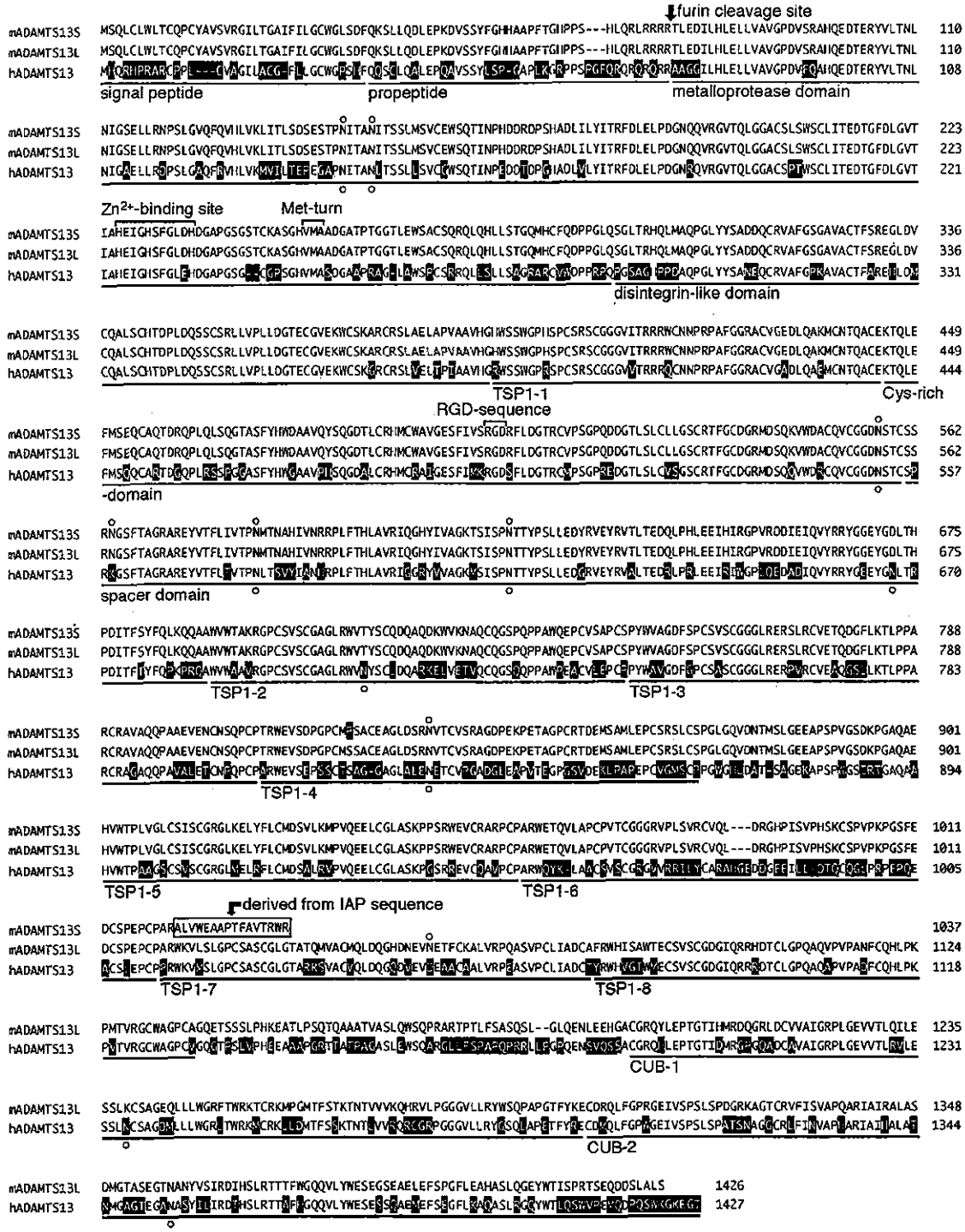

FIG. 6. **Alignment of deduced amino acid sequences of mADAMTS13 and hADAMTS13.** Sequences are represented with the *single-letter code*, and residues that differ from mADAMTS13L are *shaded*. Each structural domain is *underlined*. The predicted furin cleavage site (R*X*(K/R)R) is marked with an *arrow*. The IAP-derived 16 amino acid residues in mADAMTS13S are *boxed*. *Open circles* indicate the potential *N*-glycosylation sites.

in Fig. 4 (*B* and *C*), *Adamts13* mRNA in both strains was exclusively observed in the liver, suggesting that mADAMTS13 is primarily synthesized in the liver, similar to hADAMTS13. A single transcript of 3.5 kb was expressed in liver of the BALB/c strain with the IAP insertion. In contrast, two transcripts of 5.0 and 3.5 kb were detected in liver of the outbred Swiss Webster strain, suggesting that this strain may carry two types of alleles, like the ICR strain. Both SwissWebster and ICR strains are derived from a colony of Swiss mice.

Comparison of the Deduced Amino Acid Sequences of mADAMTS13 and hADAMTS13—The deduced amino acid sequences of mouse and human ADAMTS13 were aligned (Fig. 6). The overall sequence identity between mADAMTS13L and hADAMTS13 was ~70%. The highest identity (>80%) was observed in the disintegrin-like (81%), TSP1–1 (89%), cysteine-rich (80%), and TSP1–8 (83%) domains, whereas relatively low conservation (<60%) was observed in the signal peptide (43%), propeptide (52%), TSP1–4 (46%), and TSP1–6 (40%) domains. mADAMTS13L contained eight potential *N*-glycosylation sites, six of which were conserved in hADAMTS13. mADAMTS13L also included several motifs characteristic for each domain of hADAMTS13, such as a furin cleavage sequence at the end of

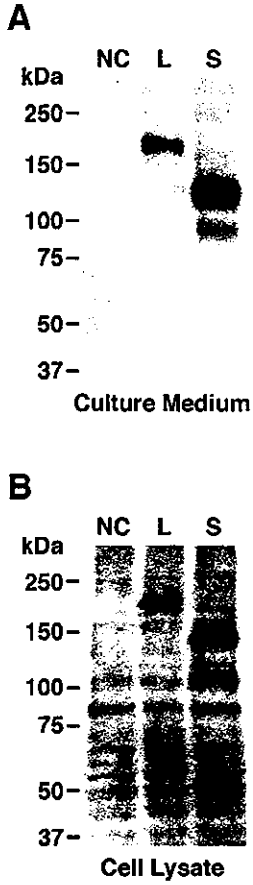

FIG. 7. **Transient expression of recombinant mADAMTS13.** *A*, mADAMTS13 in the culture medium. HeLa cells were transfected with plasmids encoding mADAMTS13L (*L*) and mADAMTS13S (*S*). The concentrated culture media were analyzed by Western blot with an anti-mADAMTS13 antibody under nonreducing conditions. *B*, mADAMTS13 in the cell lysate. The cell lysates including extracellular matrixes were analyzed by Western blot with an anti-FLAG antibody under reducing condition. *NC* is the culture medium or the lysate of untransfected cells. The size of protein markers is indicated at the *left*. The small size difference of recombinant enzymes in medium and cell lysates was due to the difference of electrophoretic condition. The faster migrating bands seen in medium and cell lysates expressing mADAMTS13S may result from proteolysis during cell culture. The typical result of three experiments is shown.

the propeptide, a zinc-binding site in the metalloprotease domain, and an RGD sequence in the cysteine-rich domain. The sequences of mADAMTS13L and mADAMTS13S were almost identical, except for the deletion of C-terminal regions.

Expression and Enzymatic Activity of Recombinant mADAMTS13L and mADAMTS13S—We transiently expressed the mADAMTS13L and mADAMTS13S proteins in HeLa cells. Western blot analysis using a polyclonal antibody against mADAMTS13 revealed that both were secreted into the culture media (Fig. 7*A*). Transient expression of mADAMTS13L produced an immunoreactive band of ~200 kDa, and mADAMTS13S exhibited a 130-kDa band. The level of mADAMTS13S in the medium was almost 10-fold higher than that of mADAMTS13L. This was not due to a preferential accumulation of mADAMTS13L on the cell surface or the extracellular matrix, because a relatively high amount of mADAMTS13S was also observed in the cell lysates (Fig. 7*B*). It was conceivable that mADAMTS13S was effectively synthesized in HeLa cells compared with mADAMTS13L in our experimental conditions. Whether mADAMTS13S is also preferentially expressed *in vivo* remains unknown. Further analysis is required to determine the plasma levels of mADAMTS13L and mADAMTS13S in mice.

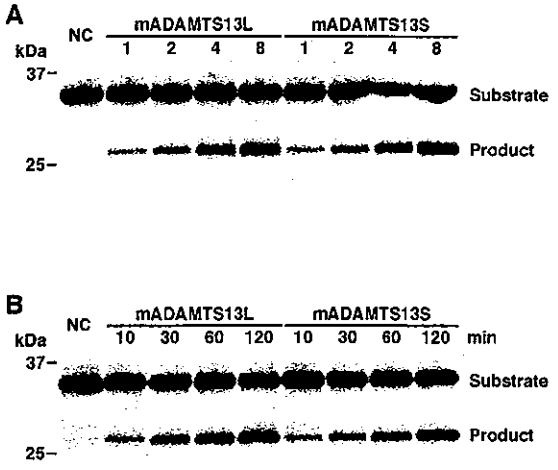

FIG. 8. **Enzymatic activity of recombinant mADAMTS13.** *A*, cleavage of GST-mVWF73-H by serial dilutions of mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S at 37 °C for 1 h. A negative control reaction using the culture medium of untransfected cells (NC) was also performed simultaneously. The products were analyzed by Western blot using an anti-GST antibody. The *numbers 1, 2, 4,* and *8* indicate relative amounts of mADAMTS13L and mADAMTS13S in the reaction mixtures. The typical result of three experiments is shown. *B*, time course of GST-mVWF73-H cleavage by mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S for the indicated time at 37 °C. The reaction mixtures contained the equivalent amounts of the recombinant enzyme. Products were analyzed by Western blot using an anti-GST antibody. The typical result of three experiments is shown.

The VWF cleaving activities of recombinant proteins were measured by the degradation of the specific recombinant substrate, GST-mVWF73-H. The relative concentration of recombinant mADAMTS13 in the culture medium was determined by chemiluminescent intensities on Western blot, and equal amounts were used for the enzymatic assay. The substrate, GST-mVWF73-H, was incubated with serial dilutions of the culture medium, and the cleavage product including the N-terminal GST tag was visualized by Western blot using anti-GST (Fig. 8). When the substrate was incubated with the medium of mADAMTS13L-transfected cells, a band appeared with the expected size of the N-terminal portion (28 kDa) in a concentration-dependent manner, indicating the cleaving activity of recombinant mADAMTS13L (Fig. 8*A*). No degradation was observed after the incubation of GST-mVWF73-H with the medium from untransfected cells. The cleaved band was also detected after incubation with the mADAMTS13S culture medium, and the chemiluminescent intensities of the product bands were almost equal to those obtained by mADAMTS13L (Fig. 8*A*). We confirmed that the degradation of GST-mVWF73-H by mADAMTS13 was also time-dependent, and the rate of the product formation by mADAMTS13S was similar to that by mADAMTS13L (Fig. 8*B*).

The VWF Cleaving Activity of Mouse Plasma—To examine the ADAMTS13 activity in plasma from various mouse strains, we collected plasma samples from five strains and carried out the enzymatic assay using GST-mVWF73-H. As shown in Fig. 9, plasma from all strains cleaved GST-mVWF73-H. Comparison of the product levels did not reveal a significant difference among strains. This suggested that the IAP insertion into the *Adamts13* gene does not affect the *in vitro* cleavage of GST-mVWF73-H by plasma.

DISCUSSION

In this study, we identified two isoforms of the mouse *Adamts13* gene that result from the strain-specific insertion of

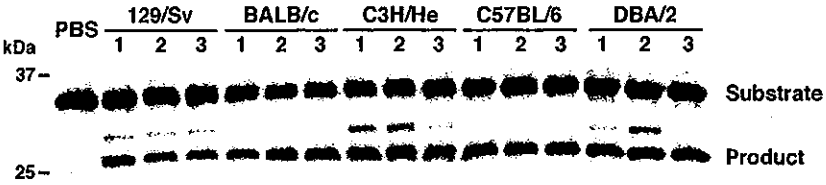

FIG. 9. **Cleavage of GST-mVWF73-H by mouse plasma.** GST-mVWF73-H was incubated with plasma samples from mice with (BALB/c, C3H/He, C57BL/6, and DBA/2) or without (129/Sv) the IAP insertion in the *Adamts13* gene. The products were analyzed by Western blot using an anti-GST antibody. The results from three animals/strain are shown.

an IAP-retrotransposon. The IAP-free *Adamts13* gene contained 29 exons, and the deduced protein sequence included 1,426 amino acid residues with the same domain organization as hADAMTS13. In contrast, the IAP-inserted *Adamts13* gene contained only 24 exons encoding 1,037 amino acids having a truncated C terminus.

The inserted IAP is one of the endogenous transposable elements, which is closely related to retroviruses and transposed via the reverse transcription of an RNA intermediate (26, 27). The IAP element contains two long terminal repeats with the signals for the initiation/regulation of transcription and for the polyadenylation of transcripts (28). IAP insertions into introns have been shown to cause formation of chimeric transcripts (29–32), similar to our findings in the *Adamts13* gene. We noted that the presence of IAP in the *Adamts13* gene induces the appearance of a cryptic splicing site followed by a premature in-frame stop codon and a polyadenylation signal derived from the IAP long terminal repeat. As a result, the insertion leads to replacement of the last 405 amino acid residues corresponding to two TSP1 motifs and two CUB domains with the IAP-encoded 16 amino acid residues.

Northern blot and RT-PCR analyses confirmed that the IAP chimeric short transcript (3.5 kb) and the IAP-free long transcript (5 kb) were expressed in a strain-specific manner. Both types of transcripts were specifically expressed in the liver, consistent with expression of the human *ADAMTS13* gene. It should be noted that the IAP insertion could not completely abolish the formation of mADAMTS13L mRNA. The RT-PCR products (540-bp; Fig. 3) characteristic of mADAMTS13L mRNA were also detectable in the strains with the IAP insertion when using a large amount of template (data not shown). A small amount of mADAMTS13L protein may be expressed in mice with the IAP-inserted *Adamts13* gene such as the BALB/c, C3H/He, C57BL/6, and DBA/2 strains. Incidentally, the RT-PCR and 3′-RACE data did not show any splicing variants that encoded mADAMTS13S-like protein in the IAP-free strains.

Recently, we developed a novel recombinant substrate, GST-VWF73-H, to measure hADAMTS13 activity (24). GST-VWF73-H is a partial region of human VWF flanked by GST and His_6 tags. Because of difficulty in isolating VWF from mouse plasma, we have also prepared the recombinant substrate, GST-mVWF73-H, based on the mouse VWF cDNA sequence. Both mouse and human plasma efficiently cleaved GST-mVWF73-H and produced a fragment of the expected size. Mouse plasma also cleaved the substrate for hADAMTS13, GST-VWF73-H (data not shown).

Both recombinant mADAMTS13L and mADAMTS13S were secreted into the culture medium of HeLa cells. This result indicates that the IAP insertion does not abolish secretion of mADAMTS13 from cells. The recombinant mADAMTS13L and mADAMTS13S cleaved GST-mVWF73-H with nearly the same efficiency. Similarly, a deletion mutant of hADAMTS13 in mimicry of mADAMTS13S was also secreted efficiently from HeLa cells and cleaved GST-VWF73-H with normal activity (data not shown). In previous reports, we and others found that deletion mutants of hADAMTS13 devoid of the C-terminal TSP1 motifs and CUB domains retained VWF cleaving activity (21, 22). Therefore, our current observation on mouse and human recombinant proteins was consistent with these previous studies. Moreover, the plasma VWF cleaving activities in mice were also comparable among the strains with or without the IAP insertion in the *Adamts13* gene. The C-terminal two TSP1 motifs and two CUB domains of mADAMTS13 may contribute to activity but are not essential for the VWF cleavage, at least *in vitro*.

The fact that several common strains of mice have a naturally truncated form of ADAMTS13 allows us to hypothesize that the truncated domains are not necessary *in vivo*. However, several mutations in TSP1–7, TSP1–8, CUB-1, and CUB-2 domains of hADAMTS13 were reported to associate with congenital thrombotic thrombocytopenic purpura (15, 17–20). It is still unclear whether these mutants are secreted from cells, as is the case with mADAMTS13S. To date, two mutations, R1123C and 4143insA, were characterized by expression analysis, and both impaired secretion of the enzyme from cells (19, 20). The C-terminal mutations found in thrombotic thrombocytopenic purpura patients may influence their synthesis or secretion.

Bernardo *et al.* (33) reported that several short peptides within the regions from TSP1–6 to the C terminus of hADAMTS13 block VWF cleavage on the endothelial cell surface under flow conditions. This finding suggests an important role for the C-terminal domains *in vivo*. Although our results clearly show that the mouse has managed without full-length ADAMTS13, the relative importance of ADAMTS13 for regulation of VWF activity may be different between human and mouse. A gene targeting technique of mouse *Adamts13* will help to clarify the physiological contribution of mADAMTS13.

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Three Novel Missense Mutations of *WNK4*, a Kinase Mutated in Inherited Hypertension, in Japanese Hypertensives

Implication of Clinical Phenotypes

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Background: Mutations in serine-threonine kinase *WNK4* with no lysine (K) at a key catalytic residue cause familial hypertension known as pseudohypoaldosteronism type II (PHAII). The objective of this study was to test whether more subtle changes of *WNK4* could be implicated in hypertension or renal failure.

Methods: We screened 956 Japanese patients with hypertension or renal failure for mutations in exons 7 and 17 in the *WNK4* gene where the mutations were identified in patients with PHAII.

Results: We identified three novel missense mutations, Met546Val (n = 2) and Pro556Thr (n = 2) in exon 7, and Pro1173Thr (n = 1) in exon 17, in a heterozygous state in addition to four single nucleotide polymorphisms including one synonymous mutation (Ala547Ala). Results of genotyping Met546Val and Pro556Thr mutations indicated that these mutations were not present in a Japanese general population (n = 1875).

Conclusions: The present study indicated that a systematic screening of *WNK4* in a large set of patients with hypertension or renal failure detected some rare genetic variants. Although substantial contribution of three novel missense mutations in exons 7 and 17 of *WNK4* to the genetics of hypertension or renal failure is still unclear, these mutations in the *WNK4* gene identified in Japanese hypertensives but not in a general population may contribute to hypertension and progression of hypertensive complications to some extent. Am J Hypertens 2004;17: 446–449 © 2004 American Journal of Hypertension, Ltd.

Key Words: Pseudohypoaldosteronism, *WNK4*, gene variants, hypertension.

Pseudohypoaldosteronism type II (PHAII), so-called Gordon syndrome,[1] is an autosomal dominant disease characterized by hypertension, hyperkalemia, normal renal glomerular filtration, and renal tubular acidosis caused by impaired renal K^+ and H^+ excretion. These physiologic abnormalities are all chloride dependent[2] and can be corrected by thiazide diuretics, specific antagonists of the Na-Cl co-transporter present at the distal convoluted tubule. It has been reported that mutations in either of two serine-threonine kinases, *WNK1* and *WNK4*, can cause PHAII.[3] The defect in the *WNK1* gene was a large deletion in intron 1, resulting in increased *WNK1* expression.[3]

Immunohistochemical analysis showed that *WNK1* is ubiquitously expressed in many tissues and organs,[4,5] and seems restricted to diverse chloride-transporting epithelia.[3,6] In the kidneys, *WNK1* is restricted to the aldosterone-sensitive distal nephron.[3,6]

WNK4 expression is predominantly limited to the distal convoluted tubule, connecting tubule, and collecting duct in the kidney.[3] In the distal convoluted tubule, *WNK4* co-localizes with XO-1, a specific tight junction protein, whereas in the cortical collecting duct, *WNK4* expression is mostly cytoplasmic, suggesting a specific function along the nephron axis.[3] Recent expression studies indicated that the co-expression of the thiazide receptor, the Na-Cl co-

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transporter (SLC12A3), with *WNK4* leads to a significant decrease in thiazide-sensitive sodium uptake.[7,8] *WNK4* was shown to consistently suppress the cell surface expression of the thiazide-sensitive Na-Cl co-transporter.

The *WNK4* mutations identified in patients with PHAII were missense mutations in the highly conserved regions of four WNK family genes.[3] Three causative missense mutations, Glu562Lys, Asp564Ala, and Gln565Glu, were present in exon 7 and another mutation, Arg1185Cys, in exon 17, all of which were distant from the catalytic kinase domain. Mutations identified in the *WNK4* gene so far have all been accompanied by charge changes, suggesting functional significance.

To test whether subtle changes of *WNK4* could be implicated in hypertension or renal failure, this study was undertaken to screen the mutations in exons 7 and 17 of *WNK4* in Japanese patients with hypertension or renal failure. Furthermore, we assessed the relevance of these mutations to the clinical phenotypes.

Methods

Hypertensive Subjects

A total of 956 hypertensive subjects (525 men and 431 women, average age: 65.0 ± 10.6 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center.

Ninety-two percent of 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 systolic blood pressure (BP) above 140 mm Hg and/or diastolic BP above 90 mm Hg or the use of antihypertensive agents. Blood pressure was measured three times in sitting position and averaged after at least 5 min of resting. In the hypertensive subjects, about one-third of them have hypertensive cardiovascular complications. In detail, 112 subjects had renal impairment (serum creatinine ≥1.4 mg/dL), 103 subjects had ischemic heart disease, and 152 subjects had episodes of stroke. Study subjects had routine laboratory tests including electrolytes, renal function, blood glucose, glycohemoglobin A1c, plasma renin activity, and plasma aldosterone concentration by radioimmunoassay.

Screening of Mutations in Exons 7 and 17 of *WNK4*

Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan).[9] The regions of exons 7 and 17 were amplified by polymerase chain reaction (PCR) using two pairs of specific primers; 5′-atatcctggagttcccaagaagg-3′ and 5′-ctagaggtggaaggcagggtaag-3′, which flank the 460-bp region containing exon 7, and 5′-tgaggagttctgggctgagctg-3′ and 5′-atgatgcctgggagcaggatg-3′, which flank the 493-bp region containing exon 17. The PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA), as described previously.[10] The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes Corp., Ann Arbor, MI), followed by visual inspection.

Genotyping of Missense Mutations for Large-scale Case-control Subjects

Missense mutations identified in hypertensives were genotyped in 1875 subjects (861 men and 1014 women, average age: 64.7 ± 11.1 years) who participated in the large cohort Suita Study. The sample selection and study design of the Suita Study have been described previously.[11–13] The subjects have been visiting the National Cardiovascular Center every 2 years for general health checkups. According to the criteria of high BP above 140 and/or 90 mm Hg or the use of antihypertensive agents, 795 subjects were diagnosed as hypertensive. This group includes 18 subjects with renal impairment (serum creatinine ≥1.4 mg/dL), 147 subjects with ischemic heart diseases, and 60 subjects with episode of strokes. In addition to performing a routine blood examination that included lipid profiles, glucose levels, BP, anthropometric measurements, a physician or nurse administered questionnaires covering personal history of cardiovascular diseases, including angina pectoris, myocardial infarction, or stroke.

Two missense mutations, Met546Val and Pro1173Thr, were genotyped by using the TaqMan-PCR method.[10] The sequences of PCR primers and probes for the TaqMan-PCR method are follows: Met546Val (*A6744G*), primers, gccaggacctccaccag, ctggctcctcaggctcag; probes, Fam-caactgtgcccgtggc-MGB (for G allele), Vic-caactgtgcccatggc-MGB (for A allele); primers, Pro1173Thr (*C15503A*), gaaacactacagacactacagaaaaaagaaa, cggctggacagcatagca; probes, Fam- cccccaacgggtat-MGB (for A allele); Vic-cccccaccgggtat-MGB (for C allele). We tried to genotype another missense mutation, Pro556Thr, by the TaqMan-PCR method, but it failed.

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

Results

We screened 956 subjects in the hypertensive group for *WNK4* gene polymorphisms. The regions of exons 7 and 17 of the *WNK4* gene were amplified from the genomic DNA and directly sequenced. In this study, we were not able to detect three causative missense mutations of PHAII—Glu562Lys, Asp564Ala, and Gln565Glu—in exon 7 and Arg1185Cys in exon 17. However, we identified three novel missense mutations, two in exon 7 and one in exon 17, of the *WNK4* gene (Table 1). Two of 956

Table 1. Summary of sequence variations of exons 7 and 17 in *WNK4* identified in Japanese patients with hypertension or renal failure

SNP Name	Region	Amino Acid Substitution	Allele 1		Allele 2	Total Number	Allele 1 Frequency	Allele 2 Frequency
			Homo	Hetero	Homo			
6744A>G	exon 7	Met546Val	941	2	0	943	0.999	0.001
6749C>T	exon 7	Ala547Ala	875	66	2	943	0.962	0.038
6774C>A	exon 7	Pro556Thr	941	2	0	943	0.999	0.001
15402C>T	intron 16	—	954	1	0	955	0.999	0.001
15503C>A	exon 17	Pro1173Thr	948	1	0	949	0.999	0.001
15677T>C	intron 17	—	923	17	0	940	0.991	0.009
15738C>A	intron 17	—	921	15	1	937	0.991	0.009

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (Hum Mut, 11, 1–3, 1998). The nucleotide sequence (GenBank Accession ID:NT_010755) was used as a reference sequence.

individuals had an A-to-G substitution at nucleotide 6744 in exon 7 leading to an amino acid substitution from Met to Val at position 546 (Met546Val). Two of 956 individuals had a C-to-A substitution at nucleotide 6774 in exon 7 leading to an amino acid substitution from Pro to Thr at position 556 (Pro556Thr). One of 956 individuals had a C-to-A substitution at nucleotide 15,503 in exon 17 leading to an amino acid substitution from Pro to Thr at position 1173 (Pro1173Thr). These identified missense mutations were found in heterozygous form. In addition, we identified one synonymous mutation (6749 C>A) encoded for Ala547 with a minor allele frequency of 3.8% and three additional rare mutations in introns 16 and 17 (Table 1).

The characteristics of five hypertensive patients with these novel missense mutations in *WNK4* are listed in Table 2. In these patients, electrolyte abnormalities such as hyperkalemia, seen in PHAII, were not recognized. Thiazide diuretics, which are very useful for BP reduction in patients with PHAII, were not administered to these patients. One patient with Pro1173Thr in exon 17 had hypertensive renal failure despite only 4 years of hypertension. Furthermore, Met546Val and Pro1173Thr mutations were genotyped in 1875 subjects for general health checkups by the TaqMan-PCR method, but none of them showed the mutations. We tried to genotype the Pro556Thr mutation, but it technically failed.

Discussion

The PHAII shows an autosomal dominant inheritance pattern as the mutations cause serious loss of function. However, if the mutations have a median effect on the function, the phenotype may not be diagnosed as PHAII or may be missed. In this study of sequence analysis for *WNK4* in 956 Japanese hypertensives, we could not detect the missense mutations previously identified in patients with fa-

Table 2. Clinical profiles of five hypertensive patients with mutations

Case	1	2	3	4	5
Polymorphism	Met546Val	Met546Val	Pro556Thr	Pro556Thr	Pro1173Thr
Age (yr)	56	54	61	38	69
Sex	Female	Male	Male	Male	Male
BMI (kg/m^2)	19.0	29.4	21.6	29.0	22.7
Diagnosis	EHT,HL CVA	EHT,NIDDM HL,CGN	EHT,HL ASO	EHT,HL	EHT CRF
HT duration (yr)	22	17	12	6	4
HT Family Hx	Father	Mother	Father,brother	None	Father
SBP (mm Hg)	138	130	132	142	150
DBP (mm Hg)	92	74	86	92	74
Medication	None	ACEI,CCB,ARB	CCB,ARB	ACEI	CCB,ARB,BB
Na (mEq/L)	142	142	139	142	138
K (mEq/L)	4.1	3.7	4.1	4.2	4.5
Cl (mEq/L)	106	107	105	106	106
Creatinine (mg/dL)	0.8	0.8	0.8	0.9	2.1
Overt proteinuria	–	+	–	–	–
PRA (ng/mL/h)	2.1	1.3	4.1	1.2	No data
PAC (ng/dL)	10.4	15.9	19.1	11.4	No data
FBS (mg/dL)	76	106	88	91	100
HbA1c (%)	5.7	5.8	5.8	5.3	5.7

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin II receptor blockade; ASO = atherosclerotic obliterance; BB = β-adrenergic blocker; BMI = body mass index; CCB = calcium channel blocker; CGN = chronic glomerulonephritis; CRF = chronic renal failure; CVA = cerebrovascular disease; DBP = diastolic blood pressure; EHT = essential hypertension; FBS = fasting blood sugar; HbA1c = glycohemoglobin A1c; HL = hyperlipidemia; HT = hypertension; Hx = history; NIDDM = non-insulin-dependent diabetes mellitus; PAC = plasma aldosterone concentration; PRA = plasma renin activity; SBP = systolic blood pressure.

milial PHAII. This result is reasonable, because there were no hypertensive patients in the study suspected of PHAII from the clinical features. Instead, we identified three novel missense mutations in exons 7 and 17 of *WNK4* in five hypertensives. Furthermore, we found that Met546Val and Pro1173Thr were not present in the group of subjects with general health checkups (n = 1875). Therefore, although the clinical features of patients with these missense mutations were unclear because of a very rare allele frequency, we could regard that missense mutations, Met546Val and Pro1173Thr, may be related to the elevation of BP or progression of hypertensive complications to some extent, because the hypertensive group was obviously including patients with severe hypertension and higher rate of hypertensive cardiovascular complications compared to the subjects with general health checkups. Functional analysis for these novel missense mutations of *WNK4* would be necessary to clarify the relevance to the clinical features including hypertension.

One hypertensive patient with *WNK4* Pro1173Thr in the present study had renal failure despite only 4 years of hypertension and no history of diabetic nephropathy or chronic glomerulonephritis. The Pro1173Thr mutation of *WNK4* may influence the progression of renal failure or accelerate renal impairment caused by hypertension, although further functional studies are required to ascertain this possibility.

Recently, Monti et al[14] reported that *WNK4* may not play an important role in BP elevation in the analysis of congenic rats focusing on chromosome 10, a region homologous to human chromosome 17 including *WNK4*. On the other hand, Erlich et al[15] indicated that 1156666 G>A at intron 10 was related to the prevalence of hypertension in whites but not in African Americans. The missense mutations of Met546Val, Pro556Thr, and Pro1173Thr were not reported, but the Ala547 polymorphism that was shown as Ala535 in their numbering system was present. The numbering is different due to the inflamed Met codon at 12 amino acids upstream. The minor allele frequencies of this polymorphism in whites, African Americans, and Japanese were 0, 0.23, and 0.04, respectively, indicating that Japanese were between whites and African Americans. They also did not detect any mutation that has previously been shown to cause PHAII.

In summary, we identified three novel missense mutations in the *WNK4* gene among hypertensive patients, but these were not found in a large set of subjects with general health checkups. Although the functional mechanisms or relevance to clinical features of these mutations are unclear, the accumulation of cases with these mutations and a follow-up survey may clarify the possible role of these mutations in hypertension and progression of hypertensive complications.

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ORIGINAL ARTICLE

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Identification of 108 SNPs in *TSC*, *WNK1*, and *WNK4* and their association with hypertension in a Japanese general population

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Abstract The deletion of thiazide-sensitive Na–Cl cotransporter (*TSC*, *SLC12A3*) causes Gitelman's syndrome characterized by low blood pressure, while deletions of the *WNK1* (*PRKWNK1*) and *WNK4* (*PRKWNK4*) genes cause familial hypertension known as pseudohypoaldosteronism type II. Recent studies have revealed that cell surface expression of TSC is regulated by WNK1 and WNK4. We hypothesized that molecular variations in *TSC*, *WNK1*, and *WNK4* could lead to an increased morbidity of hypertension. We identified 52, 35, and 21 polymorphisms in Japanese hypertensives by sequencing the entire coding regions of *TSC*, *WNK1* and *WNK4*, respectively. Twenty-one representative polymorphisms were genotyped in 1,818 Japanese individuals (771 subjects with hypertension and 1,047 controls) randomly sampled in Suita city. The results indicated that the systolic blood pressure in men with the CT+TT genotype in *WNK4* C14717T was 3.1 mmHg higher than those with the CC genotype ($p=0.042$) after adjustment with confounding factors such as age, BMI, hyperlipidemia, diabetes mellitus, antihypertensive drug use, smoking, and drinking. Multivariate logistic regression analysis (with adjustment for the same parameters) in men revealed that the odds ratio for the presence of hypertension of the CT+TT genotype in C14717T to the CC genotype was 1.62 ($p=0.010$, 95% confidence interval, 1.12–2.33). Association of *TSC* and *WNK1* with hypertension was not observed. In conclusion, our study suggests the possible involvement of *WNK4* in essential hypertension in a Japanese general population.

Keywords WNK1 · WNK4 · Thiazide-sensitive Na–Cl cotransporter · Gene variants · Hypertension

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Introduction

Several molecular variants of the thiazide-sensitive Na–Cl cotransporter (*TSC*, *SLC12A3*) relate to Gitelman's syndrome characterized by their low blood pressure (BP) sodium wasting, secondary hyperaldosteronism, hypokalemia, alkalosis, hypomagnesemia, and hypocalciuria (Mastroianni et al. 1996; Simon et al. 1996; Takeuchi et al. 1996). This syndrome is known to be heritable as autosomal recessive, and the mutations identified in *TSC* may reduce the capacity of the TSC to reabsorb salt in the distal tubules where the cotransporter is regionally expressed (Mastroianni et al. 1996). On the contrary, mutations in the *WNK1* (*PRKWNK1*) and *WNK4* (*PRKWNK4*) genes relate to familial hypertension known as pseudohypoaldosteronism type II (Wilson et al. 2001), associated with hyperkalemia (despite normal renal glomerular filtration) and renal tubular acidosis caused by impaired renal K^+ and H^+ excretion. This autosomal dominant disease includes several types of mutations; a large deletion in intron 1 of *WNK1*, missense mutations in the highly conservative regions of *WNK4* (Wilson et al. 2001). Mutations identified in *WNK4* so far were all accompanied by charge changes, assuming modification of the protein function.

Recent expression studies have revealed a close link between TSC and WNK family proteins. Coexpression of TSC with WNK4 leads to a significant decrease in thiazide-sensitive sodium uptake (Choate et al. 2003; Wilson et al. 2003). WNK4 was shown consistently to

suppress cell surface expression of TSC. Although WNK1 per se was inactive on the transporter activity, it was able to abolish the inhibitory effect of WNK4, suggesting that both proteins act on the same signaling pathway (Wilson et al. 2003; Yang et al. 2003). Thus, WNK4 functions as a negative regulator for the surface expression of Na–Cl cotransporter, and loss of this regulation can cause an inherited form of hypertension. WNK1 seems to act as a suppressor of WNK4, and gain-of-function of this gene can cause loss in WNK4 function leading to an inherited form of hypertension.

It is likely that individual BP level is influenced by several different genetic variants in a general population. A polymorphism in *WNK4* (base1156666G>A) has been reported to be associated with hypertension in a Caucasian population (Erlich et al. 2003) with a discrepancy in other studies (Benjafield et al. 2003; Speirs and Morris 2004). We hypothesized that the genetic polymorphisms in *TSC*, *WNK1*, and *WNK4* may involve changes in BP level. Among the different kinds of genetic variations, single nucleotide polymorphisms (SNP) receive much attention due to their easy genotyping. This study was undertaken to identify genetic variations, mainly SNPs, in all exons of *TSC*, *WNK1*, and *WNK4* and to examine the association of SNPs with hypertension in a Japanese general population.

Methods

Subjects

The subjects of the Suita study consisted of 14,200 men and women (30–79 years of age), who had been randomly selected from the municipal population registry considering group stratification by gender and 10-year age. They were all invited, by letter, to have a group checkup every 2 years at the Division of Preventive Cardiology, National Cardiovascular Center, Japan. DNA from the leukocytes was collected from participants who visited the National Cardiovascular Center between April 2002 and February 2003. The study protocol was approved by the ethical committees on human research of the National Cardiovascular Center and Suita city. Written informed consent was obtained from each subject for proceeding genetic analyses. In this study, the genotypes of 1,818 individuals including 771 subjects with hypertension (396 men and 375 women) and 1,047 controls (439 men and 608 women) were performed.

Measurements

BP was measured after at least 10 min of rest in a sitting position. Systolic and diastolic BPs (SBP and DBP) were the means of two measurements by well-trained doctors using a mercury sphygmomanometer (recorded in a 3 min pause). Hypertension was defined as SBP of ≥140 mmHg, DBP of ≥90 mmHg or current use of antihypertensive medication.

A physician or nurse questionnaired each patient regarding current smoking and alcohol drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke. Hypercholesterolemia was defined as total serum cholesterol levels ≥5.68 mmol/l (≥220 mg/dl) or current use of antihyperlipidemic medication. Diabetes was defined as fasting plasma glucose levels ≥7.0 mmol/l (126 mg/dl) or nonfasting glucose levels ≥11.1 mmol/l (200 mg/dl), HbA1C ≥6.5%, or current use of antidiabetic medication. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Blood samples from the subjects after 12 h of fasting were collected in EDTA-containing tubes. Total cholesterol and high density lipoprotein (HDL) cholesterol levels were measured with an autoanalyzer (Toshiba TBA-80) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.

Direct sequencing for SNP discovery and genotyping of polymorphisms

For DNA sequencing, Japanese patients with essential hypertension at the Division of Hypertension and Nephrology, National Cardiovascular Center, Japan, were recruited. Genomic DNA was extracted using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). We sequenced the 48 or 96 Japanese hypertensive samples in which hypertension-susceptive SNPs would be most concentrated. In exon 22 of *TSC* and exons 7 and 17 of *WNK4*, more than 250 Japanese hypertensive samples were sequenced (Kamide et al.

Table 1 Basic characteristics of subjects in Suita, a Japanese urban population, 2002. *HDL* high density lipoprotein cholesterol. Values are mean ± SD or percentage. Hypertension indicates SBP ≥140 mmHg and/or DBP ≥90 mmHg or antihypertensive medication. Hyperlipidemia, total cholesterol ≥5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication. Diabetes, fasting plasma glucose ≥7.0 mmol/l (126 mg/dl) or nonfasting plasma glucose ≥11.1 mmol/l (200 mg/dl) or antidiabetic medication

	Women ($n=983$)	Men ($n=835$)
Age (year)	63.3 ± 11.0	66.3 ± 11.1*
Systolic blood pressure (mmHg)	128.0 ± 19.6	131.9 ± 19.5*
Diastolic blood pressure (mmHg)	76.6 ± 9.8	79.7 ± 10.7*
Body mass index (kg/m^2)	22.3 ± 3.2	23.3 ± 3.0*
Total cholesterol (mmol/l)	5.57 ± 0.79*	5.10 ± 0.78
HDL cholesterol (mmol/l)	1.67 ± 0.40*	1.42 ± 0.36
Current smokers (%)	6.3	30.1**
Current drinkers (%)	29.3	67.0**
Present illness (%)		
Hypertension	38.2	47.4**
Hyperlipidemia	55.2**	27.4
Diabetes mellitus	5.2	12.6**

*$P<0.05$ between men and women by Student's t test
**$P<0.05$ between men and women by χ^2 test

Table 2 List of 108 polymorphisms and their allele frequencies in *TSC*, *WNK1*, and *WNK4* genes identified by direct sequencing. dbSNP ID was searched by using SNPper, a CHIP Bioinformatics Tool (Riva and Kohane 2001; http://snpper.chip.org/bio/snpper-enter, as of May 1 of 2003, that was constructed by dbSNP build 112). The apparent linkage disequilibrium (LD), defined by r^2 more than 0.5, is indicated by *a–l* in the LD column. Exon 22 of TSC and exons 7 and 17 of WNK4 were sequenced using more than 250 hypertensive samples

Gene name	Allele 1/ allele 2 SNPs	LD ($r^2 > 0.5$)	Amino acid change	Region	Allele 1 homo	Hetero	Allele 2 homo	Total	Allele frequency		Flanking sequence	dbSNP ID
									Allele 1	Allele 2		
TSC	*C-1991A*	a		Promoter	38	10	0	48	0.896	0.104	CACCACTGCCTC[C/A]CTGCAATGGCTT	
	A-950G	b		Promoter	1	19	21	41	0.256	0.744	TTTAATAGAGAC[A/G]GGGTTTCACCAT	
	C-704T			Promoter	46	1	0	47	0.989	0.011	CAGACAGCCCGG[C/T]GCCACACCCTGG	
	C-605T	a		Promoter	37	10	0	47	0.894	0.106	CACTTTGAAAAT[C/T]CCTGTCCTGTTT	
	C-553T			Promoter	26	1	0	27	0.981	0.019	AGCCCCCAGTCA[C/T]GTACCCCCTGCT	
	−544delT			Promoter	47	1	0	48	0.990	0.010	TCACGTACCCCC[T/−]GCTTGCTCAATC	
	C-213G	a		Promoter	35	8	0	43	0.907	0.093	GGGAGTGGCTGG[C/G]TTTGGGCCAGCC	
	C-142T	b		Promoter	1	20	22	43	0.256	0.744	GTGTTCTGCCTC[C/T]GGCCCTGTCCGG	
	G-141C	b		Promoter	28	15	0	43	0.826	0.174	TGTTCTGCCTCC[G/C]GCCCTGTCCGGG	
	C1784T			Intron 1	30	17	1	48	0.802	0.198	TGGATGCAGAGA[C/T]GCCGTCCCTAGC	
	A1918G		Ala122Ala	Exon 2	31	17	0	48	0.823	0.177	GGAGGGCGAGGC[A/G]GGCACCAGCAGC	rs2304479
	A2141T			Intron 2	0	8	40	48	0.083	0.917	ACAATAGATTAA[A/T]GCCTGCCGGGGA	rs2304480
	G2971A			Intron 2	47	1	0	48	0.990	0.010	TAGGGCCTAGGT[G/A]CTCGATACCCTG	
	C4527A		Thr180Lys	Exon 4	43	2	0	45	0.978	0.022	TGCTGTCGGTCA[C/A]GGTGACCTCCAT	
	C7479T		Phe341Phe	Exon 8	38	2	0	40	0.975	0.025	TGGCACCTTCTT[C/T]GGAATGTTCTCC	
	C14272T	c		Intron 10	26	18	3	47	0.745	0.255	CTGGCTCAGCCC[C/T]CACCGTGGAGTC	rs3816119
	G14277A			Intron 10	46	1	0	47	0.989	0.011	TCAGCCCCCACC[G/A]TGGAGTCCCTGA	
	C14363A		Ala464Ala	Exon 11	45	2	0	47	0.979	0.021	CATCTTCGGGGC[C/A]ACCCTCTCCTCT	
	C14366T		Thr465Thr	Exon 11	46	1	0	47	0.989	0.011	CTTCGGGGCCAC[C/T]CTCTCCTCTGCC	rs5801
	G17337A			Intron 13	44	1	0	45	0.989	0.011	GGGGTGGGAGTG[G/A]GAGGCATGGGTG	
	T18806C	d		Intron 13	6	24	18	48	0.375	0.625	GACTGGTGCCCT[T/C]GGCCCAGGGTGG	rs2304483
	C18850T		Ala569Val	Exon 14	46	2	0	48	0.979	0.021	ACAACAAGTGGG[C/T]GGCGCTGTTTGG	
	T20072C		Leu623Pro	Exon 15	46	1	0	47	0.989	0.011	GCTCCTACAACC[T/C]GGCCCTCAGCTA	
	G20088A		Ser628Ser	Exon 15	46	1	0	47	0.989	0.011	CCTCAGCTACTC[G/A]GTGGGCCTCAAT	
	C20201G			Intron 15	46	1	0	47	0.989	0.011	GAGTTTCCAAGC[C/G]TAGACCTGTCAC	
	G21421A	e		Intron 16	20	24	3	47	0.681	0.319	ATGGGGGCCCAA[G/A]GGGATGCGGAGC	
	C21500T			Intron 16	42	2	0	44	0.977	0.023	CCCTCTTGCTGG[C/T]TTCTCCCCCAGC	
	C21566G			Intron 16	43	1	0	44	0.989	0.011	CACTTTCTCCCC[C/G]ACTCCTTGTGTT	
	A21586G			Intron 16	43	1	0	44	0.989	0.011	GTGTTTTCCCTT[A/G]TCTGGGCAAAAG	
	C21822T	c	Ala714Ala	Exon 17	21	21	3	45	0.700	0.300	GGATGTCATTGC[C/T]GAGGACCTCCGC	
	C22682T			Intron 17	46	1	0	47	0.989	0.011	TCACCCCTATCC[C/T]CTGGCAGGCCGC	
	C25013T	c		Intron 18	23	22	3	48	0.708	0.292	CTGGGGGAGAAG[C/T]TGGACCTCACCT	rs3764264
	G27029A	c		Intron 20	18	25	4	47	0.649	0.351	TTTTCTTGTGAC[G/A]GTGGTGCCTGAG	
	C27646T	d		Intron 20	6	26	15	47	0.404	0.596	AAGGGGCGTTGG[C/T]GGGGCCCTGGGC	rs2278490
	T27681C[a]	d		Intron 20	5	23	18	47	0.351	0.628	TGGATGCGCGGC[T/C]GCTGGCTCTGCT	rs2278489
	A27681C[a]				0	1			0.011		TGGATGCGCGGC[A/C]GCTGGCTCTGCT	
	T27681A[a]					0					TGGATGCGCGGC[T/A]GCTGGCTCTGCT	
	T29320A		Leu849His	Exon 22	367	5	0	372	0.993	0.007	TCATTCCCTATC[T/A]CCTTGGCCGCAA	
	C29372T	c	Gly866Gly	Exon 22	23	22	3	48	0.708	0.292	TGTGTTCGTAGG[C/T]GGCCAGATTAAC	rs5804
	G34262A	f		Intron 22	44	1	3	48	0.927	0.073	TCTCAAGAAAAA[G/A]TAATAACAATAA	
	G34372A	g	Arg904Gln	Exon 23	45	3	0	48	0.969	0.031	ACCAGAACCCTC[G/A]GGCTGAGCAGTA	
	C34588T	f		Intron 23	41	3	4	48	0.885	0.115	CACAGGGCAAGG[C/T]GGCTGCAGCCCC	
	T37125C			Intron 23	46	1	0	47	0.989	0.011	CCTCAACCCACT[T/C]TCTCGTCCCCAG	
	C37210T		Asn931Asn	Exon 24	46	1	0	47	0.989	0.011	GGCCACTGTCAA[C/T]GAGATGCGGCGG	

Table 2 (Continued)

Gene name	Allele 1/ allele 2 SNPs	LD ($r^2 > 0.5$)	Amino acid change	Region	Allele 1 homo	Hetero	Allele 2 homo	Total	Allele frequency Allele 1	Allele 2	Flanking sequence	dbSNP ID
	A37311G	e		Intron 24	23	21	3	47	0.713	0.287	ACGCGACACATC[A/G]CTGGGTCAGGGA	rs2289117
	G39097A			Intron 24	29	1	0	30	0.983	0.017	GAGGCCATAGAC[G/A]TGGTGAAGGATT	
	C39119T			Intron 24	29	1	0	30	0.983	0.017	ATTGAGTGACCT[C/T]GATGATATGGGA	
	C39142T			Intron 24	40	7	0	47	0.926	0.074	GAAGTGACCACT[C/T]GGCTTTCTCCCG	rs3816118
	G39143A	g		Intron 24	44	3	0	47	0.968	0.032	AAGTGACCACTC[G/A]GCTTTCTCCCGC	rs2289116
	C39203T		Ser967Phe	Exon 25	46	1	0	47	0.989	0.011	TGCTGGATTACT[C/T]CCGAGACGCTGC	
	C39240T	g		Intron 25	43	4	0	47	0.957	0.043	GTAAGTAGTGCC[C/T]GGCTGGTGGGAG	rs2289115
	C39375T	e		Intron 25	23	20	4	47	0.702	0.298	ACATAGCCCTGG[C/T]GATTCTTAGCAT	rs2289114
	C48128T		Ile1008Ile	Exon 26	38	9	0	47	0.904	0.096	AGTCATCCTGAT[C/T]CGAGGAAACCAG	rs2289113
	A48195G		3'UTR	Exon 26	46	1	0	47	0.989	0.011	ACATCCCTGTCC[A/G]CAGCTCTGAGTG	
WNK1	*G421A*		Ala141Thr	Exon 1	89	5	0	94	0.973	0.027	CCTCCAGCCGCT[G/A]CCGCCCCTGGGG	
	C446T		Ala149Val	Exon 1	90	4	0	94	0.979	0.021	AACAGGCCGTCG[C/T]GGGCCCTGCCCC	
	C511T		Leu171Phe	Exon 1	93	1	0	94	0.995	0.005	TCCCAGCCTAGC[C/T]TTGTGGGGAGCA	
	G786A	a,b,c		Intron 1	0	15	80	95	0.079	0.921	ACTTTATTTGAC[G/A]GTCCTTTGGATC	rs3858703
	A59884G			Intron 1	88	1	0	89	0.994	0.006	TCTGAGTTACAC[A/G]TTAACAGTAAAG	
	C73737G	a,b,c		Intron 3	0	16	79	95	0.084	0.916	GACTGGCTTTCT[C/G]ACATTCCTTTTA	rs2158502
	A76571G	a,b	Ala429Ala	Exon 4	0	16	78	94	0.085	0.915	CCAAAATGCTGC[A/G]CAGATCTACCGT	
	C105668A	d		Intron 5	91	4	0	95	0.979	0.021	TTTCTTTTCCCT[C/A]TGTTTGGAAGAT	
	T105758C	d	Asp493Asp	Exon 6	91	4	0	95	0.979	0.021	AGCAGAAGAAGA[T/C]GATGGAGAAAAA	rs2286006
	G105987A			Intron 6	93	1	0	94	0.995	0.005	TGATGAAGTGCC[G/A]TGTGTGGCATAT	
	A107419G			Intron 6	75	13	0	88	0.926	0.074	TTTCAATATACT[A/G]CTGCTTAATTTA	
	C108560T	a,c	Thr665Ile	Exon 8	85	10	0	95	0.947	0.053	CCTCTGTCTTCA[C/T]AGAATCTCGAGT	rs2286007
	G124751A	e,f,g	Gln776Gln	Exon 10	4	26	56	86	0.198	0.802	GCCAGTGAGTCA[G/A]CCTCAAGCTCCA	rs1012729
	T125972A			Intron 10	92	1	0	93	0.995	0.005	TTTTTTTTTTTT[T/A]AAGCCTGTCTGT	
	G126163A	h	Gln843Gln	Exon 11	75	20	1	96	0.885	0.115	CCCTGTCTCTCA[G/A]ATTCCCATATCA	
	A128177C	i	Thr1056Pro	Exon 13	3	19	71	93	0.134	0.866	GCAGTAGCACAG[A/C]CCCAAGCTACCC	rs956868
	C128274T	e,f,g		Intron 13	60	28	5	93	0.796	0.204	GACGGTATGAAA[C/T]GCCAAACTGTCA	
	C129494T	h		Intron 16	74	20	1	95	0.884	0.116	ACAATTATGGTA[C/T]GTCTGCATTTGG	
	A129852G		Ile1172Met	Exon 16	88	4	0	92	0.978	0.022	TATTCTAGCAAT[A/G]GAGAGAGAGTCG	
	C130104T			Intron 16	90	2	0	92	0.989	0.011	GACACCCATGAC[C/T]GACAACAAACTT	
	T130917G	e,f,g,j		Intron 18	44	39	12	95	0.668	0.332	GATATTGTAGTA[T/G]GTGTTTATTTCT	
	C131195T	f,g,j,k	Asn1320Asn	Exon 19	20	47	28	95	0.458	0.542	AGAAGGACCCAA[C/T]ACAGCACCTCCA	
	C131279T	i	Thr1348Thr	Exon 19	72	19	3	94	0.867	0.133	TGGAGTCCCAAC[C/T]ACAGCAGCAGCC	
	C132236T		Ser1667Ser	Exon 19	87	2	0	89	0.989	0.011	CAGTGAACACAG[C/T]TCATCTGGAGCT	
	C132444G		Pro1737Ala	Exon 19	88	1	0	89	0.994	0.006	CAAGTTTCTACC[C/G]CAGTCAGCACTA	
	132576delT	i		Intron 19	68	17	3	88	0.869	0.131	ATCAGTTTTTTT[T/–]CTCCCTAATGAG	
	A132655G	g,j,k		Intron 19	20	36	15	71	0.535	0.465	CTTATAGTATTT[A/G]TTAAATTGACAG	
	C133634T	h		Intron 19	72	19	0	91	0.896	0.104	TTTAGCGTCTCA[C/T]GGACTTGATTTT	
	G135642T	e,f,g,j,k	Met1808Ile	Exon 21	42	42	9	93	0.677	0.323	TAGTCCAGAGAT[G/T]ATCACAGTGACT	
	T135771G			Intron 21	92	1	0	93	0.995	0.005	TTTAACATGTAT[T/G]CAGAGTTCCTGC	
	G136943A		Gln1832Gln	Exon 22	93	1	0	94	0.995	0.005	AGCAGGAACACA[G/A]CCTCAGAAGGGT	
	A141069T		Gly1858Gly	Exon 23	86	3	0	89	0.983	0.017	TTTTAAGATGGG[A/T]CGATTTCAGGTA	
	C141114T	e,f,g		Intron 23	58	27	4	89	0.803	0.197	CTTGATTCCTTC[C/T]TTTGGAGGAGTT	rs2301880
	T142439C	h		Intron 23	70	19	1	90	0.883	0.117	TGATTCTTTTTT[T/C]CCTTTTTTAAAT	
	C142763T		Arg1945Cys	Exon 24	87	6	0	93	0.968	0.032	ACCAAGGTTGGA[C/T]GTTTTCAGGTGA	
WNK4	*C163T*		Arg55Cys	Exon 1	95	1	0	96	0.995	0.005	GAGCCCCGGCCG[C/T]GCTCTTCTCGTC	
	G288A		Arg96Arg	Exon 1	95	1	0	96	0.995	0.005	TGGCCCCGCGAG[G/A]AGCCCACCGCCT	
	C383T		Pro128Leu	Exon 1	95	1	0	96	0.995	0.005	GTCCCGAGCTCC[C/T]GGACTCTGCAGT	

T2074C	Ser211Ser	Exon 2	93	1	0	94	0.995	0.005	TCGGAAACTGTC[T/C]AGAGCTGAGCGG	
C2285T		Intron 2	87	7	0	94	0.963	0.037	GATGTGTGCCCA[C/T]TGCTTCCTGAAC	
A4732G	Ile474Val	Exon 6	94	1	0	95	0.995	0.005	GACAACCAGGCC[A/G]TCGAGTTCCTGT	
A6744G	Met546Val	Exon 7	277	1	0	278	0.998	0.002	GCAACTGTGCCC[A/G]TGGCCCCCGGTC	
C6749T	a Ala567Ala	Exon 7	87	5	1	93	0.962	0.038	TGTGCCCATGGC[C/T]CCCGGTCCCCCC	
G7144T	a Ala601Ser	Exon 8	89	6	1	96	0.958	0.042	GCCTCAGACCCT[G/T]CCCTTCAGCCCC	
A7235		Intron 8	83	12	1	96	0.927	0.073	TGGGGGGCTCCC[A/DEL]GCCATTCCAAGC	
G8119A		Intron 11	95	1	0	96	0.995	0.005	GAGGGGGAGAGA[G/A]ATGAGGACAGAG	
G12806C	a	Intron 12	89	6	1	96	0.958	0.042	CGCGCCCAGCCT[G/C]ATGTTTTAAGAT	
T12948C	Ile740Thr	Exon 12	95	1	0	96	0.995	0.005	GGATTCGGGAGA[T/C]TATCCAGCGAGT	
G14139C	Gly808Ala	Exon 14	90	1	0	91	0.995	0.005	CATCTTCTCCTG[G/C]AACTCCTTTGTC	
G14440A	a Pro908Pro	Exon 14	89	6	1	96	0.958	0.042	TTTCTTTCCTCC[G/A]TGCCCCCTCCACT	rs2290042
C14597T	a Pro961Ser	Exon 14	88	6	1	95	0.958	0.042	CCTAGTCCCCCTC[C/T]CTAGCCTGCCCC	rs2290041
C14717T		Intron 14	75	19	0	94	0.899	0.101	AGGGAGACTCCA[C/T]TCTGCACTCTTC	rs2290040
C15503A	Pro1173Thr	Exon 17	278	1	0	279	0.998	0.002	AAGCAGCCCCCA[C/A]CGGGTATTGTGG	
T15677C		Intron 17	275	2	0	277	0.996	0.004	CTGTCGACTGTT[T/C]TTCTCCAGGCCC	
C15703T		Intron 17	277	1	0	278	0.998	0.002	GGGGGTCTGCCC[C/T]GGGGGAATAGAC	
C15738A		Intron 17	272	4	0	276	0.993	0.007	CACCTCCCCTTT[C/A]CTCACTTAGTGC	

[a]Triallelic polymorphism

2004). The method of direct sequencing was described previously (Okuda et al. 2002). The polymorphisms were identified by use of Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA) and confirmed by visual inspection (Takiuchi et al. 2004). SNPs having a minor allele frequency of greater than 5% were defined as candidates for genotyping using the TaqMan-PCR system (Tanaka et al. 2003). Some SNPs were not suitable for genotyping due to the presence of another SNP in the adjacent region. The representative SNPs were genotyped when they were in linkage disequilibrium (r^2 over 0.5). Since a missense mutation may directly be susceptible to hypertension, five missense SNPs with minor allele frequencies below 5%, including C4527A (Thr180Lys, *TSC*), T29320A (Leu849His, *TSC*), G34372A (Arg904Gln, *TSC*), C142763T (Arg1945Cys, *WNK1*), and C15503A (Pro1173Thr, *WNK4*), were also genotyped.

Statistical analysis

A total of 1,818 subjects who had complete genotype data were recruited for the study. Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis.

Association studies of genotypes with BP were performed through logistic regression analysis considering potential confounding variables in risk factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive drug use by gender. For multivariate risk factors, adjusted odds ratios were given with 95% confidence intervals. The associations of genotypes with hypertension were expressed in terms of odds ratios adjusted for possible confounding effects, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) by gender. All analyses were performed with SAS statistical software (release 8.2, SAS Institute, Inc., Cary, NC, USA). Linkage disequilibrium was calculated by using the SNPAlyze version 2.1 (DYNACOM Co., Ltd, Mobara, Japan).

Results

Basic characteristic of subjects

The characteristics of all 1,818 participants (835 men and 983 women) are shown in Table 1. Age, SBP, DBP, BMI, percentage of current smokers, percentage of current drinkers, and prevalence of individuals with hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, HDL cholesterol, and percentage of individuals with hyperlipidemia were significantly higher in women than in men.

Table 3 Primers and TaqMan probes for genotyping of SNPs in *TSC*, *WNK1*, and *WNK4*

Gene	SNP	Primer	Probe
TSC	*C-1991A*	CCCTGACAGCTCAAATTTCCAC	Fam-CTGCCTCCCTGCAA-MGB
		CTTGTTACCAGAGGTGCCTAAGC	Vic-CTGCCTCACTGCAA-MGB
	C-605T	GCAGAAATGAAATCCACAAGCA	Fam-TTTGAAAATCCCTGTCCTG-MGB
		CATGCACCGATCATTAGATTGG	Vic-CTTTGAAAATTCCTGTCCTG-MGB
	C-213G	GGCAGAACACCATTTGATTGTG	Fam-CTGGCCCAAAGCCAGCCACTC-TAMRA
		GAAGAGCCACTCCAGGACTCA	Vic-CTGGCCCAAACCCAGCCACTC-TAMRA
	C1784T	CGCAGTGGTGCAGGTCAGT	Fam-CAGAGACGCCGTCC-MGB
		AGGTGTCTGCCTTCCTGCTG	Vic-TGCAGAGATGCCGTC-MGB
	A1918G	CTCACCATCACCCCCTTGAC	Fam-CTGGTGCCTGCCTCGCCC-TAMRA
		CAGCAGGAAGGCAGACACCT	Vic-TGGTGCCCGCCTCGCC-TAMRA
	A2141T	GCTTCAGTTTCCCCATCTGTACA	Fam-AATAGATTAAAGCCTGCCGG-MGB
		GGTGGCTTTTTAGGGAAACACA	Vic-AATAGATTAATGCCTGCCGG-MGB
	C4527A	GATGAACGTAGGTCGCATGGT	Fam-TGTCGGTCACGGTGA-MGB
		GATGGCTGAGATGGAGAGGC	Vic-TGTCGGTCAAGGTG-MGB
	T18806C	AGCAGCTCTGGCCTAGAAAGAG	Fam-TGGTGCCCTTGGCCCAGG-TAMRA
		ACGGAGATGATAGCCCCAAAC	Vic-CTGGTGCCCTCGGCCCAG-TAMRA
	T29320A	TCACATAGTGCTCTGTCCTGAGTG	Fam-TCCCTATCTCCTTGGC-MGB
		GATCTTGCATTTGCTCCACCTC	Vic-CCTATCACCTTGGCC-MGB
	C29372T	GCAAGAGGAGGTGGAGCAAAT	Fam-TTCGTAGGCGGCCAG-MGB
		CCCTCCACACTTACGCCTTTC	Vic-TCGTAGGTGGCCAGAT-MGB
	G34372A	GGGATTCCATGAAGTCCACATC	Fam-AACCCTCGGGCTGA-MGB
		CTGGAAGCCCCAAAACAGAAC	Vic-AGAACCCTCAGGCTG-MGB
	C39375T	GAAGCAGAAGGGCCAAAGTTC	Fam-ATAGCCCTGGCGATT-MGB
		GATGCCTGGGACACGTGAG	Vic-TAGCCCTGGTGATTC-MGB
WNK1	*G786A*	GAACTGCAGGTAAAGCCCCAC	Fam-TTTGACGGTCCTTTG-MGB
		GAACTCGATCAACTGGCTTCG	Vic-TTTATTTGACAGTCCTTTG-MGB
	C108560T	CTGATGGGACGGTTGACAGTG	Fam-TCTTCACAGAATCTCGA-MGB
		CCTGTTCATGTTGGGAACCATA	Vic-TCTTCATAGAATCTCG-MGB
	A128177C	GTTGCTCCTGCAGAGCCAGT	Fam-AGTAGCACAGACCCAA-MGB
		TCTACAGAGGAAGCCAAAGTGGT	Vic-AGTAGCACAGCCCCA-MGB
	C133634T	TTGATTTGCTCTTCAGTACGCAG	Fam-AGCGTCTCACGGACT-MGB
		GCACCTACAGACAACAAAGGGAA	Vic-AGCGTCTCATGGACT-MGB
	G135642T	AAAACTTACACCAACCGCAGAAG	Fam-CTGTGATCATCTCTG-MGB
		ATTCAGTCCCAGCAACCTCTAGA	Vic-ACTGTGATAATCTCTG-MGB
	C141114T	TGGGACGATTTCAGGTAAGACAG	Fam-ATTCCTTCCTTTGGAGGA-MGB
		TTGTGTCCCAAATAGGTAGGCA	Vic-ATTCCTTCTTTTGGAGGAG-MGB
	C142763T	ACGACCCACTTTGTTTGCTGTA	Fam-CTGAAAACGTCCAACCT-MGB
		GTCAGACACTGGGCAGCCTAC	Vic-CCTGAAAACATCCAACCT-MGB
WNK4	*C14597T*	CTGGCTGTGATGACTGTGGC	Fam-TCCCCTCCCTAGCCT-MGB
		TGAAGGGCTTTCCTGGCC	Vic-TCCCCTCTCTAGCCTG-MGB
	C14717T	CACAGCTGAGGTGGAGAGTGAG	Fam-CTCCACTCTGCACTC-MGB
		GGAGGTGGTGAGGCCTAGAAA	Vic-ACTCCATTCTGCACTC-MGB

Polymorphisms of *TSC*, *WNK1*, and *WNK4*

We sequenced 96 alleles from 48 patients with hypertension in *TSC* and 192 alleles from 96 patients with hypertension in *WNK1* and *WNK4*, and identified 52, 35, and 21 polymorphisms, respectively (Table 2). There were six, nine, and nine missense mutations in *TSC*, *WNK1* and *WNK4*, respectively. Among them, missense mutations with minor allele frequencies above 5% were 0, 3, and 0, respectively, indicating that most of the missense mutations were rare. We selected SNPs with minor allele frequencies above 5% for genotyping. Five missense SNPs with the minor allele frequency below 5% were also included. We selected representative SNPs for genotyping when some of the SNPs were in linkage disequilibrium. Finally, 12, 7, and 2 SNPs, in a total of 21 SNPs, were selected for genotyping in population-based samples. The primers and probes of the TaqMan-PCR method are summarized in Table 3.

Susceptible SNPs related to hypertension

The results of the case-control study are shown in Table 4. Among 21 SNPs, the C14717T SNP of *WNK4* was significantly associated with hypertension in men ($\chi^2 = 7.53$, $p = 0.023$). SBP in men with the CT + TT genotypes was 3.1 mmHg higher than those with the CC genotype ($p = 0.042$) after adjustment for age, BMI, hyperlipidemia, diabetes mellitus, antihypertensive drug use, smoking, and drinking (Table 5). Multivariate logistic regression analysis with adjustment for age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that the odds ratio for the presence of hypertension for the CT + TT genotypes in C14717T in comparison to the CC genotype in men was 1.62 (95% confidence interval, 1.12–2.33, $p = 0.010$) (Table 6). When the controls were defined as SBP $\leq$ 120 mmHg, DBP $\leq$ 80 mmHg, or nonmedication, and the hypertensives were defined as SBP $\geq$160 mmHg, DBP $\geq$100 mmHg, or current use of antihypertensive

Table 4 Genotype distributions of 21 SNPs of TSC, WNK1, and WNK4 in normotensives and hypertensives. *n.d.* not determined

Gene	SNP	Genotypes	Women ($n=983$)				Men ($n=835$)			
			Normotensive ($n=608$)	Hypertensive ($n=375$)	χ^2	p	Normotensive ($n=439$)	Hypertensive ($n=396$)	χ^2	p
TSC	C-1991A	(CC/CA/AA)	539/67/2	337/37/1	0.359	0.836	392/45/2	359/36/1	0.571	0.752
TSC	C-605T	(CC/CT/TT)	539/67/2	337/37/1	0.359	0.836	392/45/2	359/36/1	0.571	0.752
TSC	C-213G	(CC/CG/GG)	539/67/2	337/37/1	0.359	0.836	392/45/2	359/36/1	0.571	0.752
TSC	C1784T	(CC/CT/TT)	435/161/12	289/81/5	3.754	0.153	320/112/7	293/94/9	0.800	0.670
TSC	A1918G	(AA/AG/GG)	407/175/26	240/118/17	0.900	0.638	283/133/23	253/131/12	2.945	0.229
TSC	A2141T	(AA/AT/TT)	6/114/488	4/67/304	0.131	0.936	2/85/352	3/71/322	0.579	0.749
TSC	C4527A	(CC/CA/AA)	591/17/0	362/13/0	*n.d.*	*n.d.*	427/12/0	382/14/0	*n.d.*	*n.d.*
TSC	T18806C	(TT/TC/CC)	115/285/208	63/181/131	0.703	0.704	57/210/172	50/182/164	0.435	0.804
TSC	T29320A	(TT/TA/AA)	592/16/0	360/15/0	*n.d.*	*n.d.*	428/11/0	391/5/0	*n.d.*	*n.d.*
TSC	C29372T	(CC/CT/TT)	325/242/41	199/143/33	1.475	0.478	36/186/36	213/155/28	1.645	0.439
TSC	G34372A	(GG/GA/AA)	548/59/1	334/40/1	0.362	0.835	387/50/2	347/49/0	*n.d.*	*n.d.*
TSC	C39375T	(CC/CT/TT)	342/222/44	207/146/22	1.057	0.589	231/174/34	189/161/46	4.302	0.116
WNK1	G786A	(GG/GA/AA)	9/133/466	4/93/278	1.356	0.508	7/82/350	7/82/307	0.602	0.740
WNK1	C108560T	(CC/CT/TT)	527/76/5	310/62/3	3.127	0.209	377/60/2	342/52/2	0.061	0.970
WNK1	A128177C	(AA/AC/CC)	9/135/464	4/80/291	0.430	0.807	9/102/328	8/86/302	0.280	0.869
WNK1	C133634T	(CC/CT/TT)	453/143/12	267/101/7	1.449	0.485	335/94/10	296/93/7	0.733	0.693
WNK1	G135642T	(GG/GT/TT)	244/290/74	139/182/54	1.478	0.478	196/187/56	164/182/50	1.040	0.595
WNK1	C141114T	(CC/CT/TT)	361/218/29	219/134/22	0.576	0.750	278/135/26	241/134/21	0.962	0.618
WNK1	C142763T	(CC/CT/TT)	592/16/0	362/13/0	*n.d.*	*n.d.*	421/17/1	389/7/0	*n.d.*	*n.d.*
WNK4	C14597T	(CC/CT/TT)	581/27/0	353/22/0	*n.d.*	*n.d.*	410/29/0	375/21/0	*n.d.*	*n.d.*
WNK4	C14717T	(CC/CT/TT)	466/131/11	303/67/5	2.394	0.302	367/68/4	303/84/9	7.526	0.023

medication, the C14717T polymorphism was significantly associated with hypertension in men (CC vs CT+TT, odds ratio=1.91, 95% confidence interval: 1.02–3.58, $p=0.045$) after adjustment for the confounding factors described above.

Discussion

Three genes, *TSC*, *WNK1*, and *WNK4*, are potentially strong candidates for essential hypertension (Choate et al. 2003; Wilson et al. 2003). To understand whether these genes influence BP, we sequenced these genes and identified a total of 108 SNPs. To evaluate the association of the SNPs with hypertension, we genotyped 21 representative SNPs in a large members of 1,818 individuals and identified that the C14717T polymorphism in intron 14 in *WNK4* was associated with hypertension in men. The TT genotype of this SNP increased SBP by 3.1 mmHg when compared with the CC+CT genotype (Table 5). The association of this SNP with hypertension was observed after multiple adjustments for confounding factors including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication (Table 6). Therefore, we consider that the C14717T polymorphism in intron 14 in *WNK4* was associated with hypertension in our general population.

WNK4 is located on chromosome 17q21.2. Several lines of evidence indicate a region on human chromosome 17q as a gene that influences BP (Baima et al. 1999; Jacob et al. 1991; Levy et al. 2000). A quantitative trait locus of hypertension on the rat chromosome 10, equivalent to human chromosome 17, was identified in spontaneous hypertensive rats (Jacob et al. 1991). This region was reportedly linked with hypertension using hypertensive sib pairs from the United Kingdom and France (Julier et al. 1997) and was confirmed in a study of white American hypertensive sib pairs (Baima et al. 1999). Evidence obtained from the Framingham Heart Study indicated that this region is associated with BP with LOD score of 4.7 (Levy et al. 2000). Thus, these

Table 5 Blood pressure levels on genotype of *WNK4* C14717T polymorphism. Values are means ± SDs; all adjusted for age, body mass index (BMI), antihypertensive drug use, present illness (hyperlipidemia, diabetes mellitus), and lifestyle (smoking and drinking); diastolic blood pressure (DBP) and systolic blood pressure (SBP) are expressed as mmHg

	CC	CT	TT	p	CC+CT	TT	p	CC	CT+TT	p
Men (n)	670	152	13		822	13		670	165	
DBP	79.4±0.4	81.0±0.8	81.6±2.7	0.052	79.7±0.3	81.6±2.7	0.481	79.4±0.4	81.1±0.8	0.051
SBP	131.3±0.7	133.8±1.4	140.8±4.8	0.020	131.8±0.6	140.8±4.8	0.062	131.3±0.7	134.4±1.4	0.042
Women (n)	769	198	16		967	16		769	214	
DBP	76.6±0.3	76.6±0.7	76.1±2.3	0.937	76.6±0.3	76.1±2.3	0.817	76.6±0.3	76.6±0.6	0.986
SBP	128.1±0.6	128.1±1.2	124.3±4.2	0.653	128.1±0.5	124.3±4.2	0.358	128.1±0.6	127.8±1.1	0.827

Table 6 Odds ratio for the presence of hypertension for *WNK4* C14717T genotype in a Japanese general population. *OR* odds ratio, *CI* confidence interval

Sex	Genotype	OR[a] (95% CI)	*p*	Genotype	OR[a] (95% CI)	*p*
Men	CC	1 (reference)		CC+CT	1 (reference)	
	CT+TT	1.62 (1.12–2.33)	0.010	TT	3.00(0.88–10.19)	0.079
Women	CC	1 (reference)		CC+CT	1 (reference)	
	CT+TT	0.80(0.56–1.13)	0.209	TT	0.74 (0.22–2.45)	0.621

[a]Conditional logistic analysis, adjusted for age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking

studies suggest that this region may contain a gene susceptible for BP elevation.

The C14717T polymorphism in *WNK4* associated with hypertension was found in the intron. Therefore, it is not likely that it directly affects the function of WNK4, leading to hypertension. The C14717T polymorphism may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. The functional SNP may be present in the 5′-upstream region beyond our sequencing region or in the intron, creating a new splicing site. Further analysis is needed to clarify the function of this SNP. In conclusion, our study has shown the possible involvement of *WNK4* in essential hypertension in the Japanese general population.

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Identification of gene polymorphism in lipocalin-type prostaglandin D synthase and its association with carotid atherosclerosis in Japanese hypertensive patients

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Abstract

Recent reports suggested that lipocalin-type prostaglandin D synthase (L-PGDS) is implicated in atherogenesis. In the present study, we investigated the polymorphism of the L-PGDS gene and examined its relationship with the severity of carotid atherosclerosis which is determined as the maximum intima-media thickness in the common carotid artery (C-IMT$_{max}$). We identified 6 single nucleotide polymorphisms (SNPs) of the L-PGDS gene in Japanese. A rare SNP with an amino acid change (1535C > G in exon 4, Leu79Val) and a common SNP (4111 A > C in 3′-untranslated region) were selected for genotyping in 782 Japanese hypertensive subjects. There was no significant difference among genotypes in 1535C > G, however, in 4111 A > C, serum levels of high-density lipoprotein (HDL) cholesterol were significantly higher in subjects with A/A genotype than those with A/C and C/C genotypes. C-IMT$_{max}$ was significantly smaller in subjects with A/A genotype than those with A/C and C/C. Logistic regression analysis revealed that the presence of A/A genotype significantly reduced the risk for increased C-IMT$_{max}$, even after adjustment for other known risk factors [adjusted odds ratio: 0.71 (95% CI: 0.58–0.88)]. Our results suggested that 4111 A > C polymorphism in the L-PGDS gene contributes to the development of carotid atherosclerosis in Japanese hypertensive patients.

Keywords: Lipocalin-type prostaglandin D synthase; HDL-cholesterol; Intima-media thickness; Carotid artery; Hypertension; Genetic polymorphism; Clinical genetics

Lipocalin-type prostaglandin D synthase (L-PGDS), a secretory protein of the lipocalin superfamily which synthesizes PGD_2 from PGH_2, was first identified in the central nervous system [1]. L-PGDS is abundantly contained in cerebrospinal fluid [2,3], and plays an important role in the regulation of the sleep–wake cycle [4,5] and sensitivity to tactile pain [6].

L-PGDS is also detected in the cardiovascular system. We previously reported that physiological levels of laminar shear stress induce L-PGDS mRNA expression in vascular endothelial cells [7]. Other investigators reported that L-PGDS is expressed in human heart and its secretion increased in the coronary circulation in angina patients [8]. Furthermore, serum levels of L-PGDS have been suggested to indicate the occurrence of restenosis after coronary angioplasty [9]. A recent clinical study reported that L-PGDS concentrations in both serum and urine increase in patients with essential hypertension [10].

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The downstream products of L-PGDS, PGD_2, and its naturally occurring metabolites PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) have also been suggested to work as anti-atherogenic factors. Exogenous PGD_2 suppressed mRNA expression of pro-inflammatory cytokines such as inducible nitric oxide [11] and plasminogen activator inhibitor-1 [12]. Furthermore, 15d-PGJ_2, a potent endogenous ligand for peroxisome proliferator-activated receptor-γ, exerts several anti-inflammatory effects on macrophages such as the inhibition of inducible nitric oxide synthase expression [13], inhibition of inflammatory cytokine production [14], and inhibition of matrix metalloproteinase activity [15]. We also reported that PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 strongly induce G_1 arrest and promote differentiation in vascular smooth muscle cells [16–18]. These findings suggest that the L-PGDS-mediated synthetic pathway of PGD_2/PGJ_2 plays an important role in the development of vascular disease. However, L-PGDS polymorphism and its association with atherosclerosis have not been reported.

Thus, in the present study, by sequencing all exons and a part of the introns including the promoter region, we identified polymorphisms of the L-PGDS gene in Japanese. Furthermore, we also performed high-resolution ultrasonography to determine the maximum score of the intima-media thickness of the carotid artery (C-IMT_{max}) and examined the association between the L-PGDS polymorphisms and carotid atherosclerosis in asymptomatic hypertensive patients.

Methods

Subjects. Between April 2002 and March in 2003, 813 consecutive patients with essential hypertension aged 29-82 years (mean ± SD, 65.6 ± 9.6 years), participating in an annual examination at the outpatients clinic of the Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan, were enrolled in this study. Eight patients with severe hyperlipidemia [total cholesterol ⩾ 7.8 mmol/L (300 mg/dL) or triglyceride ⩾ 4.5 mmol/L (400 mg/dL)], 11 patients with severe diabetes (HbA1c ⩾ 8.0% or under insulin treatment), and 17 patients with severe renal insufficiency [serum creatinine ⩾ 265 μmol/L (3.0 mg/dL)] were excluded. Thus, a total of 782 subjects were studied. All patients were treated with anti-hypertensive agents [angiotensin II receptor blockers (ARBs), angiotensin-converting enzyme inhibitors (ACEIs), calcium channel blockers, β-adrenergic receptor blockers, α-adrenergic receptor blockers, and diuretics]. Hypertension was defined as either SBP ⩾ 140 mm Hg, DBP ⩾ 90 mm Hg, or current use of antihypertensive agents. Hyperlipidemia was defined as total cholesterol ⩾ 5.7 mmol/L (220 mg/dL) and/or triglyceride ⩾ 1.7 mmol/L (150 mg/dL). Diabetes mellitus was diagnosed as fasting blood glucose ⩾ 7.0 mmol/L (126 mg/dL) or current use of insulin or oral antidiabetic agents. Written informed consent was obtained from all patients. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Clinical parameters. At the time of the physical examination, blood pressure, body mass index (BMI), and a hematological and biochemical profile were determined. The measurements were performed in the morning after an overnight fast. Information on age and smoking status were obtained through questionnaire and interview. Total cholesterol, HDL-cholesterol, and triglyceride levels were enzymatically determined using an autoanalyzer. LDL-cholesterol was estimated using Friedewald's formula. Fasting plasma glucose and HbA1c were determined by standard laboratory methods. High-sensitivity C-reactive protein (hs-CRP) was measured using an automatic immunonephelometer with a sensitivity of 0.2 mg/L.

Screening of mutations in the L-PGDS gene. Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan) and stored at −80 °C prior to use. We first sequenced the 48 samples from healthy individuals (we also obtained written informed consent from them). All exons and a part of the introns in the L-PGDS gene were amplified by the polymerase chain reaction (PCR) and sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Primer sequences used for PCR and sequencing are available on request. The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes Corporation, MI), followed by visual inspection. We identified 6 polymorphisms of the L-PGDS gene as indicated in Table 1. The polymorphisms were named according to the recommendation by the Nomenclature Working Group for human gene mutations [19].

Genotyping of polymorphisms. The polymorphisms were genotyped using the TaqMan-PCR system as described previously [20]. Among the 6 SNPs identified, 1 SNP having a minor allele frequency of greater than 10% (4111A > C) and 1 SNP having an amino acid change (1535C > G, Leu79Val) were selected for genotyping. The sequences of the allele-specific probes and PCR primers used for the genotyping are available on request.

Carotid artery ultrasonography. Ultrasonography of bilateral carotid arteries was performed with a high-resolution Duplex scanner (SSA-390A, Toshiba Medical, Japan) using a probe with a frequency of 7.5 MHz for the B-mode scan. The carotid arteries were examined in the supine position with regard to wall changes from various longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views. Each ultrasound image was recorded on a computer with an online digital filing system, and the intima-media complex thickness (IMT) and atherosclerotic plaques were measured off-line and analyzed. The measurement was performed by two independent sonographers blinded from the clinical data. The maximum IMT of the

Table 1
Identified polymorphisms in the human L-PGDS gene

Polymorphisms (allele frequency)	Region	Amino acid change	Change sequence	dbSNP ID
−18C > T (99:1)	Exon1 (5′-UTR)		ggccccggacac[c/t]cgctctgctgca	—
1535C > G (99:1)	Exon2 (ORF)	Leu79Val	ggtggcctcaac[c/g]tgacctccacct	—
2326G > T (99:1)	Intron 3		gaggtgaggttt[g/t]gggggctgagtc	—
3141G > A (99:1)	Intron 5		gggatctgtgca[g/a]tttgggggctca	—
3215C > T (99:1)	Intron 5		attggcctaagt[c/t]tggggttctgac	—
4111A > C (21:79)	Exon 7 (3′-UTR)		ccgccaaagca[a/c]ccctgcccactcc	rs6926

UTR, untranslated region; ORF, open reading frame.

carotid artery (C-IMT$_{max}$) was determined as the maximum IMT including plaques in bilateral carotid arteries. The intra-observer and inter-observer coefficients of variation using 50 subjects were 4.6% and 4.3%, respectively.

Statistical analysis. Values are represented as means ± SD. All statistical analyses were performed using the JMP statistical software package (SAS institute, Gary, NC). Hardy–Weinberg equilibrium was assessed by χ^2 analysis. Differences in variables between the genotypes were assessed with unpaired Student's *t* test. Predictive variables including L-PGDS genotype for increased C-IMT$_{max}$ (C-IMT$_{max}$ ⩾ 1.3 mm) were analyzed by logistic regression analysis. A value of $P < 0.05$ was considered statistically significant.

Results

We systematically sequenced the L-PGDS gene (9q34.2–q34.3) in 48 healthy volunteer subjects and identified 6 SNPs (Table 1). Only 4111A > C had been recorded in public databases (dbSNPs, http://www.ncbi.nlm.nih.gov/SNP/), the remaining 5 polymorphisms were novel. Among them, we genotyped a common SNP, 4111A > C, and a rare SNP with an amino acid change (Leu79Val), 1535C > G, using the TaqMan method in 782 hypertensive patients (Table 2). The genotype distribution of both SNPs did not significantly deviate from the Hardy–Weinberg expectation.

For 1535 C > G, no individual was found to be homozygote (G/G genotype) and furthermore, there were no significant differences in phenotypic variables between the C/C and C/G genotypes (data not shown). On the other hand, the subjects with the A/A genotype of 4111A > C had significantly greater levels of HDL-cholesterol than those with the C allele (A/C + C/C), although there was no difference in other variables (Table 2).

In a simple regression analysis, C-IMT$_{max}$ was positively correlated with age ($r = 0.302$, $P < 0.001$), sex ($r = 0.151$, $P < 0.001$), duration of hypertension ($r = 0.182$, $P < 0.001$), triglyceride ($r = 0.108$, $P = 0.003$), HbA1c ($r = 0.155$, $P < 0.001$), and serum creatinine ($r = 0.120$, $P < .001$), and inversely correlated with diastolic blood pressure ($r = -0.098$, $P = 0.009$) and HDL-cholesterol ($r = -0.121$, $P < 0.001$). No association was found between 1535C > G genotypes and C-IMT$_{max}$ or other variables (data not shown). However, the C-IMT$_{max}$ was significantly smaller in subjects with the A/A genotype of 4111A > C (0.88 ± 0.31 mm) than subjects with the C allele (A/C + C/C) (0.77 ± 0.19 mm) (Fig. 1).

In a multiple logistic regression analysis including age, sex, body mass index, duration of hypertension, blood pressure, HDL-cholesterol, LDL-cholesterol, HbA1c, serum creatinine, and treatment with ACEIs and/or ARB, the high tertile of C-IMT$_{max}$ (⩾1.3 mm) was positively associated with age, sex, duration of hypertension, and systolic blood pressure,

Table 2
Clinical characteristics of the patients classified by 4111 A > C genotype

	A/A	A/C + C/C	*P* value
No.	22	760	
Age (year)	64.2 ± 10.3	65.4 ± 10.6	0.595
Male (%)	45.5	54.7	0.389
BMI (kg/m^2)	23.9 ± 3.2	23.9 ± 4.4	0.954
Current smoking (%)	3.9	16.7	0.802
Duration of hypertension (year)	17.5 ± 10.0	18.0 ± 11.0	0.812
Systolic blood pressure (mm Hg)	136.8 ± 20.5	140.6 ± 17.8	0.366
Diastolic blood pressure (mm Hg)	84.6 ± 10.4	83.3 ± 10.9	0.597
Heart rates (beats/min)	61.4 ± 10.1	63.7 ± 10.7	0.336
Total cholesterol (mmol/L)	5.44 ± 0.51	5.19 ± 0.80	0.137
HDL cholesterol (mmol/L)	1.54 ± 0.50	1.34 ± 0.39	0.047
LDL cholesterol (mmol/L)	3.25 ± 0.56	3.19 ± 0.74	0.677
Triglycerides (mmol/L)	1.48 ± 0.67	1.44 ± 0.72	0.788
Fasting plasma glucose (mmol/L)	5.71 ± 0.86	5.71 ± 1.02	0.905
Serum creatinine (μmol/L)	74.2 ± 68.5	96.1 ± 117.2	0.386
hs-CRP (mg/L)	2.0 ± 3.1	2.1 ± 13.4	0.956
Hyperlipidemia (%)	59.1	47.0	0.262
Diabetes mellitus (%)	18.2	13.9	0.574
Renal insufficiency (%)	9.1	12.8	0.610
Anti-hypertensive agents (%)			
ARBs and/or ACEIs	45.5	50.4	0.648
CCBs	68.2	70.8	0.791
β-Blockers	31.8	36.1	0.684
α-Blockers	9.1	14.2	0.497
Diuretics	27.3	23.9	0.714

Values are represented as means ± SD or frequencies. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; ARBs, angiotensin II receptor blockers; ACEIs, angiotensin-converting enzyme inhibitors; and CCBs, calcium channel blockers.