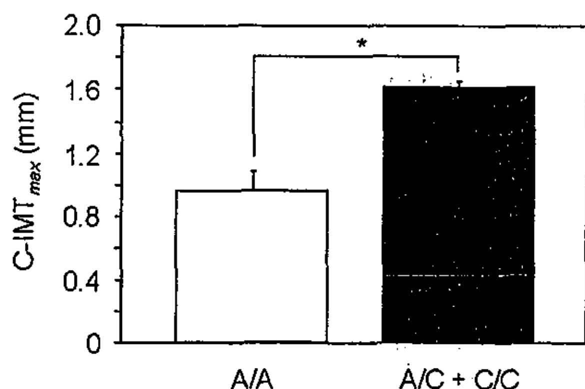

Fig. 1. Age adjusted values of $C\text{-}IMT_{max}$ in subjects without (A/A, open bar) or with (A/C + C/C, closed bar) the C allele. Error bars represent SE. $^*P < 0.05$.

Table 3
Multiple logistic regression analyses of factors affecting the high tertile of $C\text{-}IMT_{max}$ (⩾1.3 mm)

Risk factors	Adjusted OR (95% CI)	P value
Age	1.73 (1.41–2.13)	<0.001
Sex (male: 1, female: 0)	1.27 (1.06–1.54)	0.011
Body mass index	1.00 (0.84–1.20)	0.952
Duration of hypertension	1.29 (1.06–1.56)	0.011
Systolic blood pressure	1.41 (1.10–1.80)	0.008
Diastolic blood pressure	0.66 (0.50–0.87)	0.003
HDL-cholesterol	0.93 (0.77–1.11)	0.432
LDL-cholesterol	1.09 (0.92–1.29)	0.285
HbA1c	1.11 (0.91–1.34)	0.298
Serum creatinine	1.24 (0.97–1.58)	0.085
Treatment with ACEIs and/or ARB (Yes: 1, No: 0)	0.99 (0.75–1.32)	0.966
A/A genotype	0.71 (0.58–0.88)	0.002

OR, odds ratio; 95% CI, 95% confidential interval.

and inversely associated with diastolic blood pressure (Table 3). The presence of the A/A genotype independently reduced the risk of the increase in $C\text{-}IMT_{max}$, for an adjusted odds ratio of 0.71 (95% CI: 0.58–0.88).

Discussion

In the present study, we identified 6 SNPs including 1 missense SNP [1535C > G (Leu79Val)] and 1 common SNP (4111A > C) in the L-PGDS gene in Japanese. In middle-aged Japanese hypertensive patients, 1535C > G showed no correlation with the studied phenotypes. However, 4111A > C was associated with the serum levels of HDL cholesterol and $C\text{-}IMT_{max}$. The A/A genotype of 4111A > C seemed to be a possible protective factor against the increase in $C\text{-}IMT_{max}$.

To our knowledge, a polymorphism of the L-PGDS gene has never been reported. However, some investigators suggested regulatory mechanisms of enzymatic activity and gene expression of L-PGDS. Urade et al. [1] suggested that the Cys-65 residue conserved only in the human and rat enzymes but not in other species is a putative active center of this enzyme. White et al. [21] reported that thyroid hormone (T3) stimulated L-PGDS promoter activity through the thyroid hormone response element at −2576 to −2562. Fujimori et al. [22] recently reported that two NF-κB consensus elements at −1106 and −291 in the rat L-PGDS promoter were essential for the up-regulation of L-PGDS gene expression induced by interleukin-1β. Otsuki et al. [23] reported that estrogen receptor β stimulates L-PGDS promoter activity through estrogen response elements in mice. Although these sites which influence the activity and expression level are well preserved in the human L-PGDS gene, no mutation was found in the present study. In addition, we found a rare missense mutation, 1535C > G (Leu79Val), located very close to the N-glycosylation site (Asn-78) [1]. However, there was no association with the phenotypes related to atherosclerosis, such as BMI, blood pressure, lipid levels, fasting plasma glucose, and carotid atherosclerosis.

In contrast, a common mutation 4111A > C in 3′-UTR was significantly associated with the HDL-cholesterol level and severity of carotid atherosclerosis. L-PGDS belongs to the lipocalin superfamily, a group of proteins that bind and transport small lipophilic molecules. The association between the 4111A > C variant and HDL-cholesterol level found in the present study suggested that L-PGDS plays a role in lipid transport. Polymorphisms of apolipoprotein A-I [24], lipoprotein lipase [25], ATP-binding cassette transporter [26], cholesteryl ester transfer protein [27], and paraoxonase [28], all of which influence HDL-cholesterol level, have been reported to correlate with atherosclerotic diseases and therefore, mutation of the L-PGDS gene could modulate the HDL-cholesterol level resulting in the association with carotid atherosclerosis. Previous studies reported that 3′-UTR plays an important role in gene expression through the regulation of mRNA stabilization [29]. Many labile mRNAs coding for oncogenes, including c-myc and cytokines, have an AU-rich region (AUUUA repeats) in the 3′-UTR. Several investigators have identified proteins that bind to AU-rich elements and regulate mRNA stability, such as ELAV-like protein HuR, AUF1, and heat shock proteins [30–32]. However, we found no AU-rich region in the 3′-UTR of the L-PGDS gene. At this stage, we are not able to conclude whether this variant directly affects the HDL-cholesterol levels and carotid atherosclerosis. More detailed studies are required to clarify the functional mechanism of 4111A/C mutation.

Our study has several limitations. First, we sequenced all coding exons and part of the introns including the promoter region, however, it is unclear whether functional mutations exist 5′-upstream far beyond the sequenced region or in unsequenced introns that may

create a new splice site. Second, all patients were treated with anti-hypertensive agents. Several anti-hypertensive agents have been reported to suppress vascular remodeling besides BP-lowering effects, especially in ARBs [33] and ACEIs [34]. In our subjects, however, there was no significant difference among genotypes in the ratio of used anti-hypertensive agents (Table 2). Furthermore, a multiple logistic regression analysis including the treatment with ARBs and/or ACEIs also showed an independent association between the A/A genotype of 4111A > C and decreased C-IMT^{max}. Therefore, the atheroprotective effects of ARBs and ACEIs may not have influenced our results.

In conclusion, we identified six L-PGDS gene polymorphisms in Japanese and found that the frequent 4111A > C variant in the 3′-UTR is associated with increased serum levels of HDL cholesterol and the severity of carotid atherosclerosis. Although the functional mechanism of this mutation is unclear, our findings suggest the importance of common genetic variation in L-PGDS in determining the severity of carotid atherosclerosis.

Acknowledgments

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan. We are grateful to Yoko Tokunaga and Chiyako Imai for their excellent technical assistance.

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Original Article

Association of Genetic Polymorphisms of Sodium-Calcium Exchanger 1 Gene, *NCX1*, with Hypertension in a Japanese General Population

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The Na^+/Ca^{2+} exchanger (NCX) is a membrane protein involved in calcium homeostasis, catalyzing the exchange of one Ca^{2+} ion for three Na^+ ions across the cell membrane. The Na^+/Ca^{2+} exchange has been suggested to play a role in the pathogenesis of hypertension. Therefore, we examined whether genetic variations in *NCX1* were associated with hypertension. Among 15 polymorphisms identified in 96 hypertensive subjects by sequencing the entire exon and promoter regions of *NCX1*, 7 representative polymorphisms with a minor allele frequency of greater than 4% were genotyped in 1,865 individuals, of whom 787 were hypertensive and 1,072 were normotensive. These subjects were residents of Suita City and were randomly selected as a population for the Suita cohort study. Multivariate logistic regression analysis performed after adjusting for age, body mass index, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that the −23200T>C and −23181T>C polymorphisms in the 5′ upstream region of exon 1c were significantly associated with hypertension in men (−23200T>C: CC *vs.* TC+TT: odds ratio=0.61; 95% confidence intervals: 0.39 to 0.97; p=0.04) and in women (−23181T>C: CC *vs.* TC+TT: odds ratio=1.45; 95% confidence intervals: 1.04 to 2.02; p=0.03), respectively. Thus, our study suggests that *NCX1* is one of the genes related to susceptibility to essential hypertension in the Japanese general population.
(*Hypertens Res* 2004; 27: 697–702)

***Key Words*: NCX1, Na^+/Ca^{2+} exchanger, gene variants, hypertension**

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is an important membrane protein involved in calcium homeostasis in various cell types and catalyzes the electrogenic exchange of one Ca^{2+} ion for three Na^+ ions across the plasma membrane (*1–3*). The Na^+/Ca^{2+} exchange has been well demonstrated to play a role in the pathogenesis of hypertension. Blaustein *et al.* suggested that excessive Na^+ retention may secrete an ouabain-like substance that increases the cytosolic Na^+ concentration by inhibiting the plasmalemmal Na^+-pump, which increases the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by reducing Ca^{2+}-extrusion *via* Na^+/Ca^{2+} exchange (*4–6*). The increase in arteri-

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This study was supported by the Program for Promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan MPJ-3.
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Received March 2, 2004; Accepted in revised form June 4, 2004.

Table 1. Basic Characteristics of Subjects in Suita, a Japanese Urban Population, 2002

	Men (n=858)	Women (n=1,007)
Age (year)	66.3±11.1*	63.3±11.0*
Systolic blood pressure (mmHg)	131.9±19.5*	128.0±19.6*
Diastolic blood pressure (mmHg)	79.7±10.7*	76.6±10.7*
Body mass index (kg/m^2)	23.3±3.0*	22.3±3.2*
Total cholesterol (mmol/l)	5.10±0.78	5.57±0.79*
HDL-cholesterol (mmol/l)	1.42±0.36	1.67±0.40*
Current smokers (%)	30.1†	6.3†
Current drinkers (%)	67.0†	29.3†
Present illness (%)		
Hypertension	47.4†	38.2
Hyperlipidemia	27.4	55.2†
Diabetes mellitus	12.6†	5.2

Values are mean±SD or percentage. Hypertension indicates systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥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 non-fasting plasma glucose ≥11.1 mmol/l (200 mg/dl) or HbA1c ≥6.5% or antidiabetic medication. *p<0.05 between women and men by Student's t-test. † p<0.05 between women and men by χ^2 test. HDL, high-density lipoprotein.

al tone caused by high $[Ca^{2+}]_i$ would thus result in an elevation of blood pressure. Indeed, several previous studies have reported that Na^+/Ca^{2+} exchange activity was altered in the renal arterioles or arterial smooth muscle of spontaneous or salt-sensitive hypertensive rats (*7–11*). However, it is unknown whether such a mechanism relates to the occurrence of essential hypertension.

Of three isoforms (NCX1–3) derived from different genes, NCX1 is predominantly expressed in the heart, neurons and renal tubules, but is expressed at lower levels in other tissues, including the smooth muscle, skeletal muscle, lung and spleen (*1–3*). The *NCX1* gene (*SLC8A1*) is located on human chromosome 2p22.1 and includes 12 exons (*12*). There are at least 12 splice variants generated in different combinations from six exons in a tissue-specific manner (*13*). In addition, five exons encode 5′-untranslated sequences that are under the control of three tissue-specific promoters (*14–17*).

This study was undertaken to identify genetic variations in *NCX1* in a group of hypertensive subjects, and to examine the association of these variations with the presence of hypertension in a general population. In contrast to other association studies, which often focus on a limited number of polymorphisms in a gene, our study evaluated the full array of coding- and promoter-sequence polymorphisms in *NCX1*.

Methods

Subjects of the Suita Population Study

The subjects of the Suita study consisted of 14,200 men and women (30 to 79 years of age), who had been randomly selected from the municipal population registry and stratified by in consideration of gender and age (stratified in 10-year intervals). They were all invited, by letter, to receive medical and behavioral examinations 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 May 2002 and February 2003. All of the participants were Japanese. Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. In this study, the genotypes of 1,865 samples were determined. The characteristics of 1,865 participants (858 men, 1,007 women) are shown in Table 1. Routine blood examinations that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and glucose levels were performed. A physician or nurse interviewed each patient in regard to smoking and drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke.

Blood pressure was measured in a sitting position after at least 10 min of rest. Systolic and diastolic blood pressures (SBP/DBP) were taken as the means of two measurements recorded more than 3 min apart by well-trained doctors. Hypertension was defined as SBP of ≥140 mmHg, DBP of ≥90 mmHg, or the current use of antihypertensive medication (*18*). Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/l (126 mg/dl), non-fasting plasma glucose ≥11.1 mmol/l (200 mg/dl), current use of antidiabetic medication, or HbA1c ≥6.5%. Hyperlipidemia was defined as total cholesterol ≥5.68 mmol/l (220 mg/dl) or current use of antihyperlipidemia medication. Body mass index (BMI)

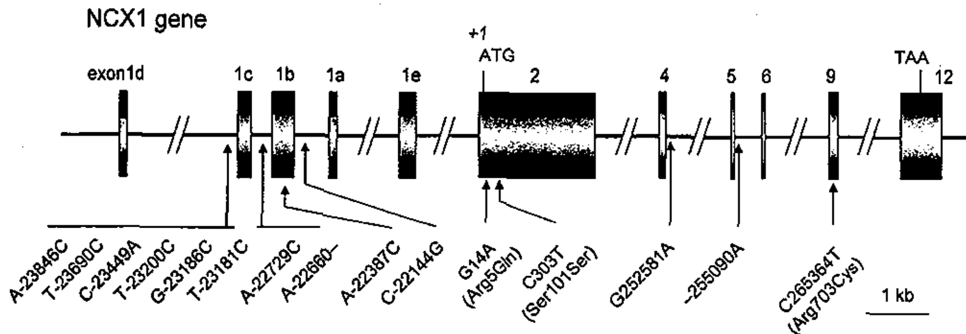

Fig. 1. *Genome structure of human* NCX1. *The* NCX1 *gene consists of sixteen exons, five (exons 1a–1e) of which direct tissue-specific transcription and eleven (exons 2–12) of which encode the open reading frame (17). The five tissue-specific transcription exons (exons 1a–1e) and the exons in which the SNPs were identified are depicted. The nucleotide changes and amino acid substitutions are also shown. The A of the ATG of the initiator Met codon is denoted nucleotide +1.*

was calculated as weight (in kg) divided by height (in m) squared.

Direct Sequencing for Single Nucleotide Polymorphism (SNP) Discovery and Genotyping of Polymorphisms

For DNA sequencing, 96 patients with essential hypertension were recruited from the Division of Hypertension and Nephrology, National Cardiovascular Center, Japan. The method of direct sequencing was described previously (*19*). Fifteen polymorphisms were identified by sequencing and 7 representative polymorphisms with a minor allele frequency of greater than 4% were genotyped by the TaqMan-polymerase chain reaction (PCR) system (*20*). Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Statistical Analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis.

Logistic regression analyses were used to examine the association between the genotypes and blood pressure in each sex with consideration for potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with 95% confidence intervals. The relationship between genotype and risk of hypertension was expressed in terms of the odds ratios adjusted for possible confounding effects including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SAS statistical software (release 8.2; SAS Institute, Cary, USA) was used for statistical analyses (*21*).

Results

Basic Characteristics of Subjects in the Suita Study

The characteristics of the 1,865 participants (858 men, 1,007 women) are summarized in Table 1. Age, SBP, DBP, BMI, percentage of current smokers, percentage of current drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, HDL-cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men.

Polymorphisms of *NCX1*

The *NCX1* gene has a complicated genome structure containing five alternative 5′ exons producing separate tissue-specific promoters and six exons encoding open reading frames (Fig. 1). We sequenced the entire exon and promoter regions of *NCX1* from 96 patients (182 alleles) with hypertension, and identified 15 polymorphisms (Table 2, Fig. 1). We identified two missense mutations, Arg5Gln in exon 2 and Arg703Cys in exon 9, in *NCX1* (Table 2). Each of the missense mutations was identified in one out of 96 individuals, indicating that their allele frequencies were rare. Two SNPs, −23200T>C and −23186G>C, were in linkage disequilibrium. Seven representative polymorphisms with a minor allele frequency of greater than 4% were genotyped for the association study.

Susceptible SNPs Related to Hypertension

Seven polymorphisms in *NCX1* were genotyped in 1,865 individuals, of whom 787 were hypertensive and 1,072 were normotensive. The primers and probes of the TagMan-PCR system and the genotyping results are summarized in Table

Table 2. List of 15 Polymorphisms and Their Allele Frequencies in the *NCX1* Gene Identified by Direct Sequencing

Allele 1/Allele 2	TaqMan	Amino acid	Region	Allele 1	Hetero	Allele 2	Total	Allele frequency		Flanking sequence
SNPs	typing	change		Homo		Homo		Allele 1	Allele 2	
−23846A>C			intron 1d	94	1	0	95	0.995	0.005	tcacactgcctt[a/c]aattcagggact
−23690T>C	typing		intron 1d	62	31	2	95	0.816	0.184	aaatttaactta[t/c]agcaaggaaaga
−23449C>A	typing		intron 1d	85	9	1	95	0.942	0.058	catactcacatt[c/a]atgtttgaggag
−23200T>C*	typing		intron 1d	0	9	86	95	0.047	0.953	attccgccccct[t/c]tttgttgcggag
−23186G>C*			intron 1d	0	9	86	95	0.047	0.953	ttgttgcggagg[g/c]aaactgaggttc
−23181T>C	typing		intron 1d	18	57	20	95	0.489	0.511	gcggaggcaaac[t/c]gaggttcctgga
−22729A>C	typing		intron 1c	71	23	1	95	0.868	0.132	taattatgagga[a/c]agtgatttattg
−22660delA			intron 1c	94	1	0	95	0.995	0.005	gattgctgcatt[a/−]ggtttttccca
−22387A>C		5′ UTR	exon 1b	93	3	0	96	0.984	0.016	attaaaaaaaaa[a/c]tcattgatatat
−22144C>G	typing		intron 1b	84	9	2	95	0.932	0.068	gcgcggccacaa[c/g]gcactgcggggc
14G>A		Arg5Gln	exon 2	95	1	0	96	0.995	0.005	tgtacaacatgc[g/a]gcgattaagtct
303C>T		Ser101Ser	exon 2	95	1	0	96	0.995	0.005	tcggttcatgtc[c/t]tctatagaagtc
252581G>A	typing		intron 4	45	40	11	96	0.677	0.323	tcttctctctcc[g/a]tgtctccctact
255089–255090insA			intron 5	94	1	0	95	0.995	0.005	tcaggtgataca[−/a]gtagctctgtga
265364C>T		Arg703Cys	exon 9	95	1	0	96	0.995	0.005	gcagaaatgggg[c/t]gccccatcctgg

The A of the ATG of the initiator Met codon is denoted nucleotide +1. * The apparent linkage disequilibrium ($r^2 \geq 0.5$). NCX1, Na^+/Ca^{2+} exchanger; SNP, single nucleotide polymorphism.

3. Multivariate logistic regression analysis after adjusting for confounding risk variables such as age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking, revealed that two polymorphisms, −23200T>C and −23181T>C, in the 5′ upstream region of exon 1c were significantly associated with hypertension in men (−23200T>C: CC *vs.* TC+TT: odds ratio=0.61; 95% confidence interval: 0.39 to 0.97; $p=0.04$) and in women (−23181T>C: CC *vs.* TC+TT: odds ratio=1.45; 95% confidence interval: 1.04 to 2.02; $p=0.03$), respectively (Table 4). When normotension was defined as SBP ≤120 mmHg, DBP ≤80 mmHg, and the absence of anti-hypertensive medication, and hypertension was defined as SBP ≥160 mmHg, DBP ≥100 mmHg, or the current use of antihypertensive medication, −23200T>C polymorphism was significantly associated with hypertension in men (CC *vs.* TC+TT: odds ratio=0.42; 95% confidence interval: 0.20 to 0.92; $p=0.03$) after adjusting for the confounding factors described above.

Discussion

In this study, we sequenced the exon and promoter regions of *NCX1* and identified 15 polymorphisms. Seven representative polymorphisms were genotyped from 1,865 subjects to examine the association of hypertension with *NCX1*. After adjustment for various confounding factors, we identified that the −23200T>C polymorphism in the 5′ upstream region of exon 1c was significantly associated with hypertension in men and the −23181T>C polymorphism in the 5′ upstream region of exon 1c was significantly associated with hypertension in women.

The *NCX1* gene has at least 12 splice variants generated in different combinations from six exons in a tissue-specific manner (*13*). In addition, three exons encode 5′-untranslated sequences that are under the control of three tissue-specific promoters (*14–16*). Exon 1c is a part of the "heart" specific transcript (*17*) and its upstream region is not likely a promoter. Therefore, the −23200T>C and −23181T>C polymorphisms present in the upstream region of exon 1c are not likely to be directly involved in transcription of *NCX1*. Rather, these polymorphisms may be in linkage disequilibrium with other polymorphisms in the region that were not examined by sequencing in this study.

In this study, the −23200T>C polymorphism in men and −23181T>C polymorphism in women were identified as SNPs conferring susceptibility for hypertension. It is well known that the greater incidence of hypertension and coronary artery disease in men is, in part, related to gender differences in possible vascular protective effects of the female sex hormones estrogen and progesterone. Furthermore, NCX1 might be related to salt-sensitive hypertension (*22*). Since there is a gender difference in salt-sensitivity and plasma renin activity (*23*, *24*), −23200T>C and −23181T>C in *NCX1* may be linked with unidentified causative genetic variations that would be influenced by the female sex hormones and/or salt-sensitivity.

In this study, we identified two missense mutations, Arg5Gln in exon 2 and Arg703Cys in exon 9, in *NCX1*. Arg5 is located within the signal peptide sequence consisting of the first N-terminal 35 amino acids of NCX1, which are removed during biosynthesis (*1*). We expressed a mutant canine NCX1 with the Arg5Gln substitution in the fibroblastic

Table 3. Genotyping Conditions and Results of *NCX1* Polymorphisms in 1,818 Individuals by TaqMan-PCR Method

SNP	Primer	Probe	Genotypes results
−23690T>C	CTCTCCCCACAGGTCATTCTG	Fam-ATTTAACTTATAGCAAGGAA-MGB	(TT/TC/CC)
	GCAGGAATCGTTCTTGCCTAA	Vic-TTAACTTACAGCAAGGAA-MGB	=(1,140/590/88)
−23449C>A	GAATCTGCAATCCCCATGTGAT	Fam-CTCACATTCATGTTTGAG-MGB	(CC/CA/AA)
	AGAACCACTGCTCTAGGCCAAT	Vic-ACTCACATTAATGTTTGAGG-MGB	=(1,542/261/15)
−23200T>C	TTCTGAGGTGCAAGGAGGGTT	Fam-CCCCCTTTTTGTTGC-MGB	(TT/TC/CC)
	GGCAGTCACCACGACTGATAGA	Vic-CCCCCTCTTTGTTG-MGB	=(4/196/1,618)
−23181T>C	GGCAGTCACCACGACTGATAGA	Fam-TCCAGGAACCTCAGTTT-MGB	(TT/TC/CC)
	AGGCTATTTCTTCCATTCCGC	Vic-CCAGGAACCTCGGTTT-MGB	=(503/869/446)
−22729A>C	GCCTGGTGCAGTGTTCCTTTA	Fam-ATTATGAGGAAAGTGATTTA-MGB	(AA/AC/CC)
	GCCCTTTCCAAGAGAAGCATTA	Vic-TATGAGGACAGTGATTTA-MGB	=(1,369/406/43)
−22144C>G	AAAAGAAAAGTTGCAGCGCCT	Fam-CCACAACGCACTGC-MGB	(CC/CG/GG)
	TTTTTCGATTTCCTGCCGG	Vic-CACAAGGCACTGCG-MGB	=(1,687/131/0)
252581G>A	AAACAAAGACATACCAGCGAGAAA	Fam-CTCTCTCCGTGTCTC-MGB	(GG/GA/AA)
	AAATTGCTAAAGCTTCAAAGGCA	Vic-TCTCTCCATGTCTCC-MGB	=(823/798/197)

PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Table 4. Odds Ratio of −23200T>C Polymorphism in Men and −23181T>C Polymorphism in Women*

Gender	SNP	OR (95% CI)		*p*	OR (95% CI)		*p*
Men	−23200T>C	CC	1 (reference)		CC+TC	1 (reference)	
		TC+TT	0.61 (0.39–0.97)	0.04	TT	—	—
Women	−23181T>C	CC	1 (reference)		CC+TC	1 (reference)	
		TC+TT	1.45 (1.04–2.02)	0.03	TT	1.39 (1.00–1.92)	0.05

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

cell line CCL39, and found that this mutant NCX1 was properly targeted into the plasma membrane and exhibited the normal Na^+/Ca^{2+} exchange activity (unpublished observations), consistent with previous reports stating that signal sequence is not essential for functional expression of the NCX1 protein (*25, 26*). On the other hand, Arg703 is located within the large cytoplasmic loop connecting the transmembrane segments 5 and 6, which are not essential for the functional expression of the NCX1 protein (*1*). Thus, the two rare mutations identified in this study would not grossly impair the function of NCX1.

In summary, we showed that the SNPs −23200T>C and −23181T>C in *NCX1* were associated with hypertension. The pathophysiological functional behaviors of these polymorphisms remain to be clarified. In future studies, it will be necessary to clarify the function of these polymorphisms or to identify the causative polymorphisms that are in linkage disequilibrium with these polymorphisms.

Acknowledgements

We would like to express our highest gratitude to Dr. Soichiro Kitamura, President of the National Cardiovascular Center, for his support of the Millennium genome project (MPJ-3). We would also like to thank Dr. Katsuyuki Kawanishi, Dr. Toshifumi Mannami, and Mr. Tadashi Fujikawa for their continuous support of our population survey in Suita City. We also thank the members of the Satsuki-Junyukai. We also thank all the staff in the Division of Preventive Cardiology for supporting medical examination and M. Banno, Y. Tokunaga, and C. Imai for their technical assistance. Finally, we are grateful to Dr. Tetsuji Yokoyama for his statistical advice.

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Original Article

The Thiazide-Sensitive Na^{+}-Cl^{-} Cotransporter Gene, *C1784T*, and Adrenergic Receptor-β3 Gene, *T727C*, May Be Gene Polymorphisms Susceptible to the Antihypertensive Effect of Thiazide Diuretics

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The response of blood pressure to thiazide diuretics (TZDs) differs among individuals. The prediction of the antihypertensive effect of TZDs is important for realizing individualized therapy in the management of hypertension. The aim of this study was to identify the single nucleotide polymorphisms (SNPs) susceptible to the antihypertensive effect of TZDs, particularly focusing on genes related to water-electrolyte absorption in the kidney. Seventy-six outpatients (mean age, 65.4±9.0 years) with essential hypertension (EHT) taking TZDs were retrospectively assessed. We defined as responders (R) those whose mean blood pressure was lowered by more than 5 mmHg after the use of TZDs. Forty-eight SNPs in 17 genes (*ADD1, GNB3, TSC* [*SLC12A3*], *MLR* [*NR3C2*], *NCX1* [*SLC8A1*], *WNK1, WNK4, AGT, ACE, AT1* [*AGTR1*], *CYP11B2, ADRB1, ADRB2, ADRB3, ADRA1A, ADRA1B, ADRA2A*) were genotyped in the 76 patients. The SNPs in *TSC, MLR, NCX1, WNK1*, and *WNK4* were identified by direct sequencing and those with minor frequencies of greater than 5% were genotyped in this study. The comparison of polymorphism prevalence between R and non-responders (NR) showed significant differences in *TSC C1784T* (*C* allele *vs. T* allele, odds ratio (OR)=3.81, *p*=0.016, confidence interval (CI): 1.25–11.63) and *ADRB3 T727C* (*Trp64Arg*) (*T* allele *vs. C* allele, OR=4.59, *p*=0.005, CI: 1.54–13.68). The blood pressure (BP) in patients homozygous for the major alleles of both *TSC C1784T* and *ADRB3 T727C* were significantly reduced by TZD treatment; however, the BP in those homozygous for the minor allele and heterozygous (*TSC C1784T*: TT+CT; *ADRB3 T727C*: CC+CT) for both SNPs were not significantly changed after TZD treatment. Both newly detected *TSC C1784T* and *ADRB3 T727C* are gene polymorphisms susceptible to the antihypertensive effect of TZDs in patients with EHT. Thus, the prediction of BP reduction by TZDs may be possible by evaluating these two SNPs.

(*Hypertens Res* 2004; 27: 821–833)

Key Words: **thiazide diuretics, gene polymorphism, essential hypertension**

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This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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Received March 3, 2004; Accepted in revised form July 20, 2004.

Introduction

Thiazide diuretics (TZDs) have been most widely used as a first line antihypertensive drug (*1*, *2*). Recently, the ALLHAT study confirmed the usefulness of TZDs for the reduction of blood pressure (BP) and cardiovascular diseases in comparison with newer antihypertensive drugs, including Ca channel blockers (CCBs) such as amlodipine and angiotensin converting enzyme inhibitors (ACEIs) such as lisinopril in about 40,000 hypertensive patients with high risk factors (*3*). TZDs are not only effective as a monotherapy for hypertension, but are also very useful for combination therapy with other antihypertensive drugs (*4*). Moreover, the use of a TZD as a drug therapy for hypertension, which is a chronic and lifelong disease, would be very good from the viewpoint of the cost of drugs, because TZDs are the cheapest of all antihypertensive drugs. However, the response of BP to TZDs differs among individuals, and TZDs often induce side effects, such as hypokalemia and lipid, glucose and uric acid metabolism abnormalities (*4*). Therefore, it would be useful to determine the individual sensitivity to a TZD before prescribing it.

Regarding previous findings about gene polymorphisms that influence TZD-sensitivity, Turner *et al.* (*5*) reported that the β3-subunit of the G protein (*GNB3*) *C825T* polymorphism was related to the antihypertensive effect of a TZD in Caucasian and African-American subjects with essential hypertension (EHT). Glorioso *et al.* (*6*) also demonstrated that the α-adducin (*ADD1*) *Gly460Trp* polymorphism is the gene conferring susceptibility to the antihypertensive effect of TZDs in Italian hypertensives. This *ADD1 Gly460Trp* polymorphism was also suggested to confer susceptibility to salt-sensitivity in Caucasians and Asians with EHT (*7*).

Mutations of causative genes have recently been detected in several monogenic electrolyte disorders, such as mutations in the thiazide-sensitive Na-Cl cotransporter (*TSC*) gene for Gitelman syndrome (*8*, *9*), the WNK1 and 4 genes for Gordon syndrome (pseudohypoaldosteronism type II) (*10*) and the mineral corticoid receptor (*MLR*) for pseudohypoaldosteronism type I (PHA I) (*11*). TZDs are commonly effective for treating Gitelman syndrome and Gordon syndrome. We also focused on the Na^+/Ca^{2+} exchanger gene (*NCX1*), because its impairment was recently reported in mesangial cells from salt-sensitive hypertensive rats (*12*). TZDs are known to be effective for salt-sensitive hypertension. It is also known that the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS) are activated in response to changes in circulating blood volume after TZD administration. Therefore, it is expected that gene polymorphisms related to the RAAS and SNS might be involved in the antihypertensive effect of TZDs. The present study investigated the gene polymorphism influencing the TZD-sensitivity by analyzing mainly single nucleotide polymorphisms (SNPs) of several water-electrolyte-related genes, including *GN3B*, *ADD1*, *TSC*, *MLR*, *NCX1*, *WNK1*, *WNK4* and RAAS- and SNS-related genes, to anticipate the effect of TZDs on BP in patients with hypertension.

Methods

Study Subjects

Peripheral blood samples for genetic analysis were collected with written informed consent from Japanese patients with EHT at an outpatient clinic of the Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan. This study protocol was approved by the Ethical Committee of the National Cardiovascular Center. Seventy-six patients, who had been newly prescribed TZDs as monotherapy or in addition to other antihypertensive agents, and whose blood-pressure data could be obtained from patients' records in 3 consecutive outpatient visits before and after starting TZDs, were retrospectively enrolled. BP 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 were the means of three physician-obtained measurements. All subjects visited the outpatient clinic every month. The TZDs were a combination of indapamide (64.5%), trichlormethiazide (26.3%), mefruside (7.9%), and hydrochlorothiazide (1.3%). We defined patients who could achieve a BP reduction greater than 5 mmHg of mean blood pressure (MBP) after taking the TZDs as responders (R), and patients who could not achieve a BP reduction greater than 5 mmHg of MBP or showed increased BP after taking TZDs as non-responders (NR), according to the common evaluation criteria of antihypertensive drug effectiveness in Japan.

DNA Studies

Direct Sequencing for Detection of Polymorphisms in TSC, MLR, WNK1, WNK4, *and* NCX1

Genomic DNA was extracted using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan) and stored at −80°C until use. We sequenced the 32–48 Japanese samples with written informed consent for genetic analysis. The methods used for the direct sequencing have been described previously (*13*). Briefly, all exons, part of the intron and an approximately 1,000-bp upstream region of exon 1, which would include the promoter regions of the *TSC*, *WNK1*, *WNK4* and *NCX* genes, were individually amplified by polymerase chain reaction and sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA). In the *MLR* gene, exon 2 was sequenced. The polymorphisms were identified using the Sequencer software package (Gene Codes Corp., Ann Arbor, USA), followed by visual inspection.

TSC gene: 16q13

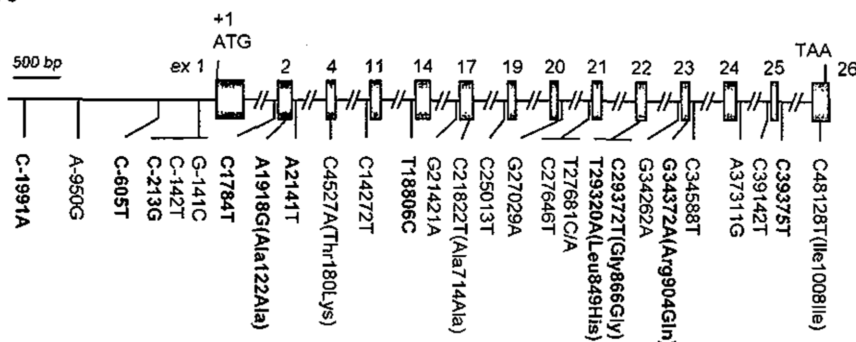

Fig. 1. *Location of genetic variants identified in* TSC. *Nucleotide numbers were counted from the initiation codon (ATG). Sequencing regions are indicated by the bars above the schematic gene structure. The initiation codon, ATG, and stop codon, TAA, are also shown. The single nucleotide polymorphisms presented in bold were genotyped in this study.*

Genotyping of Polymorphisms

The polymorphisms were genotyped using the TaqMan-polymelase chain reaction (PCR) system. Regarding genotyped SNPs, *C825T* of *GNB3* and *G29071T-Gly460Trp* of *ADD1* were selected according to previous studies (*5, 6, 14*). As the RAAS-related genes, *angiotensinogen* (*AGT: A−20C, G−6A*) (*15, 16*), *ACE* (*G12568C* for *I/D*) (*17*), *angiotensin II type 1 receptor* (*AT1: A1166C, A−153G*) (*18, 19*), and *aldosterone synthase* (*CYP11B2: C−344T*) (*20*) were tested. Furthermore, as the SNS-related genes, *adrenergic receptor β-1* (*ADRB1: A393G-Ser49Gly, G1413C-Arg389Gly*) (*21*), *β-2* (*ADRB2: C−47T, G2118A-Gly16Arg, G2151C-Glu27Gln*) (*22, 23*), *β-3* (*ADRB3:T727C-Trp64Arg*) (*24*), *α-1a* (*ADRA1A: T44653C-Arg492Cys*) (*25*), *α-1b* (*ADRA1B: G834A, G1167A*) (*26*) and *α-2a* (*ADRA2A: A3023G*) (*27*) were tested for TZD sensitivity. Regarding *ACE*, we genotyped *G12568C* instead for the *I/D* polymorphism of 287 bp in intron 16 because *G12568C* showed almost complete linkage disequilibrium (LD) with the *I/D* polymorphism, as reported previously (*17*). For the directly sequenced genes, SNPs having a minor allele frequency of greater than 5% were selected for genotyping. As a result, 11 SNPs of *TSC* (Fig. 1), 2 SNPs of *MLR*, 7 SNPs of *WNK1*, 2 SNPs of *WNK4* and 7 SNPs of *NCX1* were genotyped. The sequences of the allele-specific probes and PCR primers used for the genotyping are shown in Table A1 in Appendix.

Statistical Analysis

Values are expressed as the means±SD. Hardy-Weinberg equilibrium was assessed by χ^2 analysis. The overall distribution of alleles was analyzed by χ^2 analysis. The distribution of genotypes between R and NR was analyzed by 2×2 contingency tables with a 2-sided Fisher exact probability test. The statistical significance was established at $p<0.05$. Comparison of BP reduction between allelic variants was performed by ANOVA followed by the Fisher protected least significant difference test using Stat-View version 5.0 (SAS Institute Inc., Cary, USA). LD and haplotype analyses were performed using the SNPAlyze statistical package version 2.1 (DYNACOM Co., Ltd., Mobara, Japan). The LD between SNPs was calculated by r^2. Tight LD was regarded as $r^2 \geq 0.5$. Haplotype estimation was performed by the expectation-maximization algorithm.

Results

Group Characteristics

Overall BP was significantly reduced after TZD administration (Fig. 2). Table 1 shows the group characteristics of R and NR. Forty-five patients who showed an MBP reduction of greater than 5 mmHg were defined as R, and 31 patients were defined as NR. Neither averaged age nor body mass index (BMI) showed a significant difference between R and NR. The BP before TZD administration was significantly higher in R than in NR. After TZD treatment, the averaged BP in R was remarkably decreased; however, the averaged BP in NR was slightly higher than that at pretreatment (Table 1). Control for deviation from Hardy-Weinberg equilibrium gave non-significant results in most SNPs examined in the present study, except *ADRA1B G1167A*. In the genotyping of *ADRA1B G1167A*, all of the study subjects were homozygous for the major allele, GG. This suggests that polymorphism of *ADRA1B G1167A* might not exist in the Japanese population.

Detection of Genetic Variants

We detected 52 SNPs of *TSC*, 7 SNPs of *MLR*, 35 SNPs of *WNK1*, 22 SNPs of *WNK4* and 15 SNPs of *NCX1* (Table A2 in Appendix). We confirmed some of the identified SNPs in the public database, dbSNPs (http://www.ncbi.nlm.nih.gov/SNP/). As shown in Table A2 in Appendix, some SNPs were very rare. Therefore, we chose SNPs that had a minor allele frequency of greater than 5% for genotyping by the TaqMan

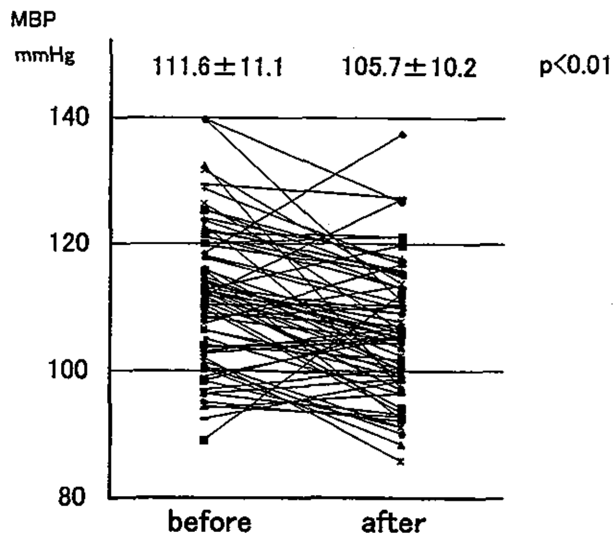

Fig. 2. *Mean blood pressure (MBP) before and after treatment with thiazide diuretics (TZDs) in all subjects. The average MBP in all subjects was significantly reduced by treatment with TZDs.*

method. Furthermore, some of these polymorphisms showed LD with other polymorphisms. Thus, we chose one SNP from among the polymorphisms with tight LD (*28*) ($r^2 \geq 0.5$) for genotyping the subjects. Therefore, genotyping by the TaqMan method was finally performed for 11 SNPs in *TSC*, 2 SNPs in *MLR*, 7 SNPs in *WNK1*, 2 SNPs in *WNK4* and 7 SNPs in *NCX1* (Table A1 in Appendix).

Association Study for the Effect of TZDs

No polymorphisms of the *MLR*, *WNK1*, *WNK4*, *NCX1*, and RAAS genes, and no polymorphisms of most of the SNS genes examined in the present study, including *C825T GNB3* and *Gly460Trp ADD1*, were significantly related to the effect of the TZDs, based on the comparison of each allele frequency between R and NR (Table 2). Two SNPs, *TSC C1784T* and *ADRB3 T727C*, showed a significant correlation with the effect of the TZDs (Table 3). The BP in patients with the homozygotes of the major allele of both *TSC C1784T* and *ADRB3 T727C* were significantly reduced by TZD treatment; however, the BP in those with the homozygotes of the minor allele and heterozygote (*TSC C1784T*: TT+CT; *ADRB3 T727C*: CC+CT) of both SNPs were not significantly changed after TZD treatment (Fig. 3). Furthermore, there was a much more significant difference in prevalence between the patients with the homozygotes of the minor alleles and heterozygote of these two SNPs, *TSC C1784T* and *ADRB3 T727C*, combined and those with the homozygotes of the major allele in R and NR than in *TSC C1784T* or *ADRB3 T727C* alone (odds ratio [OR]=5.09, $p=0.003$, confidence interval [CI]: 1.82–14.23).

Table 1. Comparison of Patients Characteristics between R and NR of TZD

	R (±SD)	NR (±SD)	*p* value
Number	45	31	
Age (years)	64.5±9.3	66.7±8.6	N.S.
Sex (male/female)	18/27	20/11	<0.05
BMI (kg/m²)	24.7±3.2	24.7±3.2	N.S.
Pre SBP (mmHg)	157.9±14.3	142.9±15.0	<0.01
Pre DBP (mmHg)	95.0±10.7	86.5±10.1	<0.01
Pre MBP (mmHg)	115.9±9.7	105.3±9.9	<0.01
Pre HR (/min)	71.0±7.9	72.4±7.9	N.S.
Post SBP (mmHg)	138.8±15.0	147.2±17.4	<0.05
Post DBP (mmHg)	87.0±9.9	88.2±11.0	N.S.
Post MBP (mmHg)	104.3±9.3	107.9±11.2	N.S.
Post HR (/min)	72.4±9.3	72.8±9.0	N.S.
Monotherapy (%)	28.9	25.8	N.S.
Kind of TZD (%)			
Indapamide	66.7	61.3	N.S.
Trichlormethiazide	26.7	25.8	N.S.
Mefruside	6.7	9.7	N.S.
Hydrochlorothiazide	0.0	3.2	N.S.

R, responder; NR, non-responder; TZD, thiazide diuretics; BMI, body mass index; Pre SBP, systolic blood pressure at pretreatment; Pre DBP, diastolic blood pressure at pretreatment; Pre MBP, mean blood pressure at pretreatment; Pre HR, heart rate at pretreatment; Post SBP, systolic blood pressure at posttreatment; Post DBP, diastolic blood pressure at posttreatment; Post MBP, mean blood pressure at posttreatment; Post HR, heart rate at posttreatment; Monotherapy, prevalence of monotherapy by thiazide diuretics; Kind of TZD, prescribed kinds of TZD; N.S., not significant.

Haplotype Analysis

We measured the LD to understand the haplotype distribution of *TSC C1784T* in Japanese. There was a strong LD between the multiple SNPs within *TSC*. *TSC* was composed of three LD blocks, and the LD block containing *C1784T* consisted of two SNPs, *C−213G* and *C1784T*. The haplotype frequency was calculated for these two SNPs and the differences in haplotype distribution were compared between R and NR (Table 4). The results showed that the haplotype H2 with the 1784T allele tended to be different between the two groups, although not significantly so ($p=0.094$).

Discussion

The present study demonstrated that *TSC C1784T* and *ADRB3 T727C* were associated with the antihypertensive effect of TZDs in Japanese patients with EHT. A hypertensive patient with the minor homozygote or heterozygote of these two SNPs is predicted to be a non-responder to TZDs.

The TSC is present in the distal convoluted tubule, which

Table 2. Comparison of Allele Frequency between R and NR to TZD

Gene	Minor allele *vs.* common allele			
	SNP	Odds ratio	*p*	95% CI
ADD1	*Gly460Trp*	1.300	0.427	0.680–2.487
GNB3	*C825T*	1.620	0.146	0.844–3.110
TSC	*C−1991A*	1.483	0.586	0.356–6.167
	C−605T	1.483	0.586	0.356–6.167
	C−213G	1.483	0.586	0.356–6.167
	C1784T	3.816	0.013	1.253–11.627
	A1918G	0.594	0.235	0.251–1.410
	A2141T	2.378	0.102	0.821–6.886
	T18806C	0.825	0.566	0.428–1.591
	T29320A	1.459	0.790	0.090–23.770
	C29372T	1.143	0.718	0.554–2.359
	G34372A	—	0.147	—
	C39375T	0.681	0.311	0.323–1.436
MLR	*C−2G*	1.147	0.731	0.524–2.509
	G538A	0.685	0.418	0.274–1.716
WNK1	*G786A*	1.021	0.965	0.407–2.561
	C108560T	1.026	0.967	0.310–3.400
	A128177C	1.124	0.791	0.473–2.673
	C133634T	1.189	0.721	0.461–3.067
	G135642T	0.950	0.881	0.484–1.864
	C141114T	0.820	0.617	0.377–1.785
	C142763T	0.967	0.971	0.157–5.961
WNK4	*C14597T*	1.467	0.704	0.201–10.700
	C14717T	1.780	0.287	0.609–5.203
NCX1	*T−23690C*	0.849	0.721	0.346–2.084
	C−23449A	0.864	0.846	0.199–3.757
	T−23200C	0.651	0.553	0.156–2.711
	T−23181C	0.850	0.633	0.436–1.656
	A−22729C	0.914	0.861	0.334–2.505
	C−22144G	2.967	0.357	0.263–33.454
	G252581A	0.906	0.779	0.456–1.802
AGT	*A−20C*	1.265	0.540	0.596–2.687
	G−6A	0.758	0.527	0.320–1.793
ACE	*G12568C (I/D)*	0.768	0.443	0.392–1.508
AT1-R	*A1166C*	0.712	0.639	0.171–2.961
	A−153G	1.172	0.818	0.302–4.552
CYP11B2	*C−344T*	1.554	0.219	0.768–3.145
ADRB1	*G1413C*	1.724	0.228	0.707–4.204
	A393G	0.692	0.432	0.276–1.738
ADRB2	*C−47T*	1.098	0.869	0.361–3.338
	G2118A	1.531	0.203	0.793–2.956
	G2151C	1.228	0.744	0.358–4.217
ADRB3	*T727C*	4.591	0.003	1.541–13.680
ADRA1A	*T44653C*	0.630	0.412	0.207–1.913
ADRA1B	*G834A*	1.381	0.333	0.718–2.657
	G1167A	—	—	—
ADRA2A	*A3023G*	1.223	0.556	0.626–2.389

SNP, single nucleotide polymorphism; R, responder; NR, non-responder; TZD, thiazide diuretics; CI, confidence interval.

has been suggested to be the principal mediator of sodium and chloride reabsorption in this segment of the nephron. Simon *et al.* (*9*) demonstrated complete linkage of Gitleman syndrome to the genetic variants of *TSC*. The loss of function of TSC in patients with *TSC* gene variants could lead to low blood pressure, hypokalemic alkalosis, hypomagnesaemia and hypocalciuria (*29*). Melander *et al.* reported that gene polymorphism of TSC may influence EHT (*30*). It has recently been reported that TSC might interact with WNK4, which is one of the causative genes of Gordon syndrome (*31*, *32*). TZDs are generally effective in patients with Gordon syndrome. This would be due to the interaction between WNK and TSC. Four kinds of TZDs were used for the present study, and the main pharmacological mechanism of the antihypertensive effect common among those four TZDs was the blockage of TSC in the distal tubule. For these reasons, we expected that the gene polymorphisms of *TSC*, *WNK1* and *WNK4* might be related to the effect of TZDs. One SNP of *TSC* showed a significant association with the effect of the TZDs; however, there were no positive SNPs in either *WNK1* or *WNK4*.

TSC consists of 26 exons and is located on *16q13*. We performed direct sequencing for *TSC*, including the promoter region, and detected 52 SNPs. We screened 11 SNPs that showed a minor allele frequency of greater than 5% for TZD sensitivity (Fig. 1). As a consequence, only *TSC C1784T* showed a significant correlation with the effect of TZDs. Since *TSC C1784T* is located in intron 1, as shown in Fig. 1, this SNP itself may not influence TSC function. Although we could not find functional polymorphisms linked with *C1784T*, there may be functional polymorphisms in much further upstream regions of the promoter or genes adjacent to *TSC*.

Administration of a TZD often induces activation of the RAAS and/or SNS as a result of circulating volume reduction. Thus, we investigated the participation of gene polymorphisms of the RAAS and SNS. The gene polymorphisms were selected from previous studies investigating the correlation between BP regulation and gene polymorphisms (*33*). Although Sciarrone *et al.* (*34*). reported that Caucasian hypertensive patients with the *I* allele of *ACE I/D* were more sensitive for hydrochrolthiazide than those with *DD*, no polymorphisms of the RAAS-related genes, including *ACE I/D*, showed a significant correlation with the effect of TZDs in the present study. Furthermore, neither *C825T* of *GNB3* (*5*) nor *Gly460Trp* of *ADD1* (*6*, *34*), which have previously been reported to influence the sensitivity to TZDs, showed a significant correlation with the effect of TZDs in the present study. It is suggested that the reason for the difference between the present findings and previous findings on the participation of the RAAS genes, *GNB3* and *ADD1*, in the effects of TZDs might be the ethnicity of the study subjects. Most studies (*5*, *34*) investigated the participation of the RAAS genes, *GNB3* and *ADD1*, in the effect of TZDs in Caucasians. In contrast, all subjects in the present study

Table 3. TZD- Sensitive Gene Polymorphisms of *TSC C1784T* and *ADRB3 T727C*

SNP	Sex	Genotype	R	NR	χ^2	*p*
TSC C1784T	M+F	CC	40	20	6.052	0.049
		CT	5	9		
		TT	0	1		
		CC	40	20	5.556	0.037
		TT+CT	5	10		
	Odds ratio=4.000, 95% CI=1.204–13.284					
		C allele	85	49	6.168	0.016
		T allele	5	11		
	Odds ratio=3.816, 95% CI=1.253–11.627					
ADRB3 T727C	M+F	CC	1	1	10.649	0.005
		CT	3	11		
		TT	40	18		
		TT	40	18	10.056	0.003
		CC+CT	4	12		
	Odds ratio=6.667, 95% CI=1.889–23.525					
		C allele	5	13	8.533	0.005
		T allele	83	47		
	Odds ratio=4.591, 95% CI=1.541–13.680					

TZD, thiazide diuretics; R, responder; NR, non-responder; SNP, single nucleotide polymorphism; M, male; F, female; CI, confidence interval.

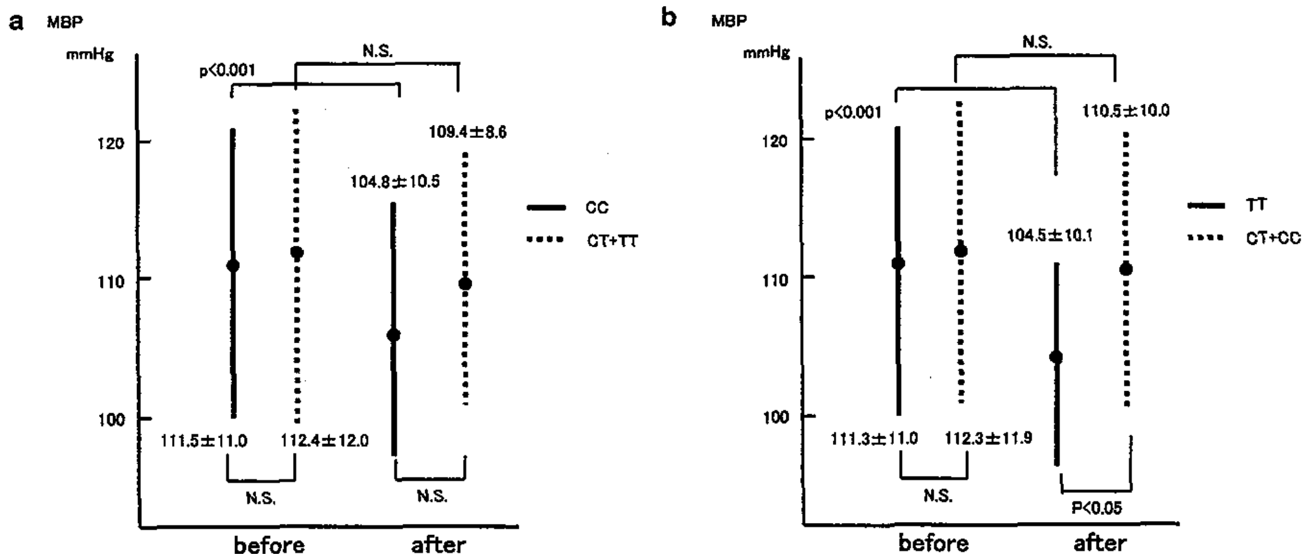

Fig. 3. *a: Comparison of the MBP changes before and after TZD therapy between homozygotes of the major allele CC and the minor homo- and heterozygote TT+CT of* TSC C1784T. *NS, not significant. b: Comparison of MBP changes before and after TZD administration between the homozygotes of the major allele TT and the minor homo- and heterozygote CC+CT of* ADRB3 T727C. *NS, not significant.*

were Japanese. The amount of salt intake in the Japanese population is generally greater than that in Caucasians (*35*). Thus, there is a possibility that Japanese may be more sensitive to TZDs than Caucasians.

The present study revealed a significant correlation between *T727C-Trp64Arg ADRB3* and the effect of TZDs. The β3 adrenergic receptors are mainly distributed in adipose tissues and involved in the regulation of lipolysis and thermogenesis. Regarding the *ADRB3* polymorphism *Trp64Arg*, it has been correlated with obesity (*36*), insulin resistance (*37*) and diabetes (*38*). In contrast, the relation between *Trp64Arg ADRB3* and hypertension is controversial (*24, 39, 40*). Re-

Table 4. Haplotype Distribution in R and NR in Two SNPs of *TSC*

Haplotype		R (n=90)	NR (n=60)	p value
H1	CC	81	47	0.622
H2	CT	5	9	0.094
H3	GC	4	2	>0.999
H4	GT	0	2	0.165

R, responder for thiazide diuretics: NR, non-responder for thiazide diuretics; SNPs, single nucleotide polymorphisms. Haplotypes were shown as combined alleles of genotyped 2 SNPs (*C−213G, C1784T*) of *TSC.*

garding the SNS activity, Shihara *et al.* (*41*) reported that subjects with the homo- and heterozygote of *64Arg ADRB3* had higher responses of the autonomic nerve activities after postural change than those with the wild-type gene. This suggests that *Trp64Arg ADRB3* may play an important role in the autonomic nervous system activities, including the activities of the SNS. To date, however, there has been no evidence that *β*3 adrenergic receptors exist and function physiologically on sympathetic nerves or the renal tubular system. Therefore, the mechanisms intervening between *ADRB3* gene polymorphism and the effectiveness of TZDs are unclear. Further investigations, including studies on the reflective activation of SNS and the worsening in insulin resistance after TZD administration, will be needed to clarify this interaction.

There is a question as to whether the contributions of *TSC C1784T* and *ADRB3 T727C* to the effects of TZDs are a TZD-specific finding. We tried to investigate the relationship between these 2 SNPs and the antihypertensive effects of an ACEI in 98 patients with EHT by the same study protocol; however, these 2 SNPs did not show any significant correlation to the effect of ACEI (unpublished data). Although investigation of other antihypertensive drugs is necessary, we speculate that the contribution of these 2 SNPs to the effect of TZDs is in fact a TZD-specific finding.

The study limitations include the retrospective design and the small sample size. The study subjects included not only patients receiving monotherapy with TZDs but also those receiving combined therapy with TZDs and other antihypertensive drugs. This issue is not considered to have much influence on the relationship between the 2 SNPs, *TSC C1784T-ADRB3 T727C,* and the effect of TZDs, because the prevalence of patients with monotherapy using TZD and the variation of the kinds of TZDs were not significantly different in between each allele of the two SNPs. However, a prospective and large-scale controlled study using TZDs is needed to confirm the importance of *TSC C1784T* and *ADRB3 T727C* on the antihypertensive effect of TZDs.

Furthermore, the BP level at pretreatment is considered an important factor in the effect of antihypertensive drugs. In the present study, BP before TZD administration was significantly higher in R than in NR. However, the BP level before TZD administration was not significantly different between TZD-sensitive and -insensitive genotypes in both *TSC C1784T* and *ADRB3 T727C-Trp64Arg*, as shown in Fig. 3. It might be possible that the BP response to TZDs was modified by the placebo effect. However, it is unlikely that *TSC C1784T* or *ADRB3 T727C* is involved in the placebo effect. A placebo-controlled prospective trial or ambulatory BP monitoring would help to confirm the significance of these SNPs in the BP-lowering effect of TZDs.

Finally, regarding the statistical approach, the Bonferroni method was not performed in this study even though multiple SNPs were investigated. The criterion for significance is p<0.001 (=0.05/48 SNPs) according to the Bonferroni method; however, *TSC C1784T* and *ADRB3 T727C* were associated with the antihypertensive effect of TZDs at p= 0.016–0.049 and 0.003–0.005, respectively (Table 3). Although this might be considered a weak correlation for this kind of genetic research, we consider these two SNPs as prominent candidates relating to the effectiveness of TZDs, because both *TSC* and *ADRB3* were suggested to play an important role in the effectiveness of TZDs in patients with EHT, as we mentioned above.

In conclusion, *TSC C1784T* and *ADRB3 T727C-Trp64Arg*, may be gene polymorphisms susceptible to the antihypertensive effect of TZDs in patients with EHT. Thus, the prediction of BP reduction by TZDs may be possible by evaluating these two SNPs. Since the publication of the JNC 7, TZDs are becoming increasingly important as first-line drugs (*1*). The prediction of the TZD sensitivity of patients may lead to the realization of individualized therapy for hypertension based on genetic background.

Acknowledgements

We are grateful to Yoko Tokunaga and Chiyoko Imai for their excellent technical assistance and Erumu Hayase, Yoko Yamakawa, Kanako Hoshino and Chikako Oku for their excellent secretarial work.

Appendix

The sequences of the allele-specific probes and PCR primers for the genotyping are shown in Table A1 and SNPs of *TSC, MLR, WNK1, WNK4* and *NCX1* are shown in Table A2.

References

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Table A1. List of Genotyping Conditions for TaqMan PCR Method

Gene name	SNP	Primer	Final conc. (nmol/l)	Probe	Final conc. (nmol/l)	96-well annealing temp. and cycle no.	384-well annealing temp. and cycle no.
ADD1	*Gly460Trp*	CACACCTTAGTCTTCGACTTGGG	800	Fam-TTCTGCCCTTCCTC-MGB	200		58°C
	(G29071T)	ACAAGATGGCTGAACTCTGGC	800	Vic-TTCTGCCATTCCTC-MGB	200		40
GNB3	*C825T*	CAGACCAGGAGCTGATCTGCTT	800	Fam-CATCACGTCCGTGGC-MGB	200		60°C
		TTGCAGTTGAAGTCGTCGTAGC	800	Vic-ATCACGTCTGTGGCCT-MGB	200		40
TSC	*C−1991A*	CCCTGACAGCTCAAATTTCCAC	800	Fam-CTGCCTCCCTGCAA-MGB	200		58°C
		CTTGTTACCAGAGGTGCCTAAGC	800	Vic-CTGCCTCACTGCAA-MGB	200		40
	C−605T	GCAGAAATGAAATCCACAAGCA	800	FAM-TTTGAAAATCCCTGTCCTG-MGB	228	62°C	58°C
		CATGCACCGATCATTAGATTGG	800	VIC-CTTTGAAAATTCCTGTCCTG-MGB	223	40	40
	C−213G	GGCAGAACACCATTTGATTGTG	800	FAM-CTGGCCCAAAGCCAGCCACTC-TAMRA	256	62°C	60°C
		GAAGAGCCACTCCAGGACTCA	800	VIC-CTGGCCCAAACCCAGCCACTC-TAMRA	282	35	40
	C1784T	CGCAGTGGTGCAGGTCAGT	800	Fam-CAGAGACGCCGTCC-MGB	200		58°C
		AGGTGTCTGCCTTCCTGCTG	800	Vic-TGCAGAGATGCCGTC-MGB	200		40
	A1918G	CTCACCATCACCCCCTTGAC	800	Fam-CTGGTGCCTGCCTCGCCC-TAMRA	200		60°C
		CAGCAGGAAGGCAGACACCT	800	Vic-TGGTGCCCGCCTCGCC-TAMRA	200		40
	A2141T	GCTTCAGTTTCCCCATCTGTACA	800	Fam-AATAGATTAAAGCCTGCCGG-MGB	200		58°C
		GGTGGCTTTTTAGGGAAACACA	800	Vic-AATAGATTAATGCCTGCCGG-MGB	200		40
	C4527A	GATGAACGTAGGTCGCATGGT	800	FAM-TGTCGGTCACGGTGA-MGB	336	60°C	58°C
		GATGGCTGAGATGGAGAGGC	800	VIC-TGTCGGTCAAGGTG-MGB	297	40	40
	T18806C	AGCAGCTCTGGCCTAGAAAGAG	800	FAM-TGGTGCCCTTGGCCCAGG-TAMRA	330	62°C	62°C
		ACGGAGATGATAGCCCCAAAC	800	VIC-CTGGTGCCCTCGGCCCAG-TAMRA	290	35	40
	T29320A	TCACATAGTGCTCTGTCCTGAGTG	800	FAM-TCCCTATCTCCTTGGC-MGB	242	62°C	60°C
		GATCTTGCATTTGCTCCACCTC	800	VIC-CCTATCACCTTGGCC-MGB	201	40	40
	C29372T	GCAAGAGGAGGTGGAGCAAAT	800	FAM-TTCGTAGGCGGCCAG-MGB	117	60°C	58°C
		CCCTCCACACTTACGCCTTTC	800	VIC-TCGTAGGTGGCCAGAT-MGB	254	40	40
	G34372A	GGGATTCCATGAAGTCCACATC	800	FAM-AACCCTCGGGCTGA-MGB	337	62°C	—
		CTGGAAGCCCCAAAACAGAAC	800	VIC-AGAACCCTCAGGCTG-MGB	329	40	—
	C39375T	GAAGCAGAAGGGCCAAAGTTC	800	FAM-ATAGCCCTGGCGATT-MGB	267	58°C	58°C
		GATGCCTGGGACACGTGAG	800	VIC-TAGCCCTGGTGATTC-MGB	84	40	40
MLR	*C−2G*	TTGTGGCTTAGCAAATGCAATT	800	Fam-TTTGTTAGCGATGGAGAC-MGB	602	62°C	
		CAGGGAGACTGTGGTAGCCTTT	800	Vic-ATTTGTTAGGGATGGAGAC-MGB	224	40	
	G538A	GGGCTTTTCTCATGACACATGATA	800	Fam-CTTTTAACAATGGCGCGC-MGB	189		60°C
		CGCCCTTGAGATCATTTATGTCT	800	Vic-TTTAACAACGGCGCGCA-MGB	361		40
NCX1	*T−23690C*	CTCTCCCCACAGGTCATTCTG	800	Fam-ATTTAACTTATAGCAAGGAA-MGB	200		58°C
		GCAGGAATCGTTCTTGCCTAA	800	Vic-TTAACTTACAGCAAGGAA-MGB	200		40
	C−23449A	GAATCTGCAATCCCCATGTGAT	800	Fam-CTCACATTCATGTTTGAG-MGB	200		56°C
		AGAACCACTGCTCTAGGCCAAT	800	Vic-ACTCACATTAATGTTTGAGG-MGB	200		40
	T−23200C	TTCTGAGGTGCAAGGAGGGTT	800	Fam-CCCCCTTTTTGTTGC-MGB	100		56°C
		GGCAGTCACCACGACTGATAGA	800	Vic-CCCCCTCTTTGTTG-MGB	100		40
	T−23181C	GGCAGTCACCACGACTGATAGA	800	Fam-TCCAGGAACCTCAGTTT-MGB	200		56°C
		AGGCTATTTCTTCCATTCCGC	800	Vic-CCAGGAACCTCGGTTT-MGB	200		40
	A−22729C	GCCTGGTGCAGTGTTCCTTTA	800	Fam-ATTATGAGGAAAGTGATTTA-MGB	200		58°C
		GCCCTTTCCAAGAGAAGCATTA	800	Vic-TATGAGGACAGTGATTTA-MGB	200		40
	C−22144G	AAAAGAAAAGTTGCAGCGCCT	800	Fam-CCACAACGCACTGC-MGB	200		56°C
		TTTTTCGATTTCCTGCCGG	800	Vic-CACAAGGCACTGCG-MGB	100		40
	G252581A	AAACAAAGACATACCAGCGAGAAA	800	Fam-CTCTCTCCGTGTCTC-MGB	200		58°C
		AAATTGCTAAAGCTTCAAAGGCA	800	Vic-TCTCTCCATGTCTCC-MGB	200		40
WNK1	*G786A*	GAACTGCAGGTAAAGCCCCAC	800	Fam-TTTGACGGTCCTTTG-MGB	200		58°C
		GAACTCGATCAACTGGCTTCG	800	Vic-TTTATTTGACAGTCCTTTG-MGB	200		40
	C108560T	CTGATGGGACGGTTGACAGTG	800	Fam-TCTTCACAGAATCTCGA-MGB	200		58°C

Table A1. (Continued)

Gene name	SNP	Primer	Final conc. (nmol/l)	Probe	Final conc. (nmol/l)	96-well annealing temp. and cycle no.	384-well annealing temp. and cycle no.
		CCTGTTCATGTTGGGAACCATA	800	Vic-TCTTCATAGAATCTCG-MGB	200		40
	A128177C	GTTGCTCCTGCAGAGCCAGT	800	Fam-AGTAGCACAGACCCAA-MGB	200		58°C
		TCTACAGAGGAAGCCAAAGTGGT	800	Vic-AGTAGCACAGCCCCA-MGB	200		40
	C133634T	TTGATTTGCTCTTCAGTACGCAG	800	Fam-AGCGTCTCACGGACT-MGB	200		58°C
		GCACCTACAGACAACAAAGGGAA	800	Vic-AGCGTCTCATGGACT-MGB	200		40
	G135642T	AAAACTTACACCAACCGCAGAAG	800	Fam-CTGTGATCATCTCTG-MGB	200		58°C
		ATTCAGTCCCAGCAACCTCTAGA	800	Vic-ACTGTGATAATCTCTG-MGB	200		40
	C141114T	TGGGACGATTTCAGGTAAGACAG	800	Fam-ATTCCTTCCTTTGGAGGA-MGB	200		58°C
		TTGTGTCCCAAATAGGTAGGCA	800	Vic-ATTCCTTCTTTTGGAGGAG-MGB	200		40
	C142763T	ACGACCCACTTTGTTTGCTGTA	800	Fam-CTGAAAACGTCCAACCT-MGB	200		58°C
		GTCAGACACTGGGCAGCCTAC	800	Vic-CCTGAAAACATCCAACCT-MGB	200		40
WNK4	*C14597T*	CTGGCTGTGATGACTGTGGC	800	Fam-TCCCCTCCCTAGCCT-MGB	200		58°C
		TGAAGGGCTTTCCTGGCC	800	Vic-TCCCCTCTCTAGCCTG-MGB	200		40
	C14717T	CACAGCTGAGGTGGAGAGTGAG	800	Fam-CTCCACTCTGCACTC-MGB	200		58°C
		GGAGGTGGTGAGGCCTAGAAA	800	Vic-ACTCCATTCTGCACTC-MGB	200		40
AGT	*A(−20)C**	CTTCTGGCATCTGTCCTTCTGG	250	Direct sequence			64°C
		CTGGTCTTATGAGAGGGGAGAGG	250				35
	*G(−6)A**	Same as *A(−20)C*		Direct sequence			
ACE	*G12568C*	AGCAGAGGTGAGCTAAGGGCT	667	Fam-CTCAAGGCATTCAA-MGB	200		58°C
	(I/D)	GGCCATCACATTCGTCAGATCT	667	Vic-CTCAAGCCATTCAA-MGB	200		40
AT1	*A(−153)G*	AACGCTGATCTGATAGTTGACACG	800	Fam-CCGTCAATATCCCGAG-MGB	200		60°C
		CTCTGTTTTGCATTCCCTCCTC	800	Vic-CCGTCAGTATCCCGA-MGB	200		40
	A1166C	AGAGAACATTCCTCTGCAGCACT	800	Fam-CAAATGAGCATTAGCT-MGB	200		60°C
		CGGTTCAGTCCACATAATGCAT	800	Vic-CAAATGAGCCTTAGCT-MGB	200		40
CYP11B2	*C(−344)T*	TGGACATTTTCTGCAGTTTTTGA	800	Fam-ATCCAAGGCTCCCTCT-MGB	100		56°C
		TCCTTTCTCCAGGGCTGAGA	800	Vic-CAAGGCCCCCTCT-MGB	100		40
ADRB1	*G1413C*	TTCTTCAACTGGCTGGGCTAC	800	Fam-CCTTCCAGGGACTGC-MGB	200		58°C
		GTCTCCGTGGGTCGCGT	800	Vic-CTTCCAGCGACTGCT-MGB	200		40
	A393G	CCGGTAACCTGTCGTCGG	800	Fam-CAGCGAAAGCCCCGA-MGB	200		58°C
		GATCACCAGCACATTGCCC	800	Vic-AGCGAAGGCCCCGAG-MGB	100		40
ADRB2	*C(−47)T*	CATTGGGTGCCAGCAAGAA	800	Fam-CGCCTCAGCGGGCGGA-TAMRA	100		56°C
		GAATGAGGCTTCCAGGCGT	800	Vic-CGCCTCAGCAGGCGGACC-TAMRA	100		40
	G2118A	CGCTGAATGAGGCTTCCAG	800	Fam-ACCCAATGGAAGCC-MGB	100		58°C
		CTGCGTGACGTCGTGGTC	800	Vic-ACCCAATAGAAGCCA-MGB	100		40
	G2151C	CCAGGACGATGAGAGACATGAC	800	Fam-TCCCTTTCCTGCGTGA-MGB	200		58°C
		CCTTCTTGCTGGCACCCA	800	Vic-TCCCTTTGCTGCGTG-MGB	200		40
ADRB3	*T727C*	CACGTTGGTCATGGTCTGGA	800	Fam-CGGAGTCCAGGCGA-MGB	200		58°C
		GAGGCAACCTGCTGGTCATC	800	Vic-TCGGAGTCCGGGCG-MGB	200		40
ADRA1A	*T44653C*	TCCAGCCAAGAGTTCAAAAAGG	800	Fam-CAGTGTCTCTGCAGAA-MGB	100		56°C
		CCAGGGCATGTTTGGAAGACT	800	AGTGTCTCCGCAGAA-MGB	200		40
ADRA1B	*G834A*	CGCACTCCTTGTCATCGTTG	800	Fam-TCCTTCCACCCAAGGA-MGB	200		58°C
		GTCTTGTCCACCGTCATCTCC	800	Vic-CTCCTTCCATCCAAGGA-MGB	200		40
	G1167A	CAAGATGAACATACCGACCACAA	800	Fam-CCCAACGTCTTAGCT-MGB	200		60°C
		CAACCCCAGGAGTTCCATAGC	800	Vic-CCCAACGTTTTAGCT-MGB	200		40
ADRA2A	*A3023G*	TCCCCTTCCATTCCCAACTC	800	Fam-TCTCTTTTTAAAGAAAAAT-MGB	200		56°C
		TTCAACATCAAAACCAAGGCC	800	Vic-TCTTTTTGAAGAAAAAT-MGB	100		40

* The genotyping for *AGT A(−20)C* and *G(−6)* polymorphisms was performed by the direct sequence method. A pair of the PCR primers was 5′-CTTCTGGCATCTGTCCTTCTGG-3′ and 5′-CTGGTCTTATGAGAGGGGAGAGG-3′.

Table A2. List of 130 Polymorphisms and Their Allele Frequency in *TSC*, *MLR*, *WNK1*, *WNK4* and *NCX1* Genes Identified by the Direct Sequence

Gene name	Allele 1/Allele 2 SNPs	Amino acid change	Region	Allele 1 Homo	Hetero	Allele 2 Homo	Total	Allele frequency Allele 1	Allele frequency Allele 2	Flanking sequence	dbSNP ID
TSC	*C−1991A*		promoter	38	0	10	48	0.792	0.208	caccactgcctc[c/a]ctgcaatggctt	
	A−950G		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		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		promoter	35	8	0	43	0.907	0.093	gggagtggctgg[c/g]tttgggccagcc	
	C−142T		promoter	1	20	22	43	0.256	0.744	gtgttctgcctc[c/t]ggccctgtccgg	
	G−141C		promoter	28	15	0	43	0.826	0.174	tgttctgcctcc[g/c]gccctgtccggg	
	C1784T		intron1	30	17	1	48	0.802	0.198	tggatgcagaga[c/t]gccgtccctagc	
	A1918G	Ala122Ala	exon2	31	17	0	48	0.823	0.177	ggagggcgaggc[a/g]ggcaccagcagc	rs2304479
	A2141T		intron2	0	8	40	48	0.083	0.917	acaatagattaa[a/t]gcctgccgggga	rs2304480
	G2971A		intron2	47	1	0	48	0.990	0.010	tagggcctaggt[g/a]ctcgataccctg	
	C4527A	Thr180Lys	exon4	43	2	0	45	0.978	0.022	tgctgtcggtca[c/a]ggtgacctccat	
	C7479T	Phe341Phe	exon8	38	2	0	40	0.975	0.025	tggcaccttctt[c/t]ggaatgttctcc	
	C14272T		intron10	26	18	3	47	0.745	0.255	ctggctcagccc[c/t]caccgtggagtc	rs3816119
	G14277A		intron10	46	1	0	47	0.989	0.011	tcagccccacc[g/a]tggagtccctga	
	C14363A	Ala464Ala	exon11	45	2	0	47	0.979	0.021	catcttcggggc[c/a]accctctcctct	
	C14366T	Thr465Thr	exon11	46	1	0	47	0.989	0.011	cttcggggccac[c/t]ctctcctctgcc	rs5801
	G17337A		intron13	44	1	0	45	0.989	0.011	ggggtgggagtg[g/a]gaggcatgggtg	
	T18806C[b]		intron13	6	24	18	48	0.375	0.625	gactggtgccct[t/c]ggcccagggtgg	rs2304483
	C18850T	Ala569Val	exon14	46	2	0	48	0.979	0.021	acaacaagtggg[c/t]ggcgctgtttgg	
	T20072C	Leu623Pro	exon15	46	1	0	47	0.989	0.011	gctcctacaacc[t/c]ggccctcagcta	
	G20088A	Ser628Ser	exon15	46	1	0	47	0.989	0.011	cctcagctactc[g/a]gtgggcctcaat	
	C20201G		intron15	46	1	0	47	0.989	0.011	gagtttccaagc[c/g]tagacctgtcac	
	G21421A		intron16	20	24	3	47	0.681	0.319	atggggcccaa[g/a]gggatgcggagc	
	C21500T		intron16	42	2	0	44	0.977	0.023	ccctcttgctgg[c/t]ttctcccccagc	
	C21566G		intron16	43	1	0	44	0.989	0.011	cactttctcccc[c/g]actccttgtgtt	
	A21586G		intron16	43	1	0	44	0.989	0.011	gtgttttccctt[a/g]tctgggcaaaag	
	C21822T	Ala714Ala	exon17	21	21	3	45	0.700	0.300	ggatgtcattgc[c/t]gaggacctccgc	
	C22682T		intron17	46	1	0	47	0.989	0.011	tcacccctatcc[c/t]ctggcaggccgc	
	C25013T[c]		intron18	23	22	3	48	0.708	0.292	ctgggggagaag[c/t]tggacctcacct	rs3764264
	G27029A		intron20	18	25	4	47	0.649	0.351	ttttcttgtgac[g/a]gtggtgcctgag	
	C27646T[b]		intron20	6	26	15	47	0.404	0.596	aaggggcgttgg[c/t]ggggccctgggc	rs2278490
	T27681C[b*]		intron20	5	23	18	47	0.351	0.628	tggatgcgcggc[t/c]gctggctctgct	rs2278489
	A27681C[*]			0	1	—		0.011	—	tggatgcgcggc[a/c]gctggctctgct	
	T27681A[*]			—	0	—		—	—	tggatgcgcggc[t/a]gctggctctgct	
	T29320A	Leu849His	exon22	367	5	0	372	0.993	0.007	tcattccctatc[t/a]ccttggccgcaa	
	C29372T[c]	Gly866Gly	exon22	23	22	3	48	0.708	0.292	tgtgttcgtagg[c/t]ggccagattaac	rs5804
	G34262A		intron22	44	1	3	48	0.927	0.073	tctcaagaaaaa[g/a]taataacaataa	
	G34372A[d]	Arg904Gln	exon23	45	3	0	48	0.969	0.031	accagaaccctc[g/a]ggctgagcagta	
	C34588T		intron23	41	3	4	48	0.885	0.115	cacagggcaagg[c/t]ggctgcagcccc	
	T37125C		intron23	46	1	0	47	0.989	0.011	cctcaacccact[t/c]tctcgtccccag	
	C37210T	Asn931Asn	exon24	46	1	0	47	0.989	0.011	ggccactgtcaa[c/t]gagatgcggcgg	
	A37311G[c]		intron24	23	21	3	47	0.713	0.287	acgcgacacatc[a/g]ctgggtcaggga	rs2289117
	G39097A		intron24	29	1	0	30	0.983	0.017	gaggccatagac[g/a]tggtgaaggatt	
	C39119T		intron24	29	1	0	30	0.983	0.017	attgagtgacct[c/t]gatgatatggga	
	C39142T		intron24	40	7	0	47	0.926	0.074	gaagtgaccact[c/t]ggctttctcccg	rs3816118
	G39143A[d]		intron24	44	3	0	47	0.968	0.032	aagtgaccactc[g/a]gctttctcccgc	rs2289116
	C39203T	Ser967Phe	exon25	46	1	0	47	0.989	0.011	tgctggattact[c/t]ccgagacgctgc	

Table A2. (Continued)

Gene name	Allele 1/Allele 2 SNPs	Amino acid change	Region	Allele 1 Homo	Hetero	Allele 2 Homo	Total	Allele frequency		Flanking sequence	dbSNP ID
								Allele 1	Allele 2		
	C39240T[d]		intron25	43	4	0	47	0.957	0.043	gtaagtagtgcc[c/t]ggctggtgggag	rs2289115
	C39375T[e]		intron25	23	20	4	47	0.702	0.298	acatagccctgg[c/t]gattcttagcat	rs2289114
	C48128T	Ile1008Ile	exon26	38	9	0	47	0.904	0.096	agtcatcctgat[c/t]cgaggaaaccag	rs2289113
	A48195G	3'UTR	exon26	46	1	0	47	0.989	0.011	acatccctgtcc[a/g]cagctctgagtg	
MLR	*C−2G*		exon2	0	20	27	47	0.213	0.787	tttatttgttag[c/g]gatggagaccaa	rs2070951
	G218A	Cys73Tyr	exon2	30	1	0	31	0.984	0.016	aactactccctt[g/a]ccttcagcaaga	rs5522
	G449A	Arg150His	exon2	45	3	0	48	0.969	0.031	gaaatggccatc[g/a]tccttccactct	
	G538A[a]	Val180Ile	exon2	0	14	34	48	0.146	0.854	gtcatgcgcgcc[g/a]ttgttaaaagcc	
	T1497C[a]	Asp499Asp	exon2	0	14	34	48	0.146	0.854	agaaccagatga[t/c]gggagctattac	rs5525
	A1661G	Asn554Ser	exon2	43	5	0	48	0.948	0.052	ttcctcctgtca[a/g]tactttagtgga	rs5527
	G1872A		intron2	45	3	0	48	0.969	0.031	gttttaaggatg[g/a]tcatatgttgct	
WNK1	*G421A*	Ala141Thr	exon1	89	5	0	94	0.973	0.027	cctccagccgct[g/a]ccgcccctgggg	
	C446T	Ala149Val	exon1	90	4	0	94	0.979	0.021	aacaggccgtcg[c/t]gggccctgcccc	
	C511T	Leu171Phe	exon1	93	1	0	94	0.995	0.005	tcccagcctagc[c/t]ttgtggggagca	
	G786A[f]		intron1	0	15	80	95	0.079	0.921	actttatttgac[g/a]gtcctttggatc	rs3858703
	A59884G		intron1	88	1	0	89	0.994	0.006	tctgagttacac[a/g]ttaacagtaaag	
	C73737G[f]		intron3	0	16	79	95	0.084	0.916	gactggctttct[c/g]acattcctttta	rs2158502
	A76571G[f]	Ala429Ala	exon4	0	16	78	94	0.085	0.915	ccaaaatgctgc[a/g]cagatctaccgt	
	C105668A[g]		intron5	91	4	0	95	0.979	0.021	tttcttttccct[c/a]tgtttggaagat	
	T105758C[g]	Asp493Asp	exon6	91	4	0	95	0.979	0.021	agcagaagaaga[t/c]gatggagaaaaa	rs2286006
	G105987A		intron6	93	1	0	94	0.995	0.005	tgatgaagtgcc[g/a]tgtgtggcatat	
	A107419G		intron6	75	13	0	88	0.926	0.074	tttcaatatact[a/g]ctgcttaattta	
	C108560T	Thr665Ile	exon8	85	10	0	95	0.947	0.053	cctctgtcttca[c/t]agaatctcgagt	rs2286007
	G124751A[h]	Gln776Gln	exon10	4	26	56	86	0.198	0.802	gccagtgagtca[g/a]cctcaagctcca	rs1012729
	T125972A		intron10	92	1	0	93	0.995	0.005	tttttttttttt[t/a]aagcctgtctgt	
	G126163A[i]	Gln843Gln	exon11	75	20	1	96	0.885	0.115	ccctgtctctca[g/a]attcccatatca	
	A128177C[j]	Thr1056Pro	exon13	3	19	71	93	0.134	0.866	gcagtagcacag[a/c]cccaagctaccc	rs956868
	C128274T[h]		intron13	60	28	5	93	0.796	0.204	gacggtatgaaa[c/t]gccaaactgtca	
	C129494T[i]		intron16	74	20	1	95	0.884	0.116	acaattatggta[c/t]gtctgcatttgg	
	A129852G	Ile1172Met	exon16	88	4	0	92	0.978	0.022	tattctagcaat[a/g]gagagagagtcg	
	C130104T		intron16	90	2	0	92	0.989	0.011	gacacccatgac[c/t]gacaacaaactt	
	T130917G[k]		intron18	44	39	12	95	0.668	0.332	gatattgtagta[t/g]gtgtttatttct	
	C131195T	Asn1320Asn	exon19	20	47	28	95	0.458	0.542	agaaggacccaa[c/t]acagcacctcca	
	C131279T[j]	Thr1348Thr	exon19	72	19	3	94	0.867	0.133	tggagtcccaac[c/t]acagcagcagcc	
	C132236T	Ser1667Ser	exon19	87	2	0	89	0.989	0.011	cagtgaacacag[c/t]tcatctggagct	
	C132444G	Pro1737Ala	exon19	88	1	0	89	0.994	0.006	caagtttctacc[c/g]cagtcagcacta	
	T132576–[j]		intron19	68	17	3	88	0.869	0.131	atcagttttttt[t/-]ctccctaatgag	
	A132655G		intron19	20	36	15	71	0.535	0.465	cttatagtattt[a/g]ttaaattgacag	
	C133634T[i]		intron19	72	19	0	91	0.896	0.104	tttagcgtctca[c/t]ggacttgatttt	
	G135642T[k]	Met1808Ile	exon21	42	42	9	93	0.677	0.323	tagtccagagat[g/t]atcacagtgact	
	T135771G		intron21	92	1	0	93	0.995	0.005	tttaacatgtat[t/g]cagagttcctgc	
	G136943A	Gln1832Gln	exon22	93	1	0	94	0.995	0.005	agcaggaacaca[g/a]cctcagaagggt	
	A141069T	Gly1858Gly	exon23	86	3	0	89	0.983	0.017	ttttaagatggg[a/t]cgatttcaggta	
	C141114T[h]		intron23	58	27	4	89	0.803	0.197	cttgattccttc[c/t]tttggaggagtt	rs2301880
	T142439C[i]		intron23	70	19	1	90	0.883	0.117	tgattctttttt[t/c]ccttttttaaat	
	C142763T	Arg1945Cys	exon24	87	6	0	93	0.968	0.032	accaaggttgga[c/t]gttttcaggtga	
WNK4	*C163T*	Arg55Cys	exon1	95	1	0	96	0.995	0.005	gagccccggccg[c/t]gctcttctcgtc	
	G288A	Arg96Arg	exon1	95	1	0	96	0.995	0.005	tggccccgcgag[g/a]agcccaccgcct	
	C383T	Pro128Leu	exon1	95	1	0	96	0.995	0.005	gtcccgagctcc[c/t]ggactctgcagt	
	T2074C	Ser211Ser	exon2	93	1	0	94	0.995	0.005	tcggaaactgtc[t/c]agagctgagcgg	
	C2285T		intron2	87	7	0	94	0.963	0.037	gatgtgtgccca[c/t]tgcttcctgaac	