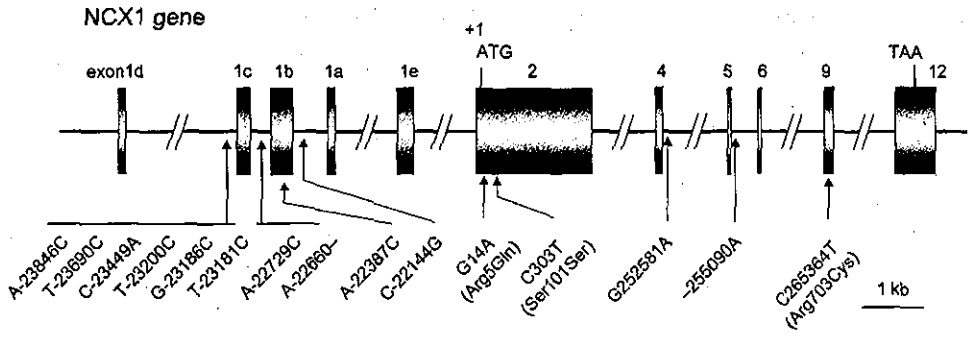

Fig. 1. *Genome structure of human* NCX1. *The* NCX1 *gene consists of sixteen exons, five (exons 1a–1e) of which direct tissue-specific transcription and eleven (exons 2–12) of which encode the open reading frame (17). The five tissue-specific transcription exons (exons 1a–1e) and the exons in which 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 SNPs	TaqMan typing	Amino acid change	Region	Allele 1 Homo	Hetero	Allele 2 Homo	Total	Allele frequency Allele 1	Allele frequency Allele 2	Flanking sequence
−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	attccgcccccct[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.

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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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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.

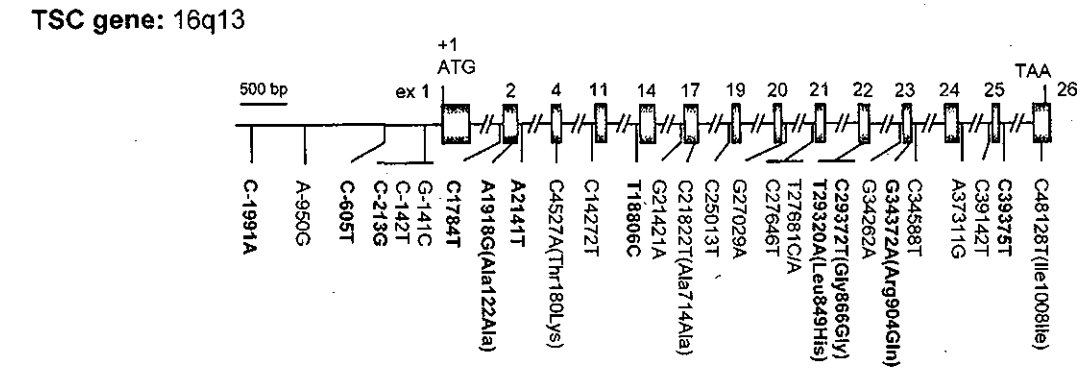

Fig. 1. *Location of genetic variants identified in* TSC. *Nucleotide numbers were counted from the initiation codon (ATG). Sequencing regions are indicated by the bars above the schematic gene structure. The initiation codon, ATG, and stop codon, TAA, are also shown. The single nucleotide polymorphisms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

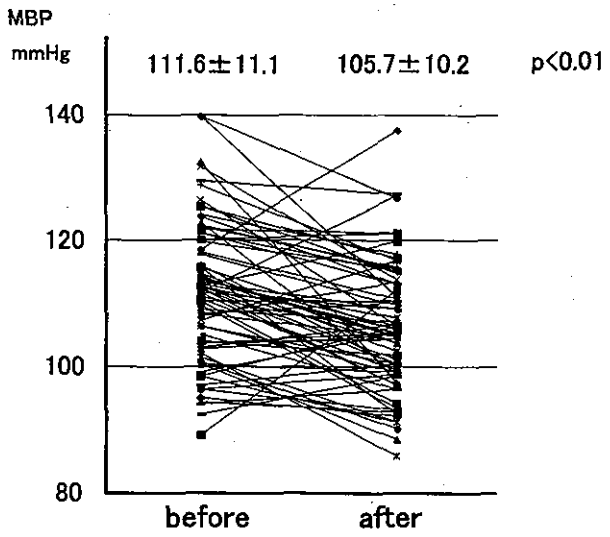

Fig. 2. *Mean blood pressure (MBP) before and after treatment with thiazide diuretics (TZDs) in all subjects. The average MBP in all subjects was significantly reduced by treatment with TZDs.*

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/m^2)	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
ADDI	*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.

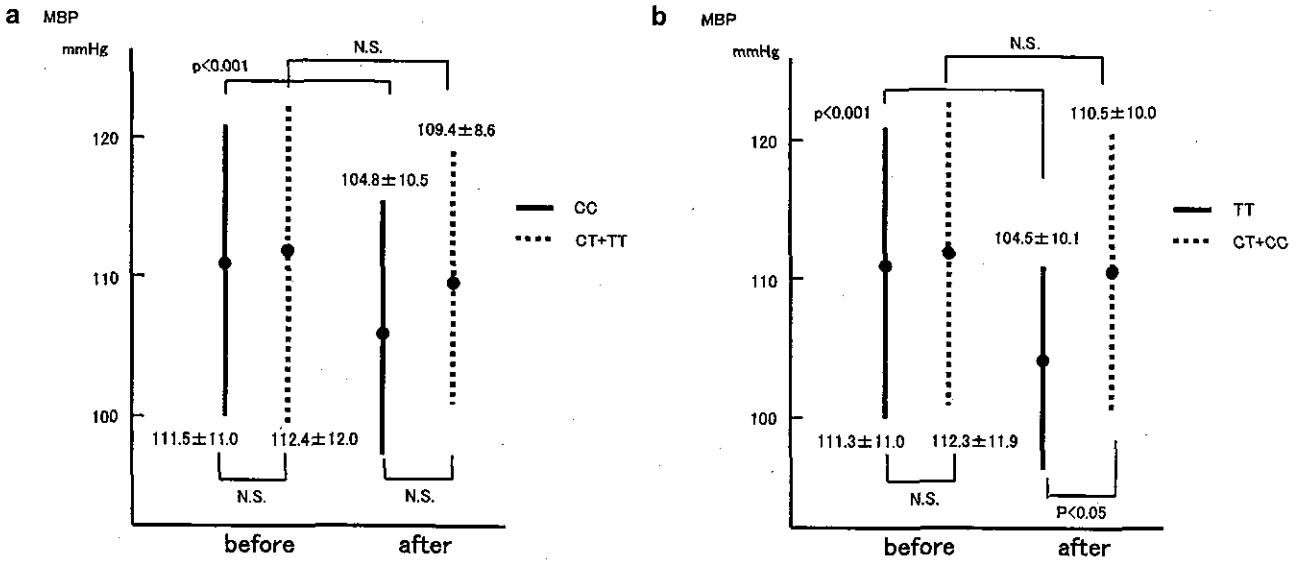

Fig. 3. *a: Comparison of the MBP changes before and after TZD therapy between homozygotes of the major allele CC and the minor homo- and heterozygote TT+CT of* TSC C1784T. *NS, not significant. b: Comparison of MBP changes before and after TZD administration between the homozygotes of the major allele 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 β3 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

1. Chobanian AV, Bakris GL, Black HR, *et al*: The seventh report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 Report. *JAMA* 2003; **289**: 2560–2572.
2. Guideline Subcommittee: 1999 World Health Organization-International Society of Hypertension guidelines for the management of hypertension. *J Hypertens* 1999; **17**:

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	tcagcccccacc[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	atgggggcccaa[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	gtgttttcccttt[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 Allele 1	Allele frequency Allele 2	Flanking sequence	dbSNP ID
	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	aactactcccttt[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]acattccttta	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	tttcttttcccct[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	ttttttttttt[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	tgattcttttttt[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	

Table A2. (Continued)

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
	A4732G	Ile474Val	exon6	94	1	0	95	0.995	0.005	gacaaccaggcc[a/g]tcgagttcctgt	
	A6744G	Met546Val	exon7	277	1	0	278	0.998	0.002	gcaactgtgccc[a/g]tggcccccggtc	
	C6749T[l]	Ala567Ala	exon7	87	5	1	93	0.962	0.038	tgtgcccatggc[c/t]cccggtcccccc	
	G7144T	Ala601Ser	exon8	89	6	1	96	0.958	0.042	gcctcagaccct[g/t]cccttcagcccc	
	A7235		intron8	83	12	1	96	0.927	0.073	tgggggctccc[a/del]gccattccaagc	
	G8119A		intron11	95	1	0	96	0.995	0.005	gaggggagaga[g/a]atgaggacagag	
	G12806C[l]		intron12	89	6	1	96	0.958	0.042	cgcgcccagcct[g/c]atgttttaagat	
	T12948C	Ile740Thr	exon12	95	1	0	96	0.995	0.005	ggattcgggaga[t/c]tatccagcgagt	
	G14139C	Gly808Ala	exon14	90	1	0	91	0.995	0.005	catcttctcctg[g/c]aactccttgtc	
	G14440A[l]	Pro908Pro	exon14	89	6	1	96	0.958	0.042	tttcttcctcc[g/a]tgcccctccact	rs2290042
	C14597T[l]	Pro961Ser	exon14	88	6	1	95	0.958	0.042	cctagtcccctc[c/t]ctagcctgcccc	rs2290041
	C14717T		intron14	75	19	0	94	0.899	0.101	agggagactcca[c/t]tctgcactcttc	rs2290040
	C15503A	Pro1173Thr	exon17	278	1	0	279	0.998	0.002	aagcagcccccca[c/a]cgggtattgtgg	
	T15677C		intron17	275	2	0	277	0.996	0.004	ctgtcgactgtt[t/c]ttctccaggccc	
	C15703T		intron17	277	1	0	278	0.998	0.002	gggggtctgccc[c/t]gggggaatagac	
	C15738A		intron17	272	4	0	276	0.993	0.007	cacctcccctttt[c/a]ctcacttagtgc	
NCX1	*A−23846C*		intron1d	94	1	0	95	0.995	0.005	tcacactgcctt[a/c]aattcagggact	
	T−23690C		intron1d	62	31	2	95	0.816	0.184	aaatttaactta[t/c]agcaaggaaaga	
	C−23449A		intron1d	85	9	1	95	0.942	0.058	catactcacatt[c/a]atgtttgaggag	
	T−23200C[m]		intron1d	0	9	86	95	0.047	0.953	attccgcccccct[t/c]tttgttgcggag	rs2301340
	G−23186C[m]		intron1d	0	9	86	95	0.047	0.953	ttgttgcggagg[g/c]aaactgaggttc	rs2301341
	T−23181C		intron1d	18	57	20	95	0.489	0.511	gcggaggcaaac[t/c]gaggttcctgga	rs2301342
	A−22729C		intron1c	71	23	1	95	0.868	0.132	taattatgagga[a/c]agtgatttattg	rs2301343
	A−22660—		intron1c	94	1	0	95	0.995	0.005	gattgctgcatt[a/-]ggtttttttccca	
	A−22387C	5′UTR	exon1b	93	3	0	96	0.984	0.016	attaaaaaaaaa[a/c]tcattgatatat	
	C−22144G		intron1b	84	9	2	95	0.932	0.068	gcgcggccacaa[c/g]gcactgcggggc	
	G14A	Arg5Gln	exon2	95	1	0	96	0.995	0.005	tgtacaacatgc[g/a]gcgattaagtct	
	C303T	Ser101Ser	exon2	95	1	0	96	0.995	0.005	tcggttcatgtc[c/t]tctatagaagtc	
	G252581A		intron4	45	40	11	96	0.677	0.323	tcttctctctcc[g/a]tgtctccctact	rs433572
	—255090A		intron5	94	1	0	95	0.995	0.005	tcaggtgataca[-/a]gtagctctgtga	
	C265364T	Arg703Cys	exon9	95	1	0	96	0.995	0.005	gcagaaatgggg[c/t]gccccatcctgg	

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). [a–m]The apparent linkage disequilibrium was indicated in the Gene name column. * Triallelic polymorphism.

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An association analysis between genetic polymorphisms of matrix metalloproteinase-3 and methylenetetrahydrofolate reductase and myocardial infarction in Japanese

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Matrix metalloproteinases (MMPs), enzymes that degrade extracellular matrix, have been extensively found in human coronary atherosclerotic plaques, which suggests that MMPs play an important role in plaque instability [1]. Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in regulating the plasma homocysteine level, and hyperhomocysteinemia confers an increased risk of coronary artery disease [2]. Polymorphisms of stromelysin-1 (*MMP3*) and *MTHFR* have been reported to be related to an increased risk of myocardial infarction (MI), but the results have been controversial [3–7]. To assess whether these polymorphisms are associated with the incidence of MI, we conducted an association study.

The study population consisted of two groups: (i) 1857 (849 male and 1008 female) controls consecutively recruited from the Suita Study between April 2002 and February 2003 [8,9], and (ii) 548 (474 male and 74 female) patients with MI recruited from the National Cardiovascular Center between May 2001 and April 2003 [10]. The *MMP3* 5A/6A and *MTHFR* C677T polymorphisms were determined by the TaqMan system (the primer and probe sequences are available on request).

Univariate analysis showed that *MMP3* 5A/6A was not associated with the incidence of MI (Table 1). Logistic analysis indicated that the 5A/5A + 5A/6A genotype of *MMP3* only tended to be more susceptible to MI than the 6A/6A genotype [$P = 0.1004$, odds ratio (OR) = 1.23, 95% confidence interval (CI) 0.96, 1.59] in male subjects. *MTHFR* C677T was not associated with the incidence of MI (Table 1). Logistic analysis indicated that the CC genotype of *MTHFR* only tended to be more susceptible to MI than the CT + TT genotype ($P = 0.0911$, OR = 1.52, 95% CI 0.93, 2.48) in female subjects. None of the genotypes significantly influenced the secondary incidence of acute coronary syndrome (Kaplan–Meier method). Moreover, none of the genotypes significantly influenced the severity of coronary atherosclerosis as assessed by the number of stenotic lesions (> 75%) by coronary arteriography. No significant deviation from Hardy–Weinberg equilibrium was observed for *MMP3* 5A/6A or *MTHFR* C677T.

It has been reported that individuals carrying the 6A/6A genotype of *MMP3* are predisposed to developing atherosclerotic plaques with significant stenosis, whereas those carrying the 5A allele are predisposed to developing unstable plaque [4]. In the Japanese population, *MMP-3* 5A/6A was initially

Table 1 Characteristics of subjects and the genotype frequencies in the present study

	Control	MI	*P*-value
Male			
N	849	474	
Age	66.2 ± 0.4	58.3 ± 0.5	< 0.0001
Body mass index (kg m^{-2})	23.2 ± 0.1	23.7 ± 0.1	0.0044
MMP3 5A/6A			
5A5A/5A6A/6A6A	18/200/619 2.2%/23.9%/74.0%	13/127/322 2.8%/27.5%/69.7%	0.2474
MTHFR C677T			
CC/CT/TT	293/411/141 34.7%/48.6%/16.7%	160/226/75 34.7%/49.0%/16.3%	0.9799
Female			
N	1008	74	
Age	63.3 ± 0.3	62.8 ± 1.3	0.7238
Body mass index (kg m^{-2})	22.3 ± 0.1	23.5 ± 0.4	0.0011
MMP3 5A/6A			
5A5A/5A6A/6A6A	16/226/755 1.6%/22.7%/75.7%	1/22/47 1.4%/31.4%/67.1%	0.2677
MTHFR C677T			
CC/CT/TT	370/467/164 37.0%/46.7%/16.4%*	33/24/13 47.1%/34.3%/18.6%	0.1202

MMP3, Matrix metalloproteinase-3; MTHFR, methylenetetrahydrofolate reductase; control, control subjects; MI, myocardial infarction. Differences in numerical data among the groups were evaluated by an unpaired *t*-test. The genotype distributions in the groups were compared by the χ^2 test. *Due to rounding, the percentages may not total 100.

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reported to be associated with the incidence of MI [3]. However, a second report showed that this association was valid in females, but not in males [5]. We also did not observe any positive association in our male subjects. Thus, in Japanese male subjects, *MMP-3* 5A/6A does not seem to predict the incidence of MI. In females, it is possible that we did not observe any positive association, at least partially, due to the relatively small number of female patients with MI, and further investigations may be needed.

Several association studies and meta-analyses have investigated the association between the *MTHFR* gene and an increased risk of MI [6]. In the Japanese population, Yamada *et al.* did not find an association between *MTHFR* C677T and the incidence of MI [5]. Considering these and our present results, it is unlikely that *MTHFR* C677T is associated with an increased risk of MI in Japanese.

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SHORT COMMUNICATION

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Genetic variants in *PCSK9* affect the cholesterol level in Japanese

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Abstract Mutations in the proprotein convertase subtilisin/kexin 9 (*PCSK9*) gene have been reported in affected members of two families with autosomal dominant hypercholesterolemia. To investigate the effects of common variants in *PCSK9* on the cholesterol level, we conducted an association study using a large cohort representing the general population in Japan ($n=1,793$). Direct sequencing in all of the exonic regions identified 21 polymorphisms. After consideration of linkage disequilibrium among these polymorphisms, we selected and genotyped nine polymorphisms by the TaqMan method. The intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with levels of total cholesterol (TC) [C(-161)T, $P=0.0285$; I474 V, $P=0.0069$] and low-density lipoprotein cholesterol (LDL-C) [C(-161)T, $P=0.0257$; I474 V, $P=0.0007$]. The distributions of these polymorphisms in subjects with miocardial infarction (MI) ($n=649$) were not different from those in the control population. These results provide the first evidence that common variants intron 1/C(-161)T and exon 9/I474 V in *PCSK9* significantly affect TC and LDL-C levels in the general population in Japan.

Keywords *PCSK9* · Cholesterol · Myocardial infarction · Polymorphisms · Association study

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Introduction

Proprotein convertase subtilisin/kexin 9 (*PCSK9*) in chromosome 1p34.1-p32 is a proprotein convertase that belongs to the subtilase subfamily (Seidah et al. 2003). A related protein is the subtilisin/kexin isoenzyme-1/site-1 protease, which plays a key role in cholesterol homeostasis by processing sterol regulatory element-binding protein (SREBP) (Brown and Goldstein 1999). The expression of PCSK9 mRNA has been reported to be down regulated by dietary cholesterol in C57BL/6 mice and to be up regulated in SREBP transgenic mice (Maxwell et al. 2003). Mutations in *PCSK9* have been reported in affected members of two families with autosomal dominant hypercholesterolemia (OMIM 603776) (Abifadel et al. 2003). These observations indicate that *PCSK9* plays an important role in cholesterol metabolism. Thus, it is possible that common genetic variations in *PCSK9* might affect the cholesterol level in the general population.

To investigate the effects of common variants in *PCSK9* on cholesterol level, we detected common variants in *PCSK9* by sequencing and conducted an association study using a large cohort representing the general population in Japan. We found that two polymorphisms, intron 1/C(-161)T and exon 9/I474V, were associated with levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). We next investigated the association between these polymorphisms and the incidence of myocardial infarction (MI).