome 1q35 and has been reported to be related to hypertension [27]. It is

interesting that *Sah* was abundantly expressed in kidney of not only SHR but also Dahl-S rats, even in the salt-unloaded condition (Fig. 1). This implies a relationship between *Sah*-dosage and susceptibility to hypertension. *Acadsb* also demonstrated a significant correlation with BPs. ACADSB is a mitochondrial enzyme involved in the metabolism of fatty acids or branched-chain amino acids. A mutation in the *ACADSB* gene causes methylbutyrylglycinuria [28]. *Acadsb* is located on rat chromosome 1q34, close to the *Sah* locus. Both the *Sah* and *Acadsb* loci are located in a QTL for BP [29]. Thus, in addition to *Sah*, *Acadsb* would be a candidate gene responsible for hypertension.

There are several limitations to the present study. We could not identify the polymorphisms in the remaining 14 genes that are differentially expressed in kidneys. Those genes were not analyzed in the present study. The number of F2 rats in the present study ($n = 101$) may have been too small for precise detection of the genes responsible for hypertension.

In conclusion, we successfully identified *Pnpo* on rat chromosome 10 as a candidate gene responsible for hypertension in Dahl-S rats. In addition, we provided evidence that *Comt* on chromosome 11 and *Sah* and *Asadsb* both on chromosome 1 are likely involved in hypertension in Dahl-S rats. The significance of these genes in hypertension in humans will be addressed in future analyses.

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Identification of 21 single nucleotide polymorphisms in human hepatocyte growth factor gene and association with blood pressure and carotid atherosclerosis in the Japanese population

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Abstract

It has been suggested that circulating concentrations of hepatocyte growth factor (HGF) are increased in individuals with vascular endothelial damage, such as in hypertensive patients and subjects with atherosclerosis. Because the influence of genetic variation of *HGF* has not been examined, we identified single nucleotide polymorphisms (SNPs) in the *HGF* gene, and investigated the association between these SNPs and blood pressure or carotid atherosclerosis in the Japanese general population. We identified 21 SNPs in the *HGF* gene by direct sequencing in a test population of 32 Japanese subjects. Among them, considering allele frequency and linkage disequilibrium, three SNPs, *C-1652T* in the promoter, *T43839A* in intron 8, and *T44222C* in intron 9, were genotyped in 2412 members of the Japanese general population randomly selected from the residents in Suita city. None of the three SNPs were significantly associated with blood pressure. After adjusting for age, smoking habits, consumption of alcohol, and the presence of diabetes mellitus and dyslipidemia, female subjects with the T allele of *T43839A* had more severe carotid atherosclerosis compared to individuals with the A allele. This study provides the first evidence that *HGF* may be a candidate susceptibility loci that affects the progression of atherosclerosis in Japanese subjects.

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1. Introduction

Endothelial cell dysfunction has been suggested as the initiating process in the development and progression of cardiovascular disease, and it is considered to be closely related to the pathophysiology of human essential hypertension. There has been accumulating evidences that hepatocyte growth factor (HGF), mesenchyme-derived pleiotropic factor, plays an important role in endothelial cell dysfunction. Since HGF was originally identified in the plasma of rats after partial hepatectomy [1], a number of investigations have suggested that HGF is a multifunctional factor implicated in tissue regeneration and angiogenesis in not only liver but also other tissues [2,3]. A local HGF system (HGF and its receptor, c-met) is expressed in vascular cells [4], and elevated serum HGF levels have been suggested to play a cardiovascular protective role in hypertensive subjects, especially those with concomitant arteriosclerosis [5–9].

Although an association between HGF and the severity of hypertension was established, few reports have investigated the association between *HGF* gene polymorphisms and blood pressure or atherosclerosis. The *HGF* gene is composed of 18 exons encompassing 70 kb on chromosome 7q11.2-q21 [10]. In the present study, we screened for sin-

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gle nucleotide polymorphisms (SNPs) in the *HGF* gene and evaluated the significance of SNPs in high blood pressure and carotid atherosclerosis determined by an ultrasonography using a large cohort, the Suita Study, which was representative of the general Japanese population.

2. Methods

2.1. Subjects

The protocol of the Suita Study, described elsewhere [11–14], was approved by the Ethics Committee of the National Cardiovascular Center. The sample consisted of 14,200 Japanese men and women ages 30–79 years stratified by gender and 10-year age groups, selected randomly from the municipal population registry. They were all invited by letter to attend regular cycles (every 2 years) of follow-up examinations. DNA from leukocytes was collected from participants who visited the Division of Preventive Medicine, National Cardiovascular Center between May 1996 and February 1998. Subjects who gave their written informed consent for genetic analyses of the genes were included in the present study. All clinical data and genotyping results were anonymous, and all data was handled in such a way that it was/will not be possible to identify an individual.

The characteristics of the subjects analyzed in the present study are summarized in Table 1. Blood pressure was measured in the subjects after at least 10 min of rest in a sitting position. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values are the mean of two

Table 1
Clinical Features of Study Participants

Variables	Men n = 1158	Women n = 1254
Age, y	61.0 ± 12.2*	58.9 ± 11.7
Body mass index, kg/m2	23.1 ± 2.8*	22.3 ± 3.1
Systolic blood pressure (SBP), mmHg	129.2 ± 10.1	128.5 ± 21.1
Diastolic blood pressure (DBP), mmHg	81.0 ± 10.9*	78.8 ± 10.6
Total cholesterol (TC), mg/dL	204.6 ± 31.9	215.9 ± 32.9*
HDL cholesterol, mg/dL	54.1 ± 14.5	64.2 ± 15.6*
Current alcohol consumer (%)	38.0†	8.6
Current smoker (%)	71.5†	29.1
Hypertension (%)	39.6†	35.5
Diabetes mellitus (%)	9.1†	3.7
Dyslipidemia (%)	35.2	51.3†

Values are means ± SDs or percentages.
Hypertension indicates SBP ≥ 140 mmHg and/or DBP ≥90 mmHg or antihypertensive medication; Dyslipidemia, TC ≥ 220 mg/dL or antidyslipidemia medication; diabetes mellitus, fasting plasma glucose ≥ 126 mg/dL or antidiabetic medication.
* $P < 0.05$ between men and women by Student's t test.
† $P < 0.05$ between men and women by χ^2 test.

physician-obtained measurements (recorded >3 min apart). Hypertension was defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg or the current use of antihypertensive medications; diabetes mellitus was defined as fasting blood glucose ≥126 mg/dl, or the current use of insulin or oral anti-diabetic agents; and dyslipidemia was defined total cholesterol ≥220 mg/dl or the current use of antidyslipidemia medication at the time of the first examination.

2.2. Evaluation of carotid atherosclerosis

Carotid ultrasonography was performed for the evaluation of atherosclerosis. Measurement methods were previously described [11,12]. Briefly, ultrasonography of both carotid arteries was performed with a high-resolution Duplex scanner (TOSHIBA SSA-250A; probe, SMA-736S mechanical sector scanner, Toshiba, Tokyo, Japan) for the B-scan. The subjects were examined in the supine position with their head slightly turned from the sonographer. All measurements were performed by two trained sonographers, who were unaware of the subjects' clinical data. The carotid arteries were carefully examined with regard to wall changes from different longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views, and measurements of thickness were performed from transverse image. Intima-medial thickness (IMT) was measured at a point 10 mm proximal from the beginning of the dilatation of the carotid bulb, and Maximum-IMT (Max-IMT) was defined as the maximum thickness of intima-media including plaques from the region branching off from the brachiocephalic artery (right) or aorta (left) to the bifurcation of the common carotid artery. Plaque Score was calculated by summing the maximum thickness of all the plaques in the bilateral carotid artery in the scanning area [11,12].

2.3. Direct sequencing for the detection of polymorphisms in the HGF genes

We obtained peripheral blood samples from 32 Japanese volunteers for direct sequencing of the *HGF* gene after obtaining written informed consent. Genomic DNA was extracted with an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). Methods of direct sequencing are described previously [15]. Briefly, all exons, a portion of introns and a region up-stream of exon 1, which included the promoter region of *HGF*, were amplified by polymerase chain reaction (PCR). The PCR products were then treated with shrimp alkaline phosphates and exonuclease I (PCR Product Pre-Sequencing Kit, USB Corporation, Cleveland, OH), and used as templates for direct single-pass sequencing using a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The reaction products were purified with a DyeEX 96 kit (QIAGEN) and analyzed on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). The obtained sequences

Table 2
Primers and TaqMan Probes for Genotype Determination

HGF SNPs	Sequence
C(−1652)T (promoter)	
Sense	5′-GGATTAGCAATAGAAACGGGTCAT-3′
Antisense	5′-CCCTGAGGTTGTGGGATATCTAGA-3′
Probe for C(−1652)	Fam-5′-AAAATAGATCCCTCAAAAG-3′-MGB
Probe for T(−1652)	Vic-5′-AATAGATCTCTCAAAAGG-3′-MGB
A43839T (intron 8)	
Sense	5′-TTCAGTAATTTGGGCAGAGTCAGT-3′
Antisense	5′-ACGTTGGTGAAGTCAGCGCTAT-3′
Probe for A43839	Fam-5′-AGTCCAAAAAGTTAGAACT-3′-MGB
Probe for T43839	Vic-5′-AGTCCAAAATGTTAGAAC-3′-MGB
C44222T (intron 9)	
Sense	5′-GCTGGCTTGCAAACAAAATCA-3′
Antisense	5′-GGCTTAGAACTGTGGCTGTCAGT-3′
Probe for C44222	Fam-5′-TTTGAAGCTGGATTTT-3′-MGB
Probe for T44222	Vic-5′-TTGAAGTTGGATTTTT-3′-MGB

were examined for the presence of a polymorphism using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection.

2.4. Genotyping of SNPs in the HGF genes

Three SNPs were genotyped using the TaqMan system [14,16]. PCR primers and probes for the TaqMan system are shown in Table 2. Fluorescence level of the reaction products was measured by use of ABI PRISM 7700 or 7900 Sequence Detection System (Applied Biosystems).

2.5. Statistical analysis

Values are expressed as the mean ± S.D. or mean ± S.E. Multiple regression and multiple logistic analyses were performed with the covariates age, body mass index, smoking, current alcohol consumption, presence of diabetes and/or dyslipidemia using the SAS version 6.0 (SAS Institute Inc., Cary, NC). Differences in frequency among the groups were tested by χ^2 analysis. Linkage disequilibrium was evaluated by obtaining r^2 values between polymorphisms using SNPAlyze ver. 2.0 (DYNACOM Co., Ltd., Shigehara, Japan).

3. Results

3.1. Detection of genetic variants in the HGF gene

We systematically searched the sequences obtained for SNPs in 32 volunteer subjects and identified 21 SNPs including 8 SNPs in the HGF promoter, 1 SNP in exon and 12 SNPs in intron regions (Table 3). Ten of these SNPs have been deposited in the public database previously, db SNPs (http://www.ncbi.nlm.nih.gov/SNP/), but 11 of the identified SNPs were novel. Thirteen SNPs had their minor allelic frequency less than 10%, therefore no further studies of these SNPs were undertaken. Six SNPs were in tight linkage disequilibrium, therefore one of them, C44222T, was selected as a representative. Thus, three SNPs, *C(-1652)T*, *A43839T* and *C44222T* were chosen for further genotyping analysis (Table 3).

Table 3
List of 21 Polymorphisms and Alele Frequency in HGF Identified in 32 Japanese Patients by Direct Sequencing

allele 1/allele 2			allele 1		allele 2		allele frequency			
SNPs	aa info.	region	homo	hetero	homo	total	allele 1	allele 2	flanking sequence	dbSNP ID
C(−2142)A		promoter	28	4	0	32	0.938	0.063	ttggaatggggt[c/a]ttatgagctacg	
G(−1965)T		promoter	31	1	0	32	0.984	0.016	atgcctcgcctt[g/t]ggggagaatgaa	
G(−1903)A		promoter	30	1	0	31	0.984	0.016	gctgattctgag[g/a]tcttcatttggg	
C(−1652)T		promoter	10	16	5	31	0.581	0.419	ataaaatagatc[c/t]ctcaaaaggaat	rs3735520
G(−1268)C		promoter	30	1	0	31	0.984	0.016	tctctgaatcaa[g/c]tgaggggtctgg	rs3735521
−(−1215)C		promoter	27	4	0	31	0.935	0.065	taggagtccccc[-/c]atgcccatacaa	
T(−955)C		promoter	31	1	0	32	0.984	0.016	ggacaatgactg[t/c]ttcttggacttt	
T(−578)C		promoter	31	1	0	32	0.984	0.016	aactagacagat[t/c]aggagctggggc	
T40171–*		intron7	0	7	25	32	0.109	0.891	taagttttttt[t/-]gttgttgttttt	rs5745686
A43839T		intron8	17	14	1	32	0.750	0.250	ctgagtccaaaa[a/t]gttagaactcta	rs2286194
C44222T*		intron9	0	7	25	32	0.109	0.891	ccaagtttgaag[c/t]tggatttttctt	rs2887069
C49065T*		intron9	0	7	25	32	0.109	0.891	acttgtaaaaaa[c/t]ctttttgtttta	
T49080C*		intron9	0	7	25	32	0.109	0.891	ttttgttttatc[t/c]gccttgatattc	
A52603G*		intron10	0	6	26	32	0.094	0.906	cctgttgtttcc[a/g]cagtcatatctt	rs1800793
G58294A		intron11	31	1	0	32	0.984	0.016	gcctgggtgaca[g/a]aatgagactctg	
T59941A		intron13	31	1	0	32	0.984	0.016	agggcacctggg[t/a]gaggcagtaaaa	rs5745739
T59984G*		intron13	26	6	0	32	0.906	0.094	tgcttgccagac[t/g]gtaagctctgga	rs2074725
G62753T		intron14	31	1	0	32	0.984	0.016	tttgctcttaag[g/t]ttataatatgta	rs5745745
G63555T	Asp543Tyr	exon15	31	1	0	32	0.984	0.016	agagacttgaaa[g/t]attatgaagctt	
A64588G		intron17	31	1	0	32	0.984	0.016	atgtgaggtaaa[a/g]aggaagttcttt	
T67183G		intron17	31	1	0	32	0.984	0.016	tttaattcctaa[t/g]aatactttgttt	rs5745767

Three SNPs underlined had the minor allele frequency over 10% and were selected for genotyping in this study.
* Six polymorphisms are in strong linkage disequilibrium (r-square > 0.5).

Table 4
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C(−1652)T Genotypes

Male	CC	CT	TT	P
n (%)	519(47.1%)	438(39.7%)	146(13.2%)	
SBP (mmHg)	129.3 ± 0.7	128.8 ± 0.8	129.3 ± 1.4	0.850
DBP (mmHg)	81.4 ± 0.5	80.6 ± 0.5	80.8 ± 0.9	0.355
%Hypertension	41.4	39.0	35.6	0.419
IMT (mm)	0.898 ± 0.005	0.903 ± 0.005	0.892 ± 0.009	0.896
Max-IMT (mm)	1.729 ± 0.035	1.745 ± 0.038	1.650 ± 0.067	0.492
Plaque Score	4.6 ± 0.2	4.9 ± 0.2	4.5 ± 0.3	0.981
Female	**CC**	**CT**	**TT**	**P**
n (%)	579(48.0%)	481(39.9%)	146(12.1%)	
SBP (mmHg)	129.1 ± 0.7	128.8 ± 0.8	127.5 ± 1.5	0.405
DBP (mmHg)	79.0 ± 0.4	79.1 ± 0.5	78.5 ± 0.8	0.727
% Hypertension	35.6	36.0	34.9	0.973
IMT (mm)	0.846 ± 0.004	0.852 ± 0.004	0.848 ± 0.008	0.601
Max-IMT (mm)	1.332 ± 0.021	1.368 ± 0.023	1.371 ± 0.042	0.257
Plaque Score	2.4 ± 0.1	2.6 ± 0.1	2.7 ± 0.2	0.143

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

3.2. Study population

Table 1 shows the clinical characteristics of the present subjects by sex. Most variables (i.e., age, body mass index, diastolic blood pressure, percentage of current alcohol drinking, smoking, diabetes, and hypertension) were significantly higher in men than in women, but percentage of dyslipidemia, serum total cholesterol and HDL cholesterol levels were significantly higher in women than in men. There were no significant differences in systolic blood pressure.

3.3. Association of three polymorphisms with blood pressure and carotid arterioscleorsis

We investigated the possible association of three SNPs in the human *HGF* gene with blood pressure and carotid atherosclerosis in a population-based sample (the Suita Study) that consisted of 2412 participants. The frequencies of each genotype are described in Tables 4-6. The genotype frequencies of all analyzed polymorphisms were consistent with Hardy-Weinberg equilibrium. There were no significant differences in the genotype frequencies of polymorphisms for either sex. Tables also show systolic and diastolic blood pressure levels, and carotid IMT and Plaque Scores in each genotype of the three polymorphisms.

After full adjustment of all confounding factors (age, body mass index, current smoking status, alcohol consumption, presence of diabetes mellitus and dyslipidemia), there was no significant association between the three genotypes and blood pressure levels or the prevalence of hypertension in all subjects and in each sex.

Table 5
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by A43839T Genotypes

Male	AA	AT	TT	P
n(%)	555(59.8%)	304(32.8%)	69(7.4%)	
SBP (mmHg)	129.2 ± 0.7	128.4 ± 0.9	130.6 ± 2.0	0.981
DBP (mmHg)	81.3 ± 0.4	80.2 ± 0.6	81.7 ± 1.2	0.550
%Hypertension	38.0	41.1	34.8	0.522
IMT(mm)	0.897 ± 0.005	0.896 ± 0.006	0.888 ± 0.013	0.643
Max-IMT (mm)	1.733 ± 0.034	1.691 ± 0.046	1.743 ± 0.098	0.703
Plaque Score	4.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.5	0.200
Female	**AA**	**AT**	**TT**	**P**
n(%)	636(61.5%)	340(32.9%)	59(5.7%)	
SBP (mmHg)	129.3 ± 0.7	127.4 ± 1.0	131.2 ± 2.3	0.578
DBP (mmHg)	79.0 ± 0.4	78.7 ± 0.5	78.9 ± 1.3	0.790
%Hypertension	36.6	32.1	44.1	0.135
IMT (mm)	0.854 ± 0.004	0.842 ± 0.005	0.837 ± 0.012	0.039
Max-IMT (mm)	1.390 ± 0.021	1.310 ± 0.028	1.232 ± 0.065	0.003
Plaque Score	2.7 ± 0.1	2.3 ± 0.2	1.8 ± 0.4	0.002

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Table 6
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C644222T Genotypes

Male	CC	CT	TT	P
n (%)	7(0.7%)	194(19.1%)	817(80.3%)	
SBP (mmHg)	125.7 ± 6.2	128.7 ± 1.2	129.3 ± 0.6	0.547
DBP (mmHg)	80.0 ± 3.9	81.2 ± 0.7	80.9 ± 0.4	0.780
%Hypertension	14.2	38.1	40.3	0.334
IMT (mm)	0.895 ± 0.039	0.885 ± 0.008	0.900 ± 0.004	0.096
Max-IMT (mm)	2.066 ± 0.287	1.690 ± 0.057	1.732 ± 0.028	0.856
Plaque Score	2.4 ± 0.9	2.4 ± 0.2	2.5 ± 0.1	0.414
Female	**CC**	**CT**	**TT**	**P**
n (%)	11(1.0%)	207(18.6%)	897(80.5%)	
SBP (mmHg)	130.2 ± 5.3	128.2 ± 1.2	129.1 ± 0.6	0.616
DBP (mmHg)	80.0 ± 3.0	78.2 ± 0.7	79.0 ± 0.3	0.439
%Hypertension	36.4	31.9	37.0	0.383
IMT (mm)	0.825 ± 0.030	0.841 ± 0.007	0.850 ± 0.003	0.161
Max-IMT (mm)	1.317 ± 0.162	1.362 ± 0.036	1.349 ± 0.017	0.844
Plaque Score	6.2 ± 1.5	4.4 ± 0.3	4.8 ± 0.1	0.414

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Although no association was found between carotid IMT, Plaque Scores and *A43839T* genotype in male subjects, women with the A allele showed significantly thicker IMT and greater Plaque Scores than those with the T allele (Table 5). There was no association between carotid IMT, Plaque Scores and genotypes of the two SNPs, *C(-1652)T* and *C44222T*, in both male and female subjects (Tables 4 and 6).

4. Discussion

Although a number of reports have suggested a strong association between the severity of hypertension and serum HGF levels, there have been few reports that investigated the association between cardiovascular disease and *HGF* gene polymorphisms by direct sequencing. Of those identified single nucleotide polymorphisms, 11 were not deposited in the public database. We have performed a large genetic epidemiological study of the Japanese general population regarding three candidate SNPs in the promoter and intron of the *HGF* gene. There was no significant association between the three *HGF* SNPs and blood pressure or the prevalence of hypertension. Interestingly, female subjects with the A allele of *A43839T* in intron 8 had more severe carotid atherosclerosis than those with the T allele.

4.1. HGF and hypertension, atherosclerosis

Clinical studies have demonstrated a positive correlation between serum HGF concentrations and blood pressure. These studies have gone on to show that serum HGF concentrations in hypertensive patients were significantly higher than those seen in normotensive control subjects [5,6]. In an experimental setting, serum HGF concentrations were significantly increased in spontaneous hypertensive rats compared to Wistar-Kyoto rats at any age, and there was a positive association between serum HGF concentration and blood pressure level [17].

A number of reports have also suggested that serum HGF concentrations are increased in proportion to the development of hypertensive target organ damage. The circulating level of HGF was elevated in patients with myocardial infarction [18] and peripheral arterial disease [9,19,20]. Furthermore, serum HGF concentrations were significantly correlated with the hyperemic response of forearm blood flow and pulse wave velocity [21]. Alternatively, it is apparent that endothelial cell dysfunction may promote abnormal vascular growth, and this vascular remodeling clearly plays an important role in the pathophysiology of atherosclerosis. In normotensive subjects, serum HGF was suggested to maintain the vascular structure and stimulate tissue regeneration in an autocrine-paracrine manner [19,22]. In patients with hypertension or diabetes mellitus, this local HGF system was disturbed by transforming growth factor-β or angiotensin II with the resultant development of abnormal vascular smooth muscle cell growth [17,23].

4.2. HGF SNPs, hypertension, and atheroscleorosis

Polymorphisms of several growth factor genes have been investigated because they may potentially play a key role in the maturation of atheromatous lesions. Among these growth factors, HGF was of particular interest because it could have cardiovascular protective effects in several disorders including hypertension, diabetes mellitus, and cardiovascular diseases.

In the present study, we identified 21 SNPs in the *HGF* gene, and determined the genotype of three of these polymorphisms in more than 2000 individuals. Our results showed that there were no significant associations between *HGF* genotypes and blood pressure levels or the prevalence of hypertension. However, the A allele of *A43839T* in intron 8 was significantly associated with increased severity

of carotid atherosclerosis in females. The reason for this sex-specific effect in our study subjects is unclear. The interaction of HGF with angiotensin II and/or transforming growth factor β could contribute to some of the difference seen in our patients population. Although still controversial [24], renin-angiotensin system related genetic variation tends to appear in male individuals in Japanese [13] and Caucasians [25,26]. Additionally, a T → C transition at nucleotide 869 of the *transforming growth factor β* gene has been reported to be one of the candidate susceptibility loci for hypertension only in the female Japanese population [27]. We assume that there might exist some link or interaction between HGF and the genetic variation of these two growth factors.

Our results suggest that the HGF gene located at chromosome 7q11.2-q21 is a candidate susceptibility locus for atherosclerosis in Japanese women. The polymorphism conferring increased susceptibility for atherosclerosis was located in the intron region without amino acid substitution. Thus, it is possible that the *A43839T* polymorphism in intron 8 of the *HGF* gene is in linkage disequilibrium with some other polymorphisms which are actually responsible for the development of atherosclerosis. To elucidate the exact mechanisms and clinical implications of the association, further functional and linkage disequilibrium analyses are required.

In conclusion, we identified 21 SNPs in the *HGF* gene including 11 SNPs that have never been reported. The present study provides the first evidence that *HGF* may be a candidate susceptibility loci that affects the progression of atherosclerosis in Japanese subjects.

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Original Article

Six Missense Mutations of the Epithelial Sodium Channel β and γ Subunits in Japanese Hypertensives

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Liddle's syndrome is an autosomal dominant disease characterized by sodium-sensitive early hypertension and mutations in either the β- or γ-subunit of the amiloride-sensitive epithelial sodium channel encoded by *SCNN1B* and *SCNN1G*. We sequenced the 381 bp-coding regions in exon 13 of *SCNN1B* and the 381 bp-coding regions in exon 12 of *SCNN1G* in 948 and 953 Japanese patients with hypertension, respectively. In the *SCNN1B* gene, we identified three missense mutations, P592S (n=3), T594M (n=2), and E632K (n=1) in a heterozygous state in addition to four synonymous ones, Ile515 (n=1), Ser520 (n=19), Ser533 (n=1), and Thr594 (n=11). In the *SCNN1G* gene, we identified three missense mutations, A578V (n=1), P603S (n=1), and L609F (n=1) in a heterozygous state in addition to two synonymous ones, Ile550 (n=1) and Leu649 (n=91, heterozygous; n=2, homozygous). We did not identify the same mutations previously reported in Liddle's syndrome kindreds. Two of the six hypertensive patients with missense mutation in the *SCNN1B* gene showed atypical renin and aldosterone levels, though one of them was diagnosed with renovascular hypertension. One patient with T594M in the *SCNN1B* gene was resistant to hypertension. The roles of these missense mutations in the *SCNN1B* or *SCNN1G* gene identified in hypertensive patients are not clear in the pathogenesis of hypertension and the regulation of electrolytes. Thus, further investigation of these mutations, including functional analyses, will be needed. (*Hypertens Res* 2004; 27: 333–338)

***Key Words*: Liddle's syndrome, *SCNN1B*, *SCNN1G*, gene variants, hypertension**

Introduction

The amiloride-sensitive epithelial sodium channel (ENaC) is responsible for the rate-limiting step of sodium reabsorption in the distal renal tubules, and thus may play a key role in the maintenance of sodium balance and blood pressure. ENaCs are composed of three homologous subunits, termed α, β, and γ, with a subunit stoichiometry of 2α:1β:1γ (*1, 2*). These three subunits show a similar topology, consisting of two transmembrane domains separated by a large extracellular domain (*3*). The α-subunit appears to be the conducting unit, and the role of the other two subunits is less certain. Neither the β or the γ subunit, when expressed alone or together, produces any measurable Na^+ current. However, co-expression with the α-subunits greatly enhances the amplitudes of the Na^+ current (*4, 5*).

Liddle's syndrome is an autosomal dominant disease characterized by sodium-sensitive early hypertension associated with hypokalemic metabolic alkalosis, low plasma renin activity, and suppressed aldosterone secretion. Several mutations have been described in Liddle's kindreds, some in the β subunit and others in the γ subunit (*5–7*). All these mutations abolish or modify a highly conserved PY motif present in their intracellular carboxyl terminal region, thereby altering the binding of ENaC to its partner, Nedd4, a ubiquitin-pro-

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tein ligase (*8–10*). This leads to a low intracellular turnover of the channel and to an increase in the number of active channels exposed at the cell membrane. The underlying hypothesis is that dysregulation of ENaC activity causes inappropriate sodium retention, with consequent volume expansion and suppression of peripheral renin activity in patients with Liddle's syndrome.

Several groups have identified other molecular variants of the β subunit of ENaC (*SCNN1B*) (*11–16*). These variants are missense mutations, and none of them affects the PY motif. Among them, the T594M mutant, which showed a prevalence of 6% to 8% in hypertensives of African descent, was well characterized (*17–20*). Whole cell voltage clamp studies indicated that this variant showed an increased response to cAMP analog (*11, 17*). This mutation has been reported to be the common in black people with essential hypertension in some studies (*18, 19*), but not another (*20*). Some studies have reported that this mutation is common among black individuals with essential hypertension (*18, 19*), but another study found no such association (*20*).

Regarding the γ subunit of ENaC (*SCNN1G*), Iwai *et al.* reported that a single nucleotide polymorphism, G(−173)A, in the promoter region of the *SCNN1G* gene was associated with blood pressure regulation in a Japanese population (*21*). They also showed that the promoter activity of the G(−173) allele was 2- to 3-fold higher than that of the A(−173) allele. This suggests that the γ subunit level is one of the determinants of the ENaC activity. In addition, it suggests that the ENaC activity may be regulated by a subtle change of each subunit protein that could be induced by the missense mutations. As mentioned above, a highly conserved PY motif present in the intracellular carboxyl terminal region of the β and γ subunits of ENaCs is highly important for their regulatory function. This suggests that the surrounding regions—encoded by exon 13 of *SCNN1B* and exon 12 of *SCNN1G*—of the PY motif are also important for the the function of ENaCs.

In the present study, we amplified the 381 bp-coding regions of both exon 13 of *SCNN1B* and exon 12 of *SCNN1G* by polymerase chain reaction (PCR) in 956 Japanese patients with hypertension and successfully sequenced the regions in 948 and 953 patients, respectively. We identified several mutations, including missense mutations, both in *SCNN1B* and *SCNN1G*. Based on our results, we also discuss the clinical profiles of the patients carrying these mutations.

Methods

Subjects

A total of 956 hypertensive subjects (525 men and 431 women; average age: 65.0±10.6 years old; body mass index: 24.2±3.4 kg/m^2) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center; all patients provided their written informed consent to participate in this study.

Ninety-two percent of the study subjects (884 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension, including renal hypertension (37 subjects), renovascular hypertension (23 subjects), primary aldosteronism (11 subjects) and hypothyroid-induced hypertension (1 subject). The hypertension criteria were blood pressure above 140 and/or 90 mmHg, or the use of antihypertensive agents. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Screening of Mutations in the 381 bp-Coding Regions in Exon 13 of the *SCNN1B* Gene and Exon 12 of the *SCNN1G* Gene

Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes by an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan) (*22*). The 381 bp-coding region in exon 13 of *SCNN1B* was amplified by PCR using a pair of specific primers: 5′-agatggtcaccccctcccgttc-3′ and 5′-taccctcccgaccctt tggagag-3′, which flank the 597-bp region. The 381 bp-coding region in exon 12 of *SCNN1G* was amplified by PCR using a pair of specific primers: 5′-tgcacagagtaagaggaaacagg-3′ and 5′-agcaggcttttggtcagagtat-3′, which flank the 677-bp region. The PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) as described previously (*23*). The obtained sequences were examined for the presence of a mutation using the Sequencher software package (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection.

Results

In a total of 956 Japanese patients with hypertension, we screened exon 13 of the *SCNN1B* gene and exon 12 of the *SCNN1G* gene. The 381 bp-coding regions were amplified from the genomic DNA and a total of 948 and 953 sample DNAs were successfully sequenced for *SCNN1B* and *SCNN1G*, respectively. Although we did not identify the same mutations previously reported in Liddle's syndrome kindreds, we identified seven mutations in the *SCNN1B* gene and five in the *SCNN1G* gene. The seven mutations in the *SCNN1B* gene consisted of three missense mutations, one of which has not previously been reported, and four synonymous mutations. The five mutations in the *SCNN1G* gene consisted of three novel missense mutations and two synonymous mutations (Tables 1 and 2).

In *SCNN1B*, three of the 948 individuals had a C-to-T substitution at nucleotide 32053, leading to an amino acid substitution from Pro to Ser at position 592 (P592S). Two of the 948 individuals had a C-to-T substitution at nucleotide 32060, leading to an amino acid substitution from Thr to Met at position 594 (T594M). This T594M mutation was previously identified only in black peoples (*11, 14, 17, 18*).

Table 1. Summary of Sequence Variations of Exon 13 in *SCNN1B* Identified in 948 Japanese Patients with Hypertension

SNP name	Region	Amino acid substitution	Allele 1		Allele 2	Total number	Allele 1	Allele 2
			Homo	Hetero	Homo			
31824C>T	exon13	Ile515Ile	937	1	0	938	0.999	0.001
31839G>C	exon13	Ser520Ser	919	19	0	938	0.990	0.010
31878T>C	exon13	Ser533Ser	940	1	0	941	0.999	0.001
32053C>T	exon13	Pro592Ser	945	3	0	948	0.998	0.002
32060C>T	exon13	Thr594Met	946	2	0	948	0.999	0.001
32061G>A	exon13	Thr594Thr	937	11	0	948	0.994	0.006
32173G>A	exon13	Glu632Lys	947	1	0	948	0.999	0.001

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomeclature Working Group (*Hum Mut*, Vol.11, pp.1–3, 1998). The nucleotide sequence (GenBank Accession ID: NT_010604) was used as a reference sequence. *SCNN1B*, β subunit of epithelial sodium channel gene; SNP, single nucleotide polymorphism.

Table 2. Summary of Sequence Variations of Exon 12 in *SCNN1G* Identified in 953 Japanese Patients with Hypertension

SNP name	Region	Amino acid substitution	Allele 1		Allele 2	Total number	Allele 1	Allele 2
			Homo	Hetero	Homo			
28898C>T	exon12	Ile550Ile	952	1	0	953	0.999	0.001
28981C>T	exon12	Ala578Val	952	1	0	953	0.999	0.001
29055C>T	exon12	Pro603Ser	952	1	0	953	0.999	0.001
29075G>C	exon12	Leu609Phe	952	1	0	953	0.999	0.001
29195C>G	exon12	Leu649Leu	860	91	2	953	0.950	0.050

The nucleotide sequence (GenBank Accession ID: NT_010393) was used as a reference sequence. *SCNN1G*, γ-subunit of the epithelial sodium channel gene; SNP, single nucleotide polymorphism.

One of the 948 individuals had a G-to-A substitution at nucleotide 32,173, leading to an amino acid substitution from Glu to Lys at position 632 (E632K). These missense mutations were found in a heterozygous form. In addition, we identified four synonymous mutations (31824C>T: $n=1$; 31839G>C: $n=19$; 31878T>C: $n=1$; 32061G>A: $n=11$) that encoded for Ile515, Ser520, Ser533 and Thr594, respectively (Table 1). Among them, two synonymous mutations (Ser520 and Thr594) were previously reported in Japanese (*13, 16, 24*).

In *SCNN1G*, one of the 953 individuals had a C-to-T substitution at nucleotide 28981, leading to an amino acid substitution from Ala to Val at position 578 (A578V). One individual had a C-to-T substitution at nucleotide 29055, leading to an amino acid substitution from Pro to Ser at position 603 (P603S). One individual had a a G-to-C substitution at nucleotide 29075, leading to an amino acid substitution from Leu to Phe at position 609 (L609F). These missense mutations were found in a heterozygous form. In addition, we identified two synonymous mutations (28898C>T: $n=1$; 29195C>G: $n=91$, heterozygous and $n=2$, homozygous) that encoded for Ile550 and Leu649, respectively (Table 2). We also identified two single nucleotide polymorphisms in intron 11, 28732G>A ($n=1$) and 28776G>A ($n=1$).

Tables 3 and 4 show the characteristics of the six patients with missense mutation in *SCNN1B* and of the three patients with missense mutation in *SCNN1G*. An essential hypertensive patient with a missense mutation of P592S in the *SCNN1B* gene (case 1, Table 3) showed high plasma renin activity (PRA; 4.8 ng/ml/h), low plasma aldosterone concentration (PAC; 1.8 ng/dl) and normokalemia (4.3 mEq/l) even while taking β blockade and spironolactone. Both the PRA and PAC in this patient were outside the normal ranges for our institute (normal PRA: 0.2–2.7 ng/ml/h; normal PAC: 2–13 ng/dl) and significantly different from the average values (PRA: 2.1 ng/ml/h; PAC: 15.6 ng/dl) among subjects of the present study who had no secondary hypertension and no missense mutation of the *SCNN1B* or *SCNN1G* genes.

One of the two patients with missense mutation of T594M in the *SCNN1B* gene (case 4, Table 3) showed severe hypertension, with blood pressure of 174/96 mmHg even while taking three kinds of antihypertensive drugs (calcium channel blockade, angiotensin II receptor antagonist and α1-adrenergic blocker).

One patient with missense mutation of E632K in the *SCNN1B* gene and renovascular hypertension (case 6, Table 3) showed much greater levels of both PRA (22 ng/ml/h) and PAC (102.9 ng/dl) compared to the other subjects with renovascular hypertension (average PRA: 6.9 ng/ml/h; average PAC: 18.7 ng/dl), as well as clear hypokalemia (2.7 mEq/l) despite spironolactone therapy.

Other hypertensive patients with missense mutations in