bute to arteriolar nitric oxide dysfunction in Dahl salt-sensitive (Dahl S) rats with salt-induced hypertension. In their experiments, abdominal aortas of Dahl S rats with a high-salt diet showed sixfold greater HO-1 protein concentrations than a low-salt group. It has also been reported that angiotensin II-induced hypertension can increase the expression of HO-1 [10]. Therefore, inducible HO-1 may play a part in hypertension.

In the present study, we screened for sequence variations in the promoter region of the HO-1 gene (*HMOX-1*) and evaluated the significance of polymorphism in essential hypertension.

Methods

Participants

We selected 1998 consecutive patients without any cardiovascular complications, from the Suita study. The selection criteria and design of the Suita study have been described previously [11]. In the present study, participants' information was made anonymous. The study was approved by the Ethics Committee of the National Cardiovascular Centre and by the Committee on Genetic Analysis and Genetic Therapy of the National Cardiovascular Centre. Informed consent to genetic analysis was obtained from about 3700 individuals, and the genotype of *HMOX-1* was determined in 1998 consecutive individuals. Participants were categorized as hypertensive when they had a systolic blood pressure greater than 140 mmHg or a diastolic blood pressure greater than 90 mmHg. Individuals who were taking antihypertensive medication were also categorized as hypertensive.

DNA studies

Genomic DNA from 96 individuals was used as a template for sequence analysis. The promoter region (up to −1.4 kb) was sequenced. (The primer sequences are available on request.) Single-nucleotide polymorphisms were determined using the TaqMan system (PE Applied Biosystems) (Table 1) and $(GT)_n$ repeat length polymorphism was determined on an ABI 3700 DNA sequencing system using GeneScan software after amplification by polymerase chain reaction with a fluorescence-labelled sense primer, P1-S (5′-AGAGCCTGC AGCTTCTCAGA-3′), and an antisense primer, P1-AS (5′-ACAAAGTCTGGCCATAGGAC-3′) (Fig. 1).

Expression study

To explore the regulatory effects of a *T(−413)A* polymorphism in the promoter region, we constructed *HMOX-1* promoter/luciferase fusion genes. The promoter region between −517 and +76 was amplified by PCR with a sense primer, P2-S (5′-GTGAGGAGG CAAGCAGTCAGCAGAGGATTC-3′) and an antisense primer, P2-AS (5′-GTGCTGGGCTCGTTCGT GCTGGCTCC-3′) (Fig. 1) and subcloned into pGL2-Enhancer DNA (Promega), which does not contain any promoter sequence. Transfection was performed in bovine aortic endothelial cells (BAECs) with PRL-CMV vector (Promega) as an internal standard. *Photinus* and *Renilla* luciferase activities were measured with a dual luciferase kit (PG-DUAL-SP, Toyo Ink, Co).

Statistical analysis

Values are expressed as mean ± SE. All statistical analyses were performed using the JMP statistical software package (SAS Institute Inc., Cary, North Carolina, USA). Multiple logistic analyses were performed with other covariates. Differences in numerical data among the groups were analysed by one-way/two-way analysis of variance and the unpaired *t*-test. Differences in frequencies among the groups and the degree of linkage disequilibrium were tested by a contingency table analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Haeme oxygenase-1 promoter polymorphisms

The nucleotide sequence of the 5′-flanking region and exon 1 of the human *HMOX-1* gene is shown in Figure 1. We found *G(−1135)A* and *T(−413)A* polymorphisms and confirmed the existence of $(GT)_n$ repeat length polymorphism in the promoter region of HO-1. The $(GT)_n$ repeat length in the *HMOX-1* gene ranged from 15 to 43. There were 22 genotypes in $(GT)_n$ repeat polymorphism with frequencies greater than 1% (Table 2).

Table 1 Primers and probes for genotype determination

Haeme oxygenase-1	Sequence
G(−*1135*)A	
Sense	5′-AGTCGAGGTGGGAAGATTGCT-3′
Antisense	5′-CCACCATGCCCAGCTAATTTA-3′
Probe for G(−*1135*)	5′-Fam-GAGACCCTGTCTCTACA-MGB-3′
Probe for A(−*1135*)	5′-Vic-AGACCCCGTCTCTACA-MGB-3′
A(−413)T	
Sense	5′-TGACATTTTAGGGAGCTGGAGACA-3′
Antisense	5′-AGGCGTCCCAGAAGGTTCCA-3′
Probe for A(−*413*)	5′-Fam-CCCACCAGGCTATTGCTCTGAGCA-Tamra-3′
Probe for T(−*413*)	5′-Tet-CCCACCAGGCTTTTGCTCTGAGC- Tamra-3′

Fig. 1

Nucleotide sequence of the 5′-flanking region and exon 1 of the human haeme oxygenase-1 gene (GenBank S58267 [12]). The fragment between primers P1-S and P1-AS was amplified by polymerase chain reaction and the $(GT)_n$ repeat length was determined. P2-S and P2-AS were used to construct haeme oxygenase-1 promoter/luciferase fusion genes.

Haplotype frequencies were estimated from data in Table 2 and are shown in Table 3. $A(-413)$-$(GT)_{30}$ and $T(-413)$-$(GT)_{23}$ were the two major alleles.

Association study

Table 4 shows characteristics of the study population. The frequency of hypertensive individuals and the use of antihypertensive drugs were significantly greater in the *AA* genotype than in other genotypes among women: 45.5, 34.2, and 35.0% ($P = 0.0099$) and 23.4, 17.5, and 15.0% ($P = 0.038$), respectively, for the *AA*, *AT*, and *TT* genotypes, respectively. Multiple logistic

Table 2 Number of *A(−413)T* genotypes in each genotype of $(GT)_n$ repeat length polymorphism

$(GT)_n$ repeat length polymorphism	*AA*	*AT*	*TT*
(21,30)	0	25	2
(22,23)	0	1	18
(22,30)	0	16	0
(23,23)	0	7	86
(23,24)	0	5	64
(23,25)	2	5	49
(23,30)	6	217	4
(23,31)	2	29	1
(23,33)	0	21	14
(23,34)	2	3	29
(24,25)	0	2	26
(24,30)	2	71	2
(25,30)	3	66	0
(25,33)	0	13	7
(26,30)	2	24	0
(30,30)	197	13	4
(30,31)	56	4	1
(30,32)	23	3	0
(30,33)	46	16	3
(30,34)	2	52	1
(30,36)	0	24	0

Table 3 Estimated allele frequency

Number of $(GT)_n$ repeats	−413	Estimated allele frequency (%)
30	*A*	42.0
23	*T*	28.0
24	*T*	7.2
25	*T*	6.4
31	*A*	4.6
33	*A*	3.6
34	*T*	2.4
33	*T*	1.9
32	*A*	1.8
22	*T*	1.4
30	*T*	1.1

Alleles with a frequency of less than 1% are not shown.

analyses indicated that the *T(−413)A* (*AA/TA+TT*) polymorphism ($P = 0.0058$), age ($P < 0.0001$), and body mass index ($P < 0.0001$) affected the occurrence of hypertension in women. The odds ratio of the *AA* genotype for hypertension in women was 1.59 ($P = 0.0058$; 95% confidence interval 1.14 to 2.20). The lack of a difference in blood pressure among the three genotypes in women may be attributable to the use of antihypertensive drugs. This association was not observed in men. No significant difference was observed in the frequency of the *G(−1135)A* genotype between normotensive and hypertensive individuals.

Functional significance of T(−413)A polymorphism

We next examined the functional significance of *T(−413)A* polymorphism *in vitro* using BAECs. As shown in Figure 2, the promoter activity of the *A(−413)*-$(GT)_{30}$ allele was significantly greater than that of the *T(−413)*-$(GT)_{23}$ allele *in vitro* ($P < 0.01$). Because the basal activity in this promoter region was low [12], we used a vector that contains an SV40 enhancer sequence. The same results were obtained without an enhancer sequence, although the activity was low.

Discussion

In the present study, we found previously unidentified sequence variations in the promoter region of HO-1. We then examined the relationship between these polymorphisms and the occurrence of hypertension. The frequency of hypertensive individuals and the use of antihypertensive drugs were significantly greater in the *AA* genotype of *T(−413)A* polymorphism of the *HMOX-1* gene than in other genotypes among women. However, this association was not observed in men.

Recently, *HMOX-1* gene promoter microsatellite polymorphism has been reported to be associated with emphysema and restenosis after percutaneous transluminal angioplasty [13,14]. In these reports, the number of $(GT)_n$ repeats was divided into three classes. However, no rational explanation was given for this classification. As *A(−413)*-$(GT)_{30}$ and *T(−413)*-$(GT)_{23}$ are the two major alleles, and our promoter assay showed that the promoter activity of the *A(−413)*-$(GT)_{30}$ allele was significantly greater than that of the *T(−413)*-$(GT)_{23}$ allele, determination of the *T(−413)A* genotype should be sufficient to decide whether there is any functional alteration in the *HMOX-1* gene.

Ever since the haeme oxygenase enzyme was isolated in 1968, research in the field has largely focused on the role of this enzyme in haeme metabolism. However, in recent years, as a result of increased awareness of the role of haeme oxygenase in a variety of biological processes, there has been growing interest in its role in maintaining cellular homeostasis. Carbon monoxide, which is one of the products of haeme oxygenase, participates in the control of cardiovascular functions, including the regulation of blood pressure. Carbon monoxide has been shown to induce the relaxation of vascular smooth muscle cells (VSMCs) by stimulating soluble guanylyl cyclase [15]. The activation of soluble guanylyl cyclase converts GTP to cGMP and intracellular cGMP regulates biological functions by activating cGMP-dependent protein kinases [16]. Thus the synchronized activities of the haeme oxygenase–carbon monoxide–soluble guanylyl cyclase–cGMP system may constitute an important metabolic pathway in the modulation of blood pressure.

A previous in-vitro investigation showed that nitric oxide and carbon monoxide activate guanylate cyclase by distinct mechanisms, carbon monoxide being far less potent than nitric oxide [7]. Because of the difference in cyclase-activating properties between these gases, carbon monoxide endogenously generated from VSMCs may modulate cyclase activity by competing with nitric oxide released from endothelial cells [6]. It has also

Table 4 Characteristics of men and women classified by haeme oxygenase-1 genotype

Characteristic	Haeme oxygenase-1 genotype			P
	AA	*AT*	*TT*	
Men ($n = 962$)	($n = 194$)	($n = 463$)	($n = 305$)	
Age (years)	61.4 ± 0.88	61.2 ± 0.57	60.7 ± 0.70	0.78
Body mass index (kg/m2)	22.7 ± 0.20	23.3 ± 0.13	23.2 ± 0.16	0.12
Waist-to-hip circumference ratio	0.91 ± 0.01	0.91 ± 0.01	0.91 ± 0.01	0.50
Alcohol consumption (ml/day)	25.2 ± 3.8	25.9 ± 1.2	25.8 ± 1.5	0.94
HbA_{1c} (mg/dl)	5.4 ± 0.06	5.4 ± 0.04	5.4 ± 0.04	0.94
HDL cholesterol (mg/dl)	64.2 ± 1.0	63.8 ± 0.7	62.5 ± 0.85	0.37
Total cholesterol (mg/dl)	201 ± 2.3	205 ± 1.5	205 ± 1.8	0.22
Triglycerides (mg/dl)	142 ± 8.6	148 ± 5.6	144 ± 6.8	0.82
Uric acid (mg/dl)	5.8 ± 0.09	5.9 ± 0.06	5.9 ± 0.07	0.66
Proteinuria (%)	14.5	15.2	17.2	0.66
Current smoking (%)	38.7	38.0	37.2	0.94
Ischaemic heart disease (%)	1.6	1.9	1.9	0.88
Cerebrovascular accident (%)	3.1	3.7	2.3	0.55
Use of antihypertensive drugs (%)	16.0	18.1	19.3	0.63
SBP (mmHg)	128.2 ± 1.4	129.2 ± 0.89	129.0 ± 1.1	0.82
DBP (mmHg)	81.0 ± 0.78	81.3 ± 0.50	80.7 ± 0.62	0.72
Pulse pressure (mmHg)	47.2 ± 1.0	47.8 ± 0.67	48.4 ± 0.83	0.70
Heart rate (beats/min)	67.3 ± 0.59	67.3 ± 0.38	67.2 ± 0.47	0.98
Age- and BMI-adjusted SBP (mmHg)	127.7 ± 1.2	129.2 ± 0.80	129.3 ± 1.0	0.89
Age- and BMI-adjusted DBP (mmHg)	81.3 ± 0.74	81.4 ± 0.48	80.3 ± 0.59	0.71
Hypertension (%)†	37.6	41.7	40.7	0.62
Women ($n = 1036$)	($n = 231$)	($n = 439$)	($n = 326$)	
Age (years)	60.0 ± 0.77	59.1 ± 0.54	58.4 ± 0.65	0.27
Body mass index (kg/m2)	22.3 ± 0.21	22.3 ± 0.14	22.5 ± 0.17	0.72
Waist-to-hip circumference ratio	0.89 ± 0.01	0.89 ± 0.01	0.90 ± 0.01	0.82
Alcohol consumption (ml/day)	5.9 ± 0.76	5.0 ± 0.5	5.4 ± 0.6	0.57
HbA_{1c} (mg/dl)	5.2 ± 0.04	5.3 ± 0.03	5.2 ± 0.03	0.29
HDL cholesterol (mg/dl)	64.2 ± 1.0	63.8 ± 0.7	62.5 ± 0.85	0.37
Total cholesterol (mg/dl)	215 ± 2.1	216 ± 1.5	216 ± 1.8	0.90
Triglycerides (mg/dl)	104 ± 5.2	111 ± 3.6	112 ± 4.4	0.47
Uric acid (mg/dl)	4.5 ± 0.07	4.4 ± 0.05	4.5 ± 0.06	0.058
Proteinuria (%)	12.1	8.1	8.6	0.22
Current smoking (%)	4.8	10.0	9.5	0.053
Ischaemic heart disease (%)	0.9	0.6	0.9	0.88
Cerebrovascular accident (%)	1.7	0.63	1.5	0.31
Use of antihypertensive drugs (%)	23.4	17.5	15.0	0.038
SBP (mmHg)	131.5 ± 1.4	128.7 ± 0.96	128.8 ± 1.2	0.22
DBP (mmHg)	79.6 ± 0.72	78.6 ± 0.50	79.0 ± 0.61	0.51
Pulse pressure (mmHg)	51.9 ± 1.0	50.1 ± 0.70	49.7 ± 0.85	0.23
Heart rate (beats/min)	68.5 ± 0.54	68.3 ± 0.37	68.3 ± 0.45	0.93
Age- and BMI-adjusted SBP (mmHg)	132.9 ± 1.4	128.0 ± 0.84	128.5 ± 1.0	0.35
Age- and BMI-adjusted DBP (mmHg)	80.1 ± 0.68	78.2 ± 0.47	79.0 ± 0.57	0.55
Hypertension (%)†	45.5	34.2	35.0	0.0099

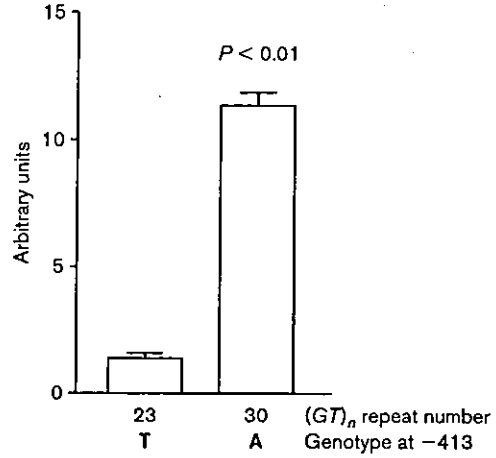
Values are mean ± SE, or %. HbA_{1c}, glycated haemoglobin; HDL, high-density lipoprotein; BMI, body mass index; SBP, DBP, systolic and diastolic blood pressures.
†Percentage of group with hypertension.

been reported that high concentrations of carbon monoxide inhibit NOS activity and nitric oxide generation [8]. These authors also examined the in-vivo effects of an HO-1 inducer, cobalt chloride ($CoCl_2$), and found that carbacol-induced release of nitric oxide from $CoCl_2$-treated rats was significantly reduced compared with that from arterial segments from control rats. In this case, high concentrations of carbon monoxide produced by haeme oxygenase may directly inhibit the generation of nitric oxide and deplete cellular stores of nitric oxide.

Recently, Johnson *et al.* [9] found that expression of vascular HO-1 and production of endogenous carbon monoxide were increased in Dahl S rats with salt-induced hypertension, but not in Dahl salt-resistant rats receiving a high-salt diet. Acute pretreatment with an inhibitor of haeme oxygenase, chromium mesoporphyrin, enhanced vascular responses to N^{ω}-nitro-L-arginine methyl ester (L-NAME) and acetylcholine in both groups, but abolished the differences between high-salt and low-salt arterioles. Therefore, their results suggest that increased concentrations of endogenous carbon monoxide contribute to arteriolar nitric oxide dysfunction *in vivo*.

As men have higher blood pressure than women, it is possible that female hormones may play a part in protecting women from developing higher blood pressures. Oestrogen has been shown to stimulate the production of nitric oxide [17,18]. Huang *et al.* [19] examined the possible effect of oestrogen on flow-induced dilatation of arterioles in four groups of rats: males, ovariectomized females, normal females, and

Fig. 2



Assessment of promoter activity. Transient transfection of haeme oxygenase-1 promoter/luciferase fusion genes was performed in bovine aortic endothelial cells. *Photinus* luciferase activity, which indicated promoter activity of the haeme oxygenase-1 gene, was divided by *Renilla* luciferase activity and expressed as relative luciferase units. The *A(−413)*-$(GT)_{30}$ allele had significantly greater promoter activity than the *T(−413)*-$(GT)_{23}$ allele ($P < 0.01$). $n = 4$ for each experiment.

ovariectomized females with oestrogen replacement. They found a greater flow-induced dilatation of arterioles in rats with high concentrations of oestrogen that was completely eliminated by 10^{-3} mol/l L-NAME. The effect of nitric oxide produced by oestrogen on blood pressure control was also observed in mice deficient in oestrogen β-receptor [20]. Thus the inhibitory effects of carbon monoxide from *HMOX-1* on guanylyl cyclase or eNOS may be more evident in females, as the vascular tone in females may be more dependent on oestrogen-mediated nitric oxide compared with that in males.

We did not observe a difference in the effect of the HO-1 genotype on blood pressure between younger and older groups (data not shown). It has been demonstrated that adaptive structural changes occur in vessels in response to the increased wall stress in hypertension [21]. As the vessel wall thickens and encroaches on the lumen, this adaptive change results in an increased vascular resistance. Therefore, it is possible that an irreversible change may already have occurred during the premenopausal period as a result of a hypertensive status induced by HO-1.

In this study, we found that the *AA* genotype of the *T(−413)A* polymorphism is associated with high blood pressure in women, possibly as a result of the high expression of HO-1. This polymorphism of *HMOX-1* may also be useful for identifying individuals with nitrate tolerance. Future studies may be required in a different group.

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Original Article

Epidemiological Evidence of an Association between *SLC6A2* Gene Polymorphism and Hypertension

Koh ONO, Yoshitaka IWANAGA, Toshifumi MANNAMI, Yoshihiro KOKUBO, Hitonobu TOMOIKE, Kazuo KOMAMURA, Keisuke SHIOJI, Naomi YASUI, Naomi TAGO, and Naoharu IWAI

Selective blockade of the norepinephrine transporter with reboxetine has been reported to induce a slight but significant increase in blood pressure. This study was designed to examine the relation of genetic variants of the norepinephrine transporter gene (solute carrier family 6, member 2; *SLC6A2*) with hypertension in a Japanese population. We genotyped five genetic variants of *SLC6A2*, three in the promoter region and two in the intronic sequence, in 1,950 subjects recruited from the Suita study. One of the variants, an A>G polymorphism in the promoter region (Promoter 3 polymorphism), was found to be associated with hypertension. Multiple logistic analysis indicated that sex (p=0.0223), age (p<0.0001), body mass index (p<0.0001), alcohol consumption (p=0.0002), and the Promoter 3 genotype (AA=1, AG+GG=2) (p=0.0090) were predictive of hypertensive status. The odds ratio of the AG+GG genotypes for hypertension was 1.35 (95% confidence interval: 1.08–1.69) over the AA genotype. *SLC6A2* may be one of the genes that contribute to hypertension in Japanese. To our knowledge, this is the first report to detect associations between *SLC6A2* genetic variants and blood pressure. (*Hypertens Res* 2003; 26: 685–689)

***Key Words*: genetics, epidemiology, norepinephrine, hypertension**

Introduction

Interactions between genetic and environmental factors are thought to play important roles in the pathogenesis of hypertension. The use of association studies in large epidemiological cohorts with a large number of single-nucleotide polymorphisms throughout the entire genome or throughout a single gene is a new strategy for identifying genes that contribute to blood pressure regulation (*1–3*). In the present study, we applied this strategy to the norepinephrine transporter gene (solute carrier family 6, member 2; *SLC6A2*) to examine whether its genetic variants influence blood pressure.

The norepinephrine transporter plays important roles in cardiovascular homeostasis. At most synapses, chemical signaling is terminated by a rapid reaccumulation of neurotransmitter into presynaptic terminals. The reuptake of noradrenaline occurs *via* a specific sodium- and chloride-dependent transporter system known as the norepinephrine transporter system. Genetic dysfunction of *SLC6A2* has been reported to cause idiopathic orthostatic intolerance, which is characterized by the absence of orthostatic hypotension but a rapid orthostatic increase in heart rate (*4*).

Interestingly, in some studies, patients with orthostatic intolerance have been reported to exhibit greater supine blood pressure values than age- and sex-matched control subjects (*5*). Selective blockade of the norepinephrine transporter by

From the National Cardiovascular Center, Suita, Japan.

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Address for Reprints: Naoharu Iwai, M.D., Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita 565-8565, Japan. E-mail: niwai@res.ncvc.go.jp

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Table 1. Genetic Variants in *SLC6A2*

Polymorphism	Adjacent sequence
Promoter 1 T>C	ggacatttgtca[c/t]gattgtccctct
Promoter 2 C>G	ccactgtcccccc[c/g]attccctcaccc
Promoter 3 A>G	tgcggaagacag[a/g]gctggtgctgca
Promoter 4 C>T	tcattcacttca[c/t]aggctggttgat
Promoter 5A>G	gggggacataaa[a/g]ttaaacaactag
Intron 5 A>G	cggtcagtgctc[a/g]gtgaccaccaag
Intron 8 C>A	atgaggtccttg[c/a]tgtttcttacag
Exon 9 G>A	ggctgtcatcac[g/a]ggcctggcagat
Intron 13 T>C	ttccctgctgtg[t/c]actgcccaaggc

reboxetine has been reported to induce a slight but significant increase in blood pressure (*6*). More direct evidence that the neuronal reuptake of norepinephrine is impaired in essential hypertension has also been reported (*7*). Thus, *SLC6A2* is a good candidate gene for human essential hypertension.

In the present study, we thoroughly searched for polymorphisms of *SLC6A2* and performed an association study using a large epidemiological cohort. To our knowledge, this is the first report to investigate associations between *SLC6A2* genetic variants and blood pressure.

Methods

Subjects

The selection criteria and design of the Suita study have been described previously (*2*). The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Genetic Therapy of the National Cardiovascular Center. Written informed consent for genetic analysis was obtained from about 3,700 subjects, and the genotype of *SLC6A2* was determined in 1,950 consecutive subjects. Subjects were categorized as hypertensives when they had a systolic blood pressure of >140 mmHg or a diastolic blood pressure of >90 mmHg. Subjects who were taking hypertensive medication were also categorized as hypertensives.

DNA Studies

DNA was isolated from peripheral leukocytes according to standard procedures. Genomic DNA from 24 subjects was used as a template for sequence analyses. The promoter (up to −1.5 kb) and coding regions (exons 1–14) were sequenced. Polymorphisms were determined by the TaqMan system. The results were analyzed using an ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, USA) using allelic discrimination software supplied by the manufacturer.

Statistical Analyses

Values are expressed as the mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc., Cary, USA). Multiple regression and multiple logistic analyses were performed with other covariates. The effects of polymorphisms on blood pressure and heart rate were assessed in subjects who were not receiving cardiovascular medications, since hypertensive subjects with excellent blood pressure control by medication may have normal blood pressure values. We excluded subjects who were receiving antihypertensive treatment, subjects who had had cerebrovascular accidents, subjects with demonstrated ischemic heart disease, and subjects with atrial fibrillation. Residuals of blood pressure values were calculated by adjusting for sex, age, ethanol consumption, and body mass index (BMI) (residuals of blood pressure values were the observed values minus the values predicted based on the sex, age, ethanol consumption, and BMI). Residuals of heart rate were calculated by adjusting for sex. Data was analyzed using a contingency table analysis and one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. The sample power was calculated using SPSS software (SPSS Inc., Chicago, USA).

Results

Detection of Genetic Variants

We sequenced the promoter region (up to −1.5kb) and coding regions (exons 1–14) and found 9 polymorphisms in *SLC6A2* (Table 1). Promoter 1, 4, and 5 polymorphisms were in complete disequilibrium among the 24 subjects sequenced, and the Promoter 1 polymorphism was selected for the association study. Intron 8, exon 9, and intron 13 polymorphisms were in tight linkage disequilibrium (45/48= 93.75% could be explained by the two haplotypes) among the 24 subjects sequenced, and the Intron 8 polymorphism was selected for the association study. Promoter 2, Promoter 3, and intron 5 were not in linkage disequilibrium with the others and were selected for the association study. The primers and probes for genotype determination are summarized in Table 2. No missense mutation was found in the exonic regions.

Association Study

An association study between the polymorphisms and blood pressure status revealed that only the Promoter 3 polymorphism had significant effects on the frequency of hypertension (Table 3). Table 4 shows characteristics of the study population according to the Promoter 3 genotype. The frequencies of the AG and GG genotypes in the Promoter 3 polymorphism were higher among hypertensives. When blood pressure values were adjusted for sex, age, BMI, and

Table 2. TaqMan Analysis of *SLC6A2* Variations

Region	Primers and probes
Promoter 1	5′-GGAACCCATACCCCTCTAGGTTT-3′
T>C	5′-TGAAGTGAATGAGTTAATGAATGAAAGAA-3′
	5′-Fam-AGAGGACATTTGTCACGATTGTCCCTCT-Tamra-3′
	5′-Vic-CAGAGGACATTTGTCATGATTGTCCCTCT-Tamra-3′
Promoter 2	5′-TTGTCTAATTGTTACCTTCTCAAATTCC-3′
C>G	5′-GAGGAAACGGGAAACATAATAAATACA-3′
	5′-Fam-CACTGTCCCCCCATTCCCTCAC-Tamra-3′
	5′-Vic-CACTGTCCCCCGATTCCCTCAC-Tamra-3′
Promoter 3	5′-CGTCAGAAAGGAGGCCAAAA-3′
A>G	5′-TTCGTTGATAAAGAAACTGACCAGATA-3′
	5′-Fam-CAGCACCAGCTCTGTCTTCCGC-Tamra-3′
	5′-Vic-CAGCACCAGCCCTGTCTTCCG-Tamra-3′
Intron 5	5′-CATCAATGCCTACCTGCACATC-3′
A>G	5′-CATCATCACCTTCTCCTGAAAACC-3′
	5′-Fam-CGGTCAGTGCTCAGTGACCACCAA-Tamra-3′
	5′-Vic-CGGTCAGTGCTCGGTGACCACC-Tamra-3′
Intron 8	5′-ATGTGCAGCTCAGACCAATGG-3′
C>A	5′-GCCTCTGGATACAGGATGAACA-3′
	5′-Fam-ATGAGGTCCTTGCTGTTTCTTACA-Tamra-3′
	5′-Vic-ATGAGGTCCTTGATGTTTCTTACA-Tamra-3′

Table 3. Genetic Variants in *SLC6A2* and the Frequency of Hypertension

Polymorphisms	Genotype frequency		*p*
	HTN	NT	
Promoter 1	47.9 : 43.7 : 8.4	47.3 : 42.8 : 9.9	ns
Promoter 2	40.8 : 46.0 : 13.2	39.5 : 47.3 : 13.2	ns
Promoter 3	71.9 : 25.7 : 2.4	76.7 : 21.2 : 2.1	0.045
Intron 5	66.3 : 31.8 : 1.9	66.3 : 30.4 : 3.3	ns
Intron 8	43.9 : 43.9 : 12.2	41.2 : 46.2 : 12.6	ns

Genotype frequency ($n=1,950$) indicates the percentages of homozygotes having the major allele : heterozygotes : homozygotes with the minor allele. HTN, hypertensive subjects; NT, normotensive subjects

ethanol consumption, all of which are reported to affect blood pressure levels, residuals of systolic blood pressure values tended to be higher in subjects with the AG and GG genotypes than in those with the AA genotype.

Multiple logistic analysis indicated that sex ($p=0.0223$), age ($p<0.0001$), BMI ($p<0.0001$), alcohol consumption ($p=0.0002$), and the Promoter 3 genotype (AA=1, AG+GG=2) ($p=0.0090$, $p=0.045$ after Bonferroni's correction for multiple tests) were predictors of hypertensive status. The odds ratio of the AG+GG genotypes for hypertension was 1.35 (95% confidence interval: 1.08–1.69) over the AA genotype. When the Promoter 3 genotype was analyzed using an A-dominant model (AA+AG=1, GG=2), the genotype could not predict hypertensive status ($p=0.55$).

Sample Power Calculation

The sample power in the present study was 0.79 for the distribution, sample size, and α value (0.05, two-tailed).

Discussion

At most synapses, chemical signaling is terminated by a rapid reaccumulation of neurotransmitters into presynaptic terminals. The reuptake of noradrenaline occurs *via* a specific Na^+- and Cl^--dependent transport system which is the target for tricyclic antidepressants such as desipramine and imipramine. Pacholczyk *et al.* isolated a cDNA encoding a human noradrenaline transporter (*SLC6A2*) (*8*). The cDNA sequence predicted a protein of 617 amino acids, with 12–13 highly hydrophobic regions compatible with membrane-spanning domains. They also suggested that analysis of the

Table 4. Characteristics of the Study Population

	AA	AG	GG	*p*
N	1,459	448	43	
% Male	47.8	48.2	44.2	ns
Age	60.1 (0.3)	59.4 (0.6)	60.4 (1.8)	ns
BMI	22.7 (0.1)	22.9 (0.1)	22.5 (0.5)	ns
Ethanol	15.0 (0.6)	15.6 (1.1)	15.6 (3.4)	ns
% Medication	17.3	19.6	18.6	ns
% CVA	2.2	1.6	0.0	ns
% MI	1.6	0.2	2.3	ns
% HTN	36.9	43.1	44.2	0.045
SBP*	124.7 (0.5)	126.5 (1.0)	126.8 (3.2)	ns
DBP*	78.7 (0.3)	78.5 (0.6)	81.1 (1.8)	ns
HR*	67.5 (0.2)	67.6 (0.4)	69.1 (1.4)	ns
R-SBP*	−0.5 (0.5)	+1.5 (0.9)	+2.6 (2.9)	0.089
R-DBP*	0.0 (0.3)	−0.3 (0.5)	+2.7 (1.7)	ns
R-HR*	−0.1 (0.2)	+0.1 (0.4)	+1.7 (1.4)	ns

The characteristics of the study population are summarized according to the Promoter 3 polymorphism. % Male, percentage of male subjects; Age, age of subjects (years); BMI, body mass index (kg/m^2); Ethanol, ethanol consumption (ml/day); % medication, percentage of subjects receiving antihypertensive medication; % CVA, percentage of subjects who have had a cerebrovascular accident; % MI, percentage of subjects who have had a myocardial infarction; % HTN, percentage of subjects with hypertension; SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); HR, heart rate per minute; R-SBP, residuals of SBP adjusting for sex, age, BMI, and ethanol consumption; R-DBP, residuals of DBP adjusting for sex, age, BMI, and ethanol consumption; R-HR, residuals of HR adjusting for sex. *Blood pressure and HR values of subjects who were receiving cardiovascular medication were excluded. The number of subjects analyzed was 1,170 subjects with the AA genotype, 356 subjects with the AG genotype, and 33 subjects with the GG genotype.

structure and function of this transporter may aid structure-based drug design for the treatment of human depression and lead to a determination of whether transporter abnormalities underlie affective disorders.

Genetic dysfunction of *SLC6A2* has been reported to cause idiopathic orthostatic intolerance, which is a syndrome characterized by lightheadedness, fatigue, altered mentation, and syncope, and is associated with postural tachycardia and plasma norepinephrine concentrations that are disproportionately high in relation to sympathetic outflow (*4*).

Interestingly, in some studies, patients with orthostatic intolerance exhibited greater supine blood pressure values than age- and sex-matched control subjects (*5*). The selective inhibition of norepinephrine reuptake by reboxetine has been reported to induce a slight but significant increase in blood pressure (*6*). The results of previous reports that involved measurement of the whole body plasma kinetics of tritiated norepinephrine suggest that neuronal norepinephrine reuptake may be impaired in essential hypertension (*9*). More direct evidence for the impaired neuronal reuptake of norepinephrine in essential hypertension has also been reported by a study using a phenotypically relevant radiotracer method (*7*). Thus, *SLC6A2* is a good candidate gene for human essential hypertension, and the present study showed that one of the promoter variants of *SLC6A2* was associated with hypertensive status.

In this study, we analyzed five polymorphisms. The association between the Promoter 3 genotype and hypertension remained significant after multiple regression analysis with Bonferroni's correction for multiple comparisons, and thus the Promoter 3 genotype was considered useful for the prediction of hypertensive status.

It has been expected that a variant of *SLC6A2* may be associated not only with blood pressure values but also with heart rate values. However, no significant effects of this genotype on heart rate were observed in this study. Heart rate and blood pressure values are both considered to be moderated by various drugs for cardiovascular diseases, including hypertension. Thus, in the present study, we excluded subjects who had received antihypertensive treatment or experienced cerebrovascular accidents, ischemic heart diseases, or atrial fibrillation from the heart rate and blood pressure value analysis. This exclusion might have blurred the effect of the Promoter 3 polymorphism on heart rate rather than blood pressure.

The sample power in this study was 0.79. This means that 79% of studies would be expected to yield a significant effect, rejecting the null hypothesis that the odds are 1.0, and suggesting that this study has adequate statistical power.

The standards of association studies have improved over the years, and it is widely anticipated that such studies will contribute to the understanding of complex traits. To date,

however, only a few association studies have been replicated (*10*). Therefore, replication in other populations will be necessary to confirm our present observations.

SLC6A2 is located at chromosome 16q12.2. *CAPNS2* (calpain small subunit 2) and *FLJ31547* (unknown function) have been reported to exist adjacent to *SLC6A2*. Moreover, the Bardet-Biedl syndrome 2 gene has also been reported to exist at this locus (*11*). The distance for tight linkage disequilibrium may vary according to the chromosomal region and race, and may be beyond 50 kb (*12*). In this sense, it may be necessary to sequence and genotype a wider range of this chromosomal region to identify the genetic variations that truly influence blood pressure.

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Association analyses between polymorphisms in the GJA4 gene cluster and myocardial infarction in Japanese

Dear Sir,

Connexin 37 (*GJA4*) is a major gap junction protein that is mainly expressed in vascular endothelial cells. Connexin 37 has been suggested to play a role in atherogenesis (1). Recently, the *C1019T* polymorphism in *GJA4* has been reported to be associated with myocardial infarction (MI) in a large-scale association study (2). However, this might be a result of linkage disequilibrium with some other truly important polymorphisms of the *GJA4* cluster. Therefore, we performed extensive association analyses between polymorphisms in the *GJA4* cluster and MI.

The *GJA4* cluster in chromosome 1p35 contains 2 related genes *(GJB3 and 5)* within a 40-kb region. Direct sequencing (36 randomly selected subjects) in this region revealed 20 polymorphisms, including the *C1019T* polymorphism that has been reported to be associated with MI (2). We genotyped all of the 20 polymorphisms by the TaqMan method. The study population consisted of 524 male patients (58 ± 10 yrs) with MI recruited from the National Cardiovascular Center, and 594 male controls (65 ± 11 yrs) consecutively recruited from the Suita Study (3).

Univariate analyses showed that the *GJB3 G1182C* (ddSNP:2236214, p=0.0040), *GJA4–1930C/T* (p=0.0162), and *I1297D* (ddSNP:3841825, p=0.0028), but not *C1019T* (p=0.1393), polymorphisms were significantly associated with MI. Logistic analysis indicated that the DD genotype of *I1297D* was more susceptible for MI than the II+ID genotype (p=0.0005, Odds=1.728, 95%CI; 1.270-2.348). No significant deviation from Hardy-Weinberg equilibrium was observed for any polymorphism. Among these three markers, *GJB3 G1182C* and *GJA4 I1297D* were almost completely concordant. Linkage disequilibrium (D'>0.9) was found between *GJA4 I1297D* and –1930C/T and between *GJA4 I1297D* and *C1019T*. The characteristics of the study subjects and genotype distributions of GJA4 polymorphisms are shown in Table 1.

Three previous studies have reported that the *C1019T* polymorphism in *GJA4* was associated with coronary artery disease

Correspondence to:
Naoharu Iwai MD, PhD
National Cardiovascular Center
5-7-1 Fujishirodai Suita
Osaka 565-8565
Japan
Tel.: +81-6-6833-5012, Fax: +81-6-6872-8091
E-mail: niwai@res.ncvc.go.jp

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Table 1: Characteristics of the study subjects and genotype distributions of GJA4 polymorphisms

	Controls (n=588)	Patients with MI (n=528)	p
Age, yrs	65(11)	58(10)	<.0001
Body mass index, kg/m2	23.3(2.9)	23.9(3.1)	<.0001
HTN, %	47.7	54.3	<.0001
DM, %	8.5	40.8	<.0001
HLP, %	13.4	56.3	<.0001
Smoker, %	35.1	65.5	<.0001
BMI (kg/m2)	22.8(3.1)	23.8(3.1)	<.0001
*GJA4 –1930C/T**	495/88/5	463/65/0	0.0162
(CC/CT/TT)	(84.2/15.0/0.9%)	(87.7/12.3/0%)	
GJA4 I1297D#	274/270/44	232/220/72	0.0028
(II/ID/DD)	(46.6/45.9/7.5%)	(44.3/42.0/13.7%)	

Values are expressed as the mean ± SD. *Number of subjects according to the GJA4 –1930C/T genotype (CC/CT/TT). *Number of subjects according to the GJA4 I1297D genotype (II/ID/DD).

in Asian populations as well as in Swedish men (4, 5). Kumari et al. investigated biophysical properties of the polymorphic variant and concluded that it may have little influence on several properties of GJA-mediated intercellular communication (6). The present findings indicate that previously reported associations between the *C1019T* polymorphism and ischemic heart disease might be due to linkage disequilibrium between the *C1019T* and *I1297D* polymorphisms. The *I1297D* polymorphism is located in the 3'-untranslated region of GJA4 mRNA and may be related to the stability of mRNA (7). Further studies are needed to elucidate the biological significance of this polymorphism.

In the present study, the MI and control groups were not age-matched. The mean age in the control group was 7 years greater than that in the MI group. Since subjects who had developed MI at a younger age were excluded from the controls, the controls in the present study may be a subset of subjects who are relatively unsusceptible to MI compared to the general population in Japan.

In conclusion, the present results suggest that the *I1297D* polymorphism is an important marker for a genetic risk of MI in a Japanese population and confirmed previous findings that the GJA4 gene contributes to MI.

Yoshitaka Iwanaga, Toshifumi Mannami, Yoichi Goto, Hiroshi Nonogi, Naoharu Iwai

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Letter to the Editor

Association analysis between polymorphisms of the lymphotoxin-α gene and myocardial infarction in a Japanese population

Recently, a genome-wide association study revealed that variants in the lymphotoxin-α gene (*LTA*) are risk factors for myocardial infarction (MI), based on the multiplex PCR-Invader assay method at 92788 randomly selected gene-based SNPs [1]. It has also been shown that, in in vitro functional analyses, these variants might have some functional significance and that *LTA* may play a role in the pathogenesis of this disorder. However, association studies are plagued by the impression that they are not consistently reproducible [2,3]. Moreover, direct evidence of the contribution of *LTA* to atherogenesis is limited in both animals and humans [4]. Therefore, we performed an association analysis between polymorphisms of *LTA* and MI in a Japanese population.

Four hundred and seventy-seven male patients with MI (<70 years old) were recruited from the National Cardiovascular Center. The mean age was 56±8 years, with a range of 25–70 years. The control group consisted of 372 unrelated Japanese males <70 years old (mean age 59±9 years, range 30–70 years) recruited from the Suita study, which represents the general population in central Japan (Osaka) [5]. From the control group we excluded all subjects with a history of vascular diseases. Genomic DNA was isolated from leukocytes according to standard procedures. Polymorphisms were determined using the TaqMan system (PE Applied Biosystems). Three polymorphisms of *LTA*, *G10A* (exon1), *A252G* (intron1), and *C804A* (exon3), and one polymorphism of nuclear factor of κ light polypeptide gene enhancer in B cells, inhibitor-like 1 (*NFKBIL1*), and *T-63A* (promoter) were genotyped. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc., USA).

The pattern of the frequency distribution of the genotypes is summarized in Table 1. These polymorphisms were almost completely concordant (i.e., the same allele frequencies and almost complete positive linkage disequilibrium). No significant deviation from Hardy–Weinberg equilibrium was observed. The −63A allele in *NFKBIL1* and the 10A, 252G, and A804 alleles in *LTA* were more common in patients than in controls. For example, in *LTA G10A*, a multiple logistic regression analysis, while adjusting for age and the prevalence

Table 1
Distribution of LTA genotypes in MI patients and controls

SNPs			Controls ($n = 372$)	MI patients ($n = 477$)	P
NFKBIL1 (A-63T)	Genotype	TT	166 (44.6%)	160 (33.6%)	0.004
		TA	157 (42.2%)	236 (49.6%)	
		AA	49 (13.2%)	80 (16.8%)	
	Allele frequency	T	0.66	0.58	0.002
		A	0.34	0.42	
LTA (G10A)	Genotype	GG	166 (44.7%)	160 (33.5%)	0.004
		GA	156 (42.1%)	235 (49.3%)	
		AA	49 (13.2%)	82 (17.2%)	
	Allele frequency	G	0.66	0.58	0.001
		A	0.34	0.42	
LTA (A252G)	Genotype	AA	163 (44.9%)	159 (33.4%)	0.003
		AG	153 (42.2%)	236 (49.6%)	
		GG	47 (13.0%)	81 (17.0%)	
	Allele frequency	A	0.66	0.58	0.001
		G	0.34	0.42	
LTA (A804C)	Genotype	CC	164 (44.8%)	161 (33.7%)	0.004
		CA	153 (41.8%)	236 (49.4%)	
		AA	49 (13.4%)	81 (17.0%)	
	Allele frequency	C	0.66	0.58	0.002
		A	0.34	0.42	

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of smoking, diabetes mellitus and hypercholesterolemia, revealed that the frequency of the A allele was significantly higher in patients with MI than in controls. An analysis which assumed that the A allele had dominant effects showed a significant association ($AA + AG$ versus GG: $P = 0.0025$, odds ratio 1.7, 95% CI 1.2–2.3). Although Ozaki et al. reported a significant association between the risk of MI and these polymorphisms, the distribution of genotypes was different (for example, in *LTA G10A*, GG/GA/AA (%): 39.4/49.1/11.5 in Control versus 36.7/44.5/18.8 in MI) and as a result, a recessive association model was assumed [1]. It is well known that one of the weaknesses of a case–control study is the selection of the control subjects, and this might explain the difference between the two studies [6].

Although the precise in vivo mechanism by which *LTA* influences the susceptibility to MI is unknown, the present study supports the notion that this gene is one of the most important genetic determinants of susceptibility to MI that has been detected so far.

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Yoshitaka Iwanaga
Koh Ono
Shuichi Takagi
Masahiro Terashima
Yoshiaki Tsutsumi
Toshifumi Mannami
Naomi Yasui
Yoichi Goto
Hiroshi Nonogi
Naoharu Iwai*
National Cardiovascular Center, Suita, Japan
*Corresponding author. *National Cardiovascular Center*
5-7-1 Fujishirodai Suita, Osaka 565-8565, Japan
Tel.: +81-6-6833-5012; fax: +81-6-6872-8091
E-mail address: niwai@res.ncvc.go.jp (N. Iwai)

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SHORT COMMUNICATION

Keisuke Shioji · Toshifumi Mannami
Yoshihiro Kokubo · Nozomu Inamoto · Shuichi Takagi
Yoichi Goto · Hiroshi Nonogi · Naoharu Iwai

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

K. Shioji · N. Iwai (✉)
Department of Epidemiology,
Research Institute,
National Cardiovascular Center,
5-7-1 Fujishirodai, Suita,
Osaka 565-8565, Japan
E-mail: niwai@res.ncvc.go.jp
Tel.: +81-6-68335012
Fax: +81-6-68722088

K. Shioji
The Organization for Pharmaceutical
Safety and Research of Japan,
Tokyo, Japan

T. Mannami · Y. Kokubo · N. Inamoto
Department of Preventive Cardiology,
National Cardiovascular Center,
Osaka, Japan

S. Takagi · Y. Goto · H. Nonogi
Division of Cardiology,
National Cardiovascular Center,
Osaka, Japan

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).

Subjects and Methods

Subjects

1. The Suita population: Selection criteria and design of the Suita Study have been described previously (Shioji et al. 2004, in press; Mannami et al. 1997). The sample consisted of 14,200 men and women aged 30–79 years, stratified by gender and 10-year age groups, who were selected randomly from the municipal population registry. They were all invited by letter to attend regular cycles of follow-up examinations (every 2 years). The basic population sampling started in 1989 with a cohort study base, and 51.7% ($n = 7,347$) of the subjects responded to the invitation letter and had paid their initial visit to the National Cardiovascular Center by February 1997. The participants visited the center every 2 years for regular health checkups. DNA from leukocytes was initially collected from participants who visited the center between May 1996 and February 1998. In the present study, the genotypes were determined in 1,880 consecutive subjects who visited the center between April 2002 and February 2003 ($n = 1,880$, Table 1). Subjects with ischemic heart disease were excluded.
2. The MI group: Selection criteria and design of the MI group have been described previously (Takagi et al. 2002). This group consisted of 649 patients with MI (553 men and 96 women) who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003 (Table 2).

Written informed consent was obtained from each subject after a full explanation of the study, which was approved by the Ethics Committee and the Committee on Genetic Analysis and Genetic Therapy of the National Cardiovascular Center.

Table 1 Suita population characteristics. *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PR* pulse rate, *% CVA* percentage of subjects with cerebrovascular accident, *% OMI* percentage of subjects with old myocardial infarction, *% HT* percentage of subjects with hypertension, *% DM* percentage of subjects with diabetes mellitus, *% HLP* percentage of subjects with hyperlipidemia, *% drinking* percentage of subjects with a drinking habit, *% smoking* percentage of subjects with a smoking habit

Parameter	Men	Women	*P* value
Number	867	1013	
Age (years)	66.3 ± 0.4	63.3 ± 0.3	< 0.0001
BMI (kg/m^2)	23.2 ± 0.1	22.3 ± 0.1	< 0.0001
SBP (mmHg)	131.8 ± 0.7	128.1 ± 0.6	< 0.0001
DBP (mmHg)	79.7 ± 0.3	76.6 ± 0.3	< 0.0001
PR (beats/min)	66.0 ± 0.3	66.0 ± 0.3	0.9334
Total cholesterol (mmol/l)	5.13 ± 0.03	5.58 ± 0.02	< 0.0001
HDL cholesterol (mmol/l)	1.43 ± 0.01	1.68 ± 0.01	< 0.0001
Triglycerides (mmol/l)	1.38 ± 0.03	1.07 ± 0.03	< 0.0001
Blood glucose (mmol/l)	5.74 ± 0.04	5.30 ± 0.04	< 0.0001
% CVA	3.6	1.4	0.0018
% OMI	2.1	0.5	0.0015
% HT	45.9	37.2	< 0.0001
% DM	11.4	4.5	< 0.0001
% HLP	14.8	24.0	< 0.0001
% drinking	67.0	29.5	< 0.0001
% smoking	29.9	6.3	< 0.0001

P value was calculated by the unpaired *t*-test

Table 2 Miocardial infarction (MI) group characteristics. *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PR* pulse rate, *% CVA* percentage of subjects with cerebrovascular accident, *% OMI* percentage of subjects with old myocardial infarction, *% HT* percentage of subjects with hypertension, *% DM* percentage of subjects with diabetes mellitus, *% LP* percentage of subjects with hyperlipidemia

Parameter	Men	Women	*P* value
Number	553	96	
Age (years)	61.3 ± 0.5	64.8 ± 1.1	0.0028
BMI (kg/m^2)	23.7 ± 0.1	23.6 ± 0.3	0.7056
Total cholesterol (mmol/l)	5.17 ± 0.05	5.43 ± 0.11	0.0400
HDL cholesterol (mmol/l)	1.08 ± 0.02	1.23 ± 0.04	0.0006
Triglycerides (mmol/l)	1.55 ± 0.04	1.21 ± 0.09	0.0010
Blood glucose (mmol/l)	7.45 ± 0.67	6.75 ± 1.59	0.6832
% HT	53.5	61.5	0.1448
% DM	41.7	58.1	0.0034
% HLP	57.9	58.3	0.9402

P value was calculated by the unpaired *t*-test

DNA studies

All 12 exonic regions were sequenced for polymorphisms in 48 healthy subjects. Selected polymorphisms were determined by the TaqMan method. Detailed information will be provided upon request.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Since the distribution of triglyceride (TG) values was skewed, a logarithmic transformation was used for the statistical test; however, untransformed means are shown in Tables 1, 2, 5, 6. LDL-C was calculated by Friedewald's formula [(LDL-C) = (TC) - (HDL-cholesterol) - (TG/5). We excluded those whose HDL-cholesterol (HDL-C) or TG levels were ≥2.6 mM or 4.53 mM respectively]. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc.). Values of $P < 0.05$ were considered to indicate statistical significance. The residuals of lipid levels were calculated by adjusting for gender, age, body mass index (BMI), smoking (cigarettes/day), and consumption of alcohol (ethanol g/week). Data were analyzed using a contingency table analysis and Student's *t*-test. Hardy-Weinberg equilibrium was calculated by a chi-square test. *R*-square values between polymorphisms were analyzed using the SNPAlyze statistical package (Dynacom Inc.).

Results

Direct sequencing identified 21 polymorphisms (Table 3). We regarded r^2-0.5 as tight linkage (Table 4). Polymorphisms with frequencies of ≤ 0.03 in the intronic region and 3'-untranslated region were neglected in further analyses. Polymorphisms that were not accompanied by an amino acid change in the exonic regions were also neglected. Accordingly, we selected and genotyped nine polymorphisms for the following association study.

As shown in Table 5, intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with levels of

Table 3 Polymorphisms and nucleotide sequence in *PCSK9*

Region	Polymorphism	Allele frequency	Sequence
Exon 1	C(-64)A (5'-UTR)	0.13	CCCACCGCAAGGCTCAAGGCGCCGC[C/A]GGCGTGGACCGCGCACGGCCTCTAG
	V4I	0.01	CTCTCCCCTGGCCCTCATGGGCACC[G/A]TCAGCTCCAGGCGGTCCTGGTGGCC
	15–16 ins (+L)	0.13	GCGGTCCTGGTGGCCGCTGCCACTG[CTG/-]CTGCTGCTGCTGCTGCTGCTCCTGG
	A53V	0.13	TTGCGTTCCGAGGAGGACGGCCTGG[C/T]CGAAGCACCCGAGCACGGAACCACA
Intron 1	**C(-161)T**	0.04	TAATAATAGTTGGCCTATATGAGTT[C/T]TTTAATTTGCTTTTTGGTCCGCATT
Exon 2	L112L	0.05	GCCGGGGATACCTCACCAAGATCCT[G/A]CATGTCTTCCATGGCCTTCTTCCTG
Intron 2	**T357C**	0.13	GCACAGTAACTACTGGCTTTCTGTA[T/C]AGAATTCCCTTTAAGCCTGGCCATG
Intron 3	G(-10)A	0.04	CATTCCCTCCTCTCCCACAAATGTC[G/A]CCTTGGAAAGACGGAGGCAGCCTGG
Intron 4	**G-36A**	0.05	CTGATTTGTTATAGGGTGGAGGGGG[G/A]GTCTTTCTCATGTGGTCCTTGTGTT
Exon 6	Q275Q	0.01	GCCTGGAGTTTATTCGGAAAAGCCA[G/A]CTGGTCCAGCCTGTGGGGCCACTGG
	P331P	0.01	GCCTCTACTCCCCAGCCTCAGCTCC[C/T]GAGGTAGGTGCTGGGGCTGCTGCCC
Exon 8	**I424V**	0.01	GATCCACTTCTCTGCCAAAGATGTC[A/G]TCAATGAGGCCTGGTTCCCTGAGGA
Intron 8	T276C	0.03	TCCCTTGTCTGTGTAAGGAGGATGA[T/C]GCCACCTTAAATAGGATTAAATGAG
	T(-57)C	0.03	CTCTCCTACCATGAACTAAAGATTT[T/C]TGTGGAGGTCCCCTCACTCCCAGCA
Exon 9	V460V	0.03	GTTGGCAGCTGTTTTGCAGGACTGT[G/A]TGGTCAGCACACTCGGGGCCTACAC
	I474V	0.03	GGGGCCTACACGGATGGCCACAGCC[A/G]TCGCCCCGCTGCGCCCCAGATGAGGA
Intron 10	**A241G**	0.11	CTTTCTCCTTATGCACCCACTGCCC[G/A]CGAGGCTTGGTCCTCACAAGTGTGA
Exon 12	G67A (3'-UTR)	0.02	CAGTGCCCTCCCTGGGACCTCCCAC[G/A]TCCTGGGGGCCTACGCCGTAGACAA
	C291T (3'-UTR)	0.03	AGCTTTAAAATGGTTCCGACTTGTC[C/T]CTCTCTCAGCCCTCCATGGCCTGGC
	C448T (3'-UTR)	0.03	GTGGAGGTGCCAGGAAGCTCCCTCC[C/T]TCACTGTGGGGCATTTCACCATTCA
	T787C (3'-UTR)	0.07	TCTAGCCAGAGGCTGGAGACAGGTG[T/C]GCCCCTGGTGGTCACAGGCTGTGCC

Bolded polymorphisms were genotyped by the TaqMan method
Allele frequencies described are based on TaqMan data (*bolded* polymorphisms, the Suita population, 1,793 subjects) or sequence data (48 subjects)

Table 4 Linkage disequilibrium among polymorphisms in *PCSK9*

Polymorphism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
C(-64)A	1	*0.80*	*1.00*	*1.00*	0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.07
V4I	2		*0.80*	*0.80*	0.00	0.40	*0.80*	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.02	0.00	0.20
15–16 ins (+L)	3			*1.00*	0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
A53V	4				0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
C(-161)T	5					0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.09	0.09	0.09	0.09	0.15	0.09	0.08	0.03
L112L	6						0.38	0.02	0.01	0.19	0.00	0.00	0.06	0.06	0.06	0.06	0.05	0.00	0.04	0.00	0.00
T357C	7							0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
G(-10)A	8								*0.79*	0.00	0.00	0.00	0.06	0.06	0.06	0.06	0.05	0.00	0.06	0.00	0.03
G-36A	9									0.00	0.00	0.00	0.04	0.04	0.04	0.04	0.03	0.00	0.04	0.00	0.01
Q275Q	10										0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P331P	11											0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
I424V	12												0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.33	0.00
T276C	13													*1.00*	*1.00*	*1.00*	0.10	0.00	*1.00*	0.00	0.00
T(-57)C	14														*1.00*	*1.00*	0.10	0.00	*1.00*	0.00	0.00
V460V	15															*1.00*	0.10	0.00	*1.00*	0.00	0.00
I474V	16																0.10	0.00	*1.00*	0.00	0.00
A241G	17																	0.00	0.09	0.00	0.36
G67A	18																		0.00	*0.66*	0.00
C291T	19																			0.00	0.00
C448T	20																				0.00
T787C	21																				

R^2 values are shown (*italics* indicates $r^2 > 0.5$)
Values are based on the genotypes of 48 subjects used for sequence analyses
Bold polymorphisms were selected for genotyping
All values refer to the variant allele indicated in the table

Table 5 Lipid levels among the *PCSK9* polymorphisms (Suita population). *BMI* body mass index, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol. *TG* triglycerides, *LDL-C* low-density lipoprotein cholesterol, *% drinking* percentage of subjects with a drinking habit, *% smoking* percentage of subjects with a smoking habit

	Intron 1/C(-161)T		*P* value	Exon 9/I474V		*P* value
	CC	CT+TT		II	IV+VV	
Number (%)	1,665 (92.9)	128 (7.1)		1,704 (95.0)	89 (5.0)	
Men/women	754/911	54/74		772/932	38/51	
Age[a]	64.4±0.3	62.8±1.0	0.1054	64.3±0.3	64.1±1.2	0.8125
BMI (kg/m^2)[a]	22.7±0.1	22.9±0.3	0.5178	22.8±0.1	22.5±0.3	0.4568
TC (mM)[b]	5.36±0.02	5.24±0.08	0.0285	5.38±0.02	5.14±0.09	0.0069
HDL-C (mM)[b]	1.57±0.01	1.56±0.04	0.4431	1.56±0.01	1.63±0.04	0.1324
TG (mM)[b]	1.20±0.02	1.21±0.08	0.8826	1.20±0.02	1.15±0.10	0.7617
LDL-C (mM)[b]	3.29±0.02	3.14±0.07	0.0257	3.29±0.02	3.01±0.08	0.0007
% drinking[c]	46.8	45.3	0.1238	46.8	44.9	0.7277
Ethanol (g/week)[a]	75.7±3.2	86.0±11.6	0.3953	77.4±3.2	60.6±14.0	0.2404
% smoking[c]	17.1	22.7	0.7472	17.4	19.1	0.6891
Cigarettes (day)[a]	8.3±0.3	7.5±1.1	0.5378	8.2±0.3	7.9±1.4	0.8145

Values are expressed as the mean±SEM.
The formula for calculating LDL-C is described in "Subjects and methods"
Student's *t*-test was performed on residual values adjusted for age, gender BMI, smoking (cigarettes/day), and alcohol consumption (ethanol, g/week)
For triglyceride values, although a logarithmic transformation was applied for the statistical test, untransformed values are shown

[a] Student's *t*-test was performed
[b] Subjects receiving hypolipidemic medication were excluded (intron 1/C-161T: CC $n=1512$, CT+TT$n=122$; exon 9/I474 V: II$n=1,550$, IV+VV $n=83$)
[c] Chi-square test was performed

TC and LDL-C in the Suita population. Since we only found one subject each who was homozygous for minor alleles, these subjects were categorized as heterozygotes. A gender-based subanalysis indicated that the exon 9/I474 V polymorphism significantly influenced the LDL-C level in both male and female subjects (Table 6). TC level in the IV(+VV) genotype of exon 9/I474 V was also lower than that in the II genotype in both male ($P=0.1656$) and female subjects ($P=0.0133$). Although *P*-values were not statistically significant, partially due to low statistical power, TC and LDL-C levels in the CT(+TT) genotype of intron 1/C(-161)T were lower than those in the CC genotype in both male and female subjects. No significant deviation from Hardy-Weinberg equilibrium was observed in these polymorphisms [C(-161)T: $P=0.8290$, I474 V: $P=0.9971$].

We next evaluated whether intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with the incidence of MI. Distribution of these polymorphisms in subjects with MI were no different from those in the Suita population (Table 7). A gender-based subanalysis indicated that these polymorphisms did not influence the incidence of MI in either male or female subjects (data not shown), nor were they associated with lipid levels in

Table 6 Lipid levels among the PCSK9 polymorphisms (gender-based subanalysis). *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglycerides, *LDL-C* low-density lipoprotein cholesterol

	Intron 1/C(-161)T			Exon 9/I474V		
Men	CC	CT+TT	*P* value	II	IV+VV	*P* value
Number (%)	742 (93.1)	55 (6.9)		757 (95.0)	40 (5.0)	
TC (mM)	5.10±0.03	4.98±0.10	0.1769	5.10±0.03	4.95±0.12	0.1656
HDL-C (mM)	1.43±0.01	1.43±0.05	0.9723	1.42±0.01	1.45±0.06	0.2599
TG (mM)	1.36±0.04	1.43±0.15	0.9598	1.37±0.04	1.41±0.17	0.7717
LDL-C (mM)	3.09±0.03	2.89±0.09	0.0554	3.08±0.03	2.88±0.11	0.0317
Women	CC	CT	*P* value	II	IV	*P* value
Number (%)	770 (92.0)	67 (8.0)		793 (94.9)	43 (5.1)	
TC (mM)	5.58±0.03	5.40±0.10	0.1042	5.59±0.03	5.26±0.12	0.0133
HDL-C (mM)	1.68±0.01	1.65±0.05	0.2716	1.67±0.01	1.77±0.06	0.3345
TG (mM)	1.04±0.02	1.03±0.07	0.7957	1.05±0.02	0.91±0.09	0.1487
LDL-C (mM)	3.44±0.03	3.30±0.10	0.1964	3.45±0.03	3.09±0.12	0.0081

Values are expressed as the mean±SEM
The formula for calculating LDL-C is described in "Subjects and methods"
Subjects receiving hypolipidemic medication were excluded
Student's *t*-test was performed on residual values adjusted for age, BMI, smoking (cigarettes/day), and alcohol consumption (ethanol, g/week)
For triglyceride values, although a logarithmic transformation was applied for the statistical test, untransformed values are shown in the table

Table 7 Association between *PCSK9* polymorphisms and the incidence of myocardial infarction (MI)

[a]Genotype distributions in the Suita population and patients with MI were compared using the chi-square test

	Intron 1/C(-161)T		*P* value	Exon 9/I474V		*P* value
	CC	CT+TT		II	IV+VV	
Suita population, number (%)	1665 (92.9)	128 (7.1)		1704 (95.0)	89 (5.0)	
Patients with MI, number (%)	593 (92.2)	50 (7.8)	0.5943[a]	609 (95.9)	26 (4.1)	0.3684[a]

patients with MI. One possible reason for this lack of association may be that a substantial proportion of the MI group had dyslipidemia and had been treated with hypolipidemic drugs.

Discussion

While C(-161)T and I474 V polymorphisms have been reported previously (Abifadel et al. 2003), association studies have not been reported. The present study clarified that the C(-161)T and I474V polymorphisms were significantly associated with TC and LDL-C levels in the total population. Even in a gender-based subanalysis, the I474V polymorphism significantly influenced the LDL-C level in both male and female subjects. It is unclear whether these polymorphisms are functional variations or just in linkage disequilibrium with other important variants, and this question requires further investigation. Since Ile at amino acid number 474 was not conserved in either rats or mice, another polymorphism in tight linkage with I474 V may be influential. In fact, a polymorphism in the polypyrimidine-rich tract in intron 8/T(-57)C was almost completely concordant with I474V ($r^2 = 1.00$, Tables 3 and 4).

The minor allele frequencies of intron 1/C(-161)T and exon 9/I474 V polymorphisms were low. However, variances between residuals of TC in genotypes [C(-161)T: CC versus CT+TT, I474 V: II versus IV+VV] were similar [C(-161)T: F-ratio=0.2368, $P=0.6266$; I474 V: F-ratio=2.418, $P=0.1201$ (Levene's test)]. Variances between residuals of LDL-C in the genotypes were also similar [C(-161)T: F ratio=0.1060, $P=0.7448$; I474 V: F ratio=0.4436, $P=0.5055$]. The sample power was 0.9234 (α-value: 0.05, sigma: 27.70, delta: 2.35, adjusted power: 0.8990, confidence limit: 0.2978–0.9996). Thus, these associations were thought to have adequate statistical power. It has been recommended that a single, nominally significant association should be viewed as tentative until it has been independently replicated at least once and preferably twice (Ioannidis et al. 2001). Accordingly, it will be necessary to verify the association between these *PCSK9* polymorphisms and the levels of TC and LDL-C using a larger number of subjects from the Suita cohort or another population.

We found two polymorphisms that were associated with TC and LDL-C levels among nine polymorphisms of *PCSK9* in the Suita population. However, if we apply Bonferroni's correction for multiple tests, only exon 9/I474 V polymorphism can be considered significantly associated with the HDL level [intron 1/C(-161)T, TC: $P=0.2565$, LDL-C: $P=0.2313$; exon 9/I474 V, TC: $P=0.0621$, LDL-C: $P=0.0063$, *P*-values are corrected by multiplying by 9 (nine polymorphisms)]. Again, it will be necessary to verify the association between these *PCSK9* polymorphisms and the levels of TC and LDL-C using a larger number of subjects from the Suita cohort or another population.

A high LDL-C level is a well-known coronary risk factor (Kannel et al. 1979). Although *PCSK9* polymorphisms affected the LDL cholesterol level, they did not affect the incidence of MI. The intron 1/C(-161)T polymorphism was inversely associated with LDL-C level and incidence of MI, although these associations were not significant. This was thought to be due, at least in part, to the low statistical power. A much larger group of MI subjects might be necessary to detect the influence of these variants on the incidence of MI.

In conclusion, the present study provides 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 Japanese population.

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