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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

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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** (CC/CT/TT)	495/88/5 (84.2/15.0/0.9%)	463/65/0 (87.7/12.3/0%)	0.0162
GJA4 I1297D# (II/ID/DD)	274/270/44 (46.6/45.9/7.5%)	232/220/72 (44.3/42.0/13.7%)	0.0028

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.

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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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Original Article

Association between Hypertension and the α-Adducin, β1-Adrenoreceptor, and G-Protein β3 Subunit Genes in the Japanese Population; the Suita Study

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This study focused on 3 genetic polymorphisms that have previously been implicated in hypertension: the α-adducin (*ADD1*/Gly460Trp), β1-adrenoreceptor (*ADRB1*/Arg389Gly), and G-protein β3 subunit (*GNB3*/C825T) gene polymorphisms. We determined genetic variants using the TaqMan system in a large cohort representing the general population in Japan (867 males, 1,013 females). Logistic analysis indicated that the *ADD1*/G460W polymorphism was associated with hypertension in female subjects. The odds ratio of the WW genotype for hypertension was 1.53 (95%CI, 1.12–2.08) over the WG+GG genotype (p=0.0070, p corrected (p_c) =0.0420 corrected by the Bonferroni method). The *ADRB1*/R389G polymorphism tended to be associated with hypertensive status in male subjects (p=0.0117, p_c=0.0702). The odds ratio of the GG genotype for hypertension was 0.38 (95%CI, 0.167–0.780) over the RR+RG genotype. The *GNB3*/C825T polymorphism was not associated with hypertensive status in either male or female subjects. The present results do not agree with those in previous reports. Almost all common variants may have only a modest effect on common diseases, and a single study even employing 1,880 subjects may lack the statistical power to detect a real association. Accordingly, it will be necessary to verify the association between these three genes and hypertension using a larger number of subjects from the Suita cohort or another population.
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***Key Words*: hypertension, α-adducin, β1-adrenoceptor, G-protein β3 subunit**

Introduction

Essential hypertension is a multifactorial disorder that is influenced by both genetic and environmental factors. Single nucleotide polymorphisms (SNPs) are mostly biallelic, more stable, and more frequent than microsatellite markers, making them suitable for association studies (*1*). Over the past few years, many SNPs on candidate genes have been separately tested for their association with hypertension, with controversial results, not only due to the inadequate sample sizes but also due to ethnic differences. If a study shows that there are no functional changes for SNPs in candidate genes, the practical implications of such a study depend on the reproducibility of the findings (*2*).

Recently, three specific mutations have been reported to be associated with hypertension: the G460W polymorphism in the α-adducin (*ADD1*) gene; the R389G polymorphism in

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Table 1. Accession Numbers, Nucleotide Sequences, TaqMan Probes and Primers of *ADD1*, *ADRB1* and *GNB3*

Region	Accession No.	Sequence/primers/probes
ADD1/G460W	IMS-JST010969	CGGGGCGACGAAGCTTCCGAGGAA[G/T]GGCAGAATGGAAGCAGTCCCAAGT
		5′-GCTCCCCACTCAGACACAGTTTT-3′ (sense)
		5′-AGAGACTGCAGCAAGGGTTTCAC-3′ (antisense)
		5′-VIC-ATTCTGCCATTCCTCGGA-MGB-3′
		5′-FAM-TTCTGCCCTTCCTCGG-MGB-3′
ADRB1/R389G	rs1801253	CCCGACTTCCGCAAGGCCTTCCAG[G/C]GACTGCTCTGCTGCGCGCGCAGGG
		5′-CCGCAGCCCCGACTTC-3′ (sense)
		5′-GCCGGTCTCCGTGGGT-3′ (antisense)
		5′-VIC-CTTCCAGGGACTGC-MGB-3′
		5′-FAM-TTCCAGCGACTGCT-MGB-3′
GNB3/C825T	IMS-JST057355	AAGCATCATCTGCGGCATCACGTC[C/T]GTGGCCTTCTCCCTCAGTGGCCGC
		5′-CTCCCACGAGAGCATCATCTG-3′ (sense)
		5′-TCGTCGTAGCCAGCGAATAGTAG-3′ (antisense)
		5′-VIC-CACGTCCGTGGCC-MBG-3′
		5′-FAM-ACGTCTGTGGCCTT-MGB-3′

ADD1, α-adducin; *ADRB1*, β1-adrenoceptor; *GNB3*, G-protein β3 subunit, G, glycine; W, tryptophan; C, cysteine; T, threonine.

the β1-adrenoreceptor (*ADRB1*) gene; and the C825T polymorphism in the G-protein β3 subunit (*GNB3*) gene, a substitution of cytosine (C) for thymine (T) at nucleotide position 825 of *GNB3* cDNA. However, there are inconsistencies among the previous association studies (*3–17*). In response to these controversial results, we investigated the associations between hypertension and the *ADD1*/G460W, *ADRB1*/R389G, and *GNB3*/C825T polymorphisms using a large cohort representing the general population in Japan (total, $n=1{,}880$: 867 males, 1,013 females).

Methods

Subjects

The selection criteria and design of the Suita Study have been previously described (*18–20*). 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. The genotypes were determined in 1,880 consecutive subjects, who visited the National Cardiovascular Center between April 2002 and February 2003. All subjects provided their written informed consent.

DNA Studies

DNA was isolated from peripheral leukocytes according to standard procedures. Polymorphisms were determined by the TaqMan system. The primers and probes are summarized in Table 1. 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 Analysis

Values are expressed as the mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc., Cary, USA). Multiple linear regression (blood pressure value) and multiple logistic (presence or absence of hypertension) analyses were performed with other covariates. Subjects were categorized as hypertensive subjects (HTN) when they had a systolic pressure of 140 mmHg or higher and/or a diastolic pressure of 90 mmHg or higher. Subjects who were currently taking hypertensive medication were also categorized as HTN. The effects of polymorphisms on blood pressure and heart rate values were assessed in subjects who were not receiving cardiovascular medications, since HTN with excellent blood pressure control by medication may have normal blood pressure values. We also excluded subjects who were receiving anti-hypertensive treatment, subjects who had had cerebrovascular accidents, subjects with demonstrated ischemic heart disease, and subjects with atrial fibrillation. Differences in numerical data among the groups were calculated by one-way analysis of variance (ANOVA) or the unpaired t-test. The difference in genotype or allelic distribution between normotensive subjects (NT) and HTN, and Hardy-Weinberg equilibrium was analyzed by a χ^2 test. In some settings, the probability (p) values were corrected (p_c) by multiplying 6 ([3 SNPs]×[2 genders], Bonferroni). Values of $p<0.05$ were considered to indicate statistical significance.

Results

Subjects

The characteristics of the study population are given in Table

Table 2. Characteristics of Study Participants

Parameter	NT	HTN	*p*	Male	Female	*p*
n	1,105	775		867	1,013	
Age (years)	61.9±0.3	68.7±0.4	<0.0001	66.3±0.4	63.3±0.3	<0.0001
BMI (kg/m^2)	22.2±0.1	23.5±0.1	<0.0001	23.2±0.1	22.3±0.1	<0.0001
SBP (mmHg)	118.3±0.4	146.2±0.5	<0.0001	131.8±0.7	128.1±0.6	<0.0001
DBP (mmHg)	73.9±0.3	83.8±0.3	<0.0001	79.7±0.3	76.6±0.3	<0.0001
PR (beats/min)	65.3±0.2	67.0±0.3	<0.0001	66.0±0.3	66.0±0.3	0.9334
Creatinine (μmol/l)	60.8±0.5	66.4±0.6	<0.0001	73.9±0.5	53.9±0.4	<0.0001
Total cholesterol (mmol/l)	5.36±0.02	5.39±0.03	0.3482	5.13±0.03	5.58±0.02	<0.0001
HDL cholesterol (mmol/l)	1.59±0.01	1.52±0.01	<0.0001	1.43±0.01	1.68±0.01	<0.0001
Triglycerides (mmol/l)	1.14±0.03	1.32±0.03	<0.0001	1.38±0.03	1.07±0.03	<0.0001
Blood glucose (mmol/l)	5.34±0.04	5.72±0.04	<0.0001	5.74±0.04	5.30±0.04	<0.0001
%CVA	1.3	4.0	0.0001	3.6	1.4	0.0018
%OMI	0.5	1.2	0.0014	2.1	0.5	0.0015
%HT	—	—	—	45.9	37.2	<0.0001
%drinking	44.7	49.8	0.0292	67.0	29.5	<0.0001
%smoking	19.0	14.6	0.0117	29.9	6.3	<0.0001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PR, pulse rate; HDL, high-density lipoprotein; %CVA, percentage of subjects with cerebrovascular accident; %OMI, percentage of subjects with old myocardial infarction; %HT, percentage of subjects with hypertension; %drinking, percentage of subjects who have drinking habit; %smoking, percentage of subjects who have smoking habit; NT, normotensive subjects; HTN, subjects with hypertension. *p* was calculated by unpaired *t*-test.

Table 3. Genotype Distribution of *ADD1*/G460W, *ADRB1*/R389G, and *GNB3*/C825T in NT and HTN

Polymorphisms	All subjects			Male			Female		
	Genotype frequency (%)		*p* value	Genotype frequency (%)		*p* value	Genotype frequency (%)		*p* value
	NT	HTN	(p_c)	NT	HTN	(p_c)	NT	HTN	(p_c)
ADD1/G460W	GG/GW/WW	GG/GW/WW		GG/GW/WW	GG/GW/WW		GG/GW/WW	GG/GW/WW	
	21.8/50.7/27.6	20.5/48.6/30.9	0.7088	20.7/49.2/30.1	22.9/50.1/27.0	0.2408	22.5/51.8/25.7	17.9/47.0/35.0	0.0238
			(1.0000)			(1.0000)			(0.1428)
ADRB1/R389G	RR/RG/GG	RR/RG/GG		RR/RG/GG	RR/RG/GG		RR/RG/GG	RR/RG/GG	
	63.7/30.8/5.5	68.0/28.4/3.7	0.0291	63.1/31.0/5.9	66.3/31.1/2.6	0.0398	64.0/30.7/5.3	69.7/25.4/4.9	0.0516
			(0.1746)			(0.2388)			(0.3096)
GNB3/C825T	CC/CT/TT	CC/CT/TT		CC/CT/TT	CC/CT/TT		CC/CT/TT	CC/CT/TT	
	26.0/48.5/25.5	22.8/49.7/27.5	0.3953	24.6/51.0/24.4	23.5/48.5/28.0	0.4588	26.9/46.8/26.3	22.1/50.9/27.0	0.2897
			(1.0000)			(1.0000)			(1.0000)

NT, normotensive subjects; HTN, subjects with hypertension; *ADD1*, α-adducin; *ADRB1*, β1-adrenoreceptor; *GNB3*, G-protein β3 subunit; G, glycine; W, tryptophan; R, arginine; C, cysteine; T, threonine; BMI, body mass index. Logistic analysis with age and BMI as covariates was performed. *P*-values were corrected (p_c) by multiplying 6 ([3 SNPs]×[2 genders], Bonferroni).

2. The observed genotype and allele frequencies in the Suita population were in accordance with Hardy-Weinberg equilibrium (*ADD1*/G460W, $p=0.9897$; *ADRB1*/R389G, $p=0.2073$; *GNB3*/C825T, $p=0.8307$).

ADD1/G460W

The effects of the three polymorphisms on hypertensive status, blood pressure values, and pulse rate are shown in Tables 3–5. Logistic analysis with age and body mass index (BMI) as covariates indicated that the *ADD1*/G460W polymorphism (WW=1, WG+GG=2) was associated with hypertension only in female subjects ($p=0.0070$, $p_c=0.0420$, Table 4). The WW genotype of the *ADD1*/G460W polymorphism was more frequent in HTN. The odds ratio of the WW genotype for hypertension was 1.53 (95% CI, 1.12–2.08) over the WG+GG genotype.

There were no differences in blood pressure values between NT and HTN (Table 5). In female subjects, the *ADD1*/G460W polymorphism tended to be associated with pulse rate ($p=0.0144$, $p_c=0.0864$). The neglect of subjects receiving anti-hypertensive medication may have obscured

Table 4. Odds Ratio of the *ADD1*, *ADRB1*, and *GNB3* Genotypes

	All subjects				Male				Female			
	GG+GW	WW	*p* value	(p_c)	GG+GW	WW	*p* value	(p_c)	GG+GW	WW	*p* value	(p_c)
ADD1/G460W	1	1.1 (0.88–1.36)	0.4137	(1.0000)	1	0.77 (0.56–1.05)	0.0959	(0.5736)	1	1.53 (1.12–2.08)	0.0070	0.0420
	RR+RG	GG	*p* value	(p_c)	RR+RG	GG	*p* value	(p_c)	RR+RG	GG	*p* value	(p_c)
ADRB1/R389G	1	0.63 (0.71–1.11)	0.0651	(0.3906)	1	0.38 (0.17–0.78)	0.0117	(0.0702)	1	1.00 (0.52–1.92)	0.995	(1.0000)
	CC	CT+TT	*p* value	(p_c)	CC	CT+TT	*p* value	(p_c)	CC	CT+TT	*p* value	(p_c)
GNB3/C825T	1	0.87 (0.69–1.10)	0.2368	(1.0000)	1	0.97 (0.70–1.35)	0.8599	(1.0000)	1	0.77 (0.55–1.07)	0.1247	0.7482

Odds ratio with its 95% confidential interval is shown. Logistic analysis with age and BMI as covariates was performed. *P* values were corrected (p_c) by multiplying 6 ([3 SNPs]×[2 genders], Bonferroni). *ADD1*, α-addcin; *ADRB1*, β1-adrenoceptor; *GNB3*, G-protein β3 subunit; R, arginine; G, glycine; C, cysteine; T, threonine; BMI, body mass index.

the relationship between the polymorphism and blood pressure values.

ADRB1/R389G

Logistic analysis with age and BMI as covariates indicated that the *ADRB1*/R389G polymorphism (GG=1, RR+RG=2) tended to be associated with hypertension only in male subjects (p=0.0117, p_c=0.0702, Table 4). The odds ratio of the GG genotype for hypertension was 0.38 (95% CI, 0.17–0.78) over the RR+RG genotype. Alcohol consumption is a well-known determinant of blood pressure level, especially in male subjects. The *ADRB1*/R389G polymorphism (GG=1, RR+RG=2) tended to be associated with hypertensive status when also adjusted for alcohol consumption (p=0.0109, p_c=0.0654, odds ratio=0.37 [95% CI, 0.16–0.48]). There were no differences in blood pressure values between NT and HTN (Table 5). Again, the neglect of subjects receiving anti-hypertensive medication may have obscured the relationship between the polymorphism and blood pressure values.

GNB3/C825T

We did not find any association between the *GNB3*/C825T polymorphism and hypertensive status, systolic blood pressure, or diastolic blood pressure in all subjects, male subjects, or female subjects (Tables 3–5).

Discussion

In the present study, we investigated the associations between hypertension and three polymorphisms, *ADD1*/G460W, *ADRB1*/R389G, and *GNB3*/C825T, in a population-based sample (the Suita Study) consisting of 1,880 subjects. Our results indicate that the WW genotype of the *ADD1* gene may be involved in hypertension in female subjects.

The *ADD1* gene in humans is highly homologous to that in rats. Known point mutations, one each in the α- and β-adducin subunits, account for up to 50% of the difference in blood pressure between the Milan hypertensive and normotensive rat strains (*21*). Based on initial case-control and linkage analyses, the *ADD1*/G460W polymorphism was implicated in the genetic component of hypertension in Italian and French populations (*3*). In addition, a group of Italian hypertensive subjects with the W allele had lower plasma renin levels and showed a significantly greater fall in blood pressure with sodium restriction or diuretic treatment (*3*). On the other hand, this association was not confirmed by two different studies in Scottish populations (*6*, *8*). In the Japanese population, while Tamaki *et al.* reported that the *ADD1*/G460W polymorphism was involved in hypertension (*5*), Kato *et al.* did not support this association (*7*). Sugimoto *et al.* demonstrated that this polymorphism is associated with low renin hypertension in younger subjects (*22*). The present study indicated that the *ADD1*/G460W polymorphism was influential in female subjects, but the corrected *p* value (p_c=0.045) was marginal. Accordingly, additional independent replications in the Japanese population are required to confirm the present association.

In the present study, the *ADD1*/G460W polymorphism was associated with hypertension only in female subjects, but not in male subjects. It remains to be clarified why these polymorphisms do not equally contribute to hypertension in both sexes. Recently, low renin hypertension has been reported to be a significant predictor of systolic sodium sensitivity in females only (*23*). Izawa *et al.* also demonstrated a gender difference in genetic polymorphisms and hypertension (*15*). Accordingly, these results may suggest that sex-based differences should be considered in the association between genetic polymorphisms and hypertension.

Two common polymorphisms, S49G and R389G, were identified in the *ADRB1* gene (*24*). The R389G polymor-

Table 5. Blood Pressure Values and Pulse Rates among Genotypes of *ADD1*/G460W, *ADRB1*/R389G, and *GNB3*/C825T Polymorphism

Variables	*ADD1*/G460W			*p* value	(p_c)	*ADRB1*/R389G			*p* value	(p_c)	*GNB3*/C825T			*p* value	(p_c)
	GG	GW	WW			RR	RG	GG			CC	CT	TT		
All subjects															
N	293	663	393			864	400	69			347	647	351		
PR (beats/min)	65.7±0.4	65.7±0.3	64.9±0.4	0.1578	(0.9468)	65.3±0.3	65.7±0.4	65.1±0.9	0.5977	(1.0000)	65.4±0.4	65.6±0.3	65.2±0.4	0.6827	(1.0000)
SBP (mmHg)	124.8±1.1	124.6±0.7	126.7±0.9	0.1735	(1.0000)	125.6±0.6	125.4±0.9	122.3±2.2	0.3774	(1.0000)	124.3±1.0	125.5±0.7	125.6±1.0	0.5610	(1.0000)
DBP (mmHg)	76.2±0.6	76.8±0.4	76.8±0.5	0.6273	(1.0000)	76.8±0.3	76.9±0.5	75.2±1.2	0.4277	(1.0000)	76.0±0.5	77.1±0.4	76.7±0.5	0.2963	(1.0000)
Res. SBP (mmHg)	−0.5±1.0	−0.3±0.6	0.7±0.8	0.5433	(1.0000)	0.4±0.6	−0.2±0.8	−3.2±2.0	0.2209	(1.0000)	−0.9±0.9	0.1±0.7	0.4±0.9	0.5743	(1.0000)
Res. DBP (mmHg)	−0.5±0.6	0.2±0.4	−0.1±0.5	0.6109	(1.0000)	0.1±0.3	0.0±0.5	−1.5±0.2	0.4350	(1.0000)	−0.7±0.5	0.3±0.4	0.0±0.5	0.3561	(1.0000)
Male															
N	126	282	176			366	185	27			144	284	156		
PR (beats/min)	65.0±0.7	65.5±0.5	65.5±0.6	0.8213	(1.0000)	65.5±0.4	65.1±0.6	64.1±1.5	0.6239	(1.0000)	65.1±0.6	65.7±0.5	64.9±0.6	0.4875	(1.0000)
SBP (mmHg)	129.0±1.6	126.2±1.1	128.0±1.6	0.3077	(1.0000)	127.3±1.0	128.6±1.4	121.1±3.5	0.1407	(0.8442)	125.9±1.5	126.9±1.0	129.2±1.5	0.2610	(1.0000)
DBP (mmHg)	79.1±0.9	78.2±0.6	78.5±0.8	0.7363	(1.0000)	78.6±0.6	78.8±0.8	75.3±2.0	0.2699	(1.0000)	78.4±0.9	78.4±0.9	78.4±0.8	0.9992	(1.0000)
Res. SBP (mmHg)	1.5±1.5	−0.5±1.0	−0.3±1.3	0.5435	(1.0000)	−0.1±0.9	1.5±1.3	−7.7±3.3	0.0326	(0.1956)	−1.5±1.4	−0.5±1.0	2.2±1.4	0.1469	(1.0000)
Res. DBP (mmHg)	0.9±0.9	−0.1±0.6	−0.4±0.8	0.5304	(1.0000)	0.0±0.5	0.6±0.7	−2.7±1.9	0.2712	(1.0000)	−0.1±0.8	0.0±0.6	0.0±0.8	0.9907	(1.0000)
Female															
N	167	381	217			498	215	42			203	363	195		
PR (beats/min)	66.3±0.6	65.9±0.4	64.4±0.5	0.0144	(0.0864)	65.2±0.3	66.2±0.5	65.8±1.1	0.1950	(1.0000)	65.6±0.4	65.6±0.4	65.5±0.5	0.9869	(1.0000)
SBP (mmHg)	121.7±1.4	123.3±0.9	125.6±1.2	0.1000	(0.6000)	124.3±0.8	122.7±1.3	123.1±2.8	0.5399	(1.0000)	123.2±1.3	124.4±1.0	122.7±1.3	0.5142	(1.0000)
DBP (mmHg)	74.0±0.7	75.9±0.5	75.5±0.7	0.1213	(0.7278)	75.5±0.4	75.2±0.7	75.1±1.5	0.9136	(1.0000)	74.3±0.7	76.0±0.5	75.3±0.7	0.1427	(1.0000)
Res. SBP (mmHg)	−1.8±1.3	−0.2±0.8	1.4±1.1	0.1508	(0.9048)	0.7±0.7	−1.6±1.1	0.0±2.5	0.2495	(1.0000)	−0.4±1.1	0.6±0.9	−1.1±1.2	0.4864	(1.0000)
Res. DBP (mmHg)	−1.4±0.7	0.4±0.5	0.1±0.6	0.1051	(0.6306)	0.2±0.4	−0.5±0.6	−0.2±1.5	0.7206	(1.0000)	−1.0±0.7	0.5±0.5	−0.0±0.7	0.1882	(1.0000)

PR, pulse rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Res. SBP and Res. DBP, residuals of SBP and DBP for age and body mass index, respectively. We excluded subjects who were receiving anti-hypertensive or cardiovascular medication, subjects who had had a cerebrovascular accident, subjects with demonstrated ischemic heart diseases, and subjects with atrial fibrillation. *P* was calculated by ANOVA. *P* values were corrected (p_c) by multiplying 6 [(3 SNPs)×(2 genders), Bonferroni].

phism is located in the intracellular cytoplasmic tail near the seventh transmembrane region of the receptor, which is the putative Gs-protein binding domain. The R389 variant mediates a higher level of isoproterenol-stimulated adenylate cyclase activity than the G389 *variant in vitro* (*25*). In accordance with this *in vitro* study, Bengtsson *et al.* reported that homozygotes for the *ADRB1*/R389 allele had an increased risk of developing hypertension in a case-control study (*9*). Their genotype-discordant sibling pair analysis demonstrated that siblings who were homozygotes for the R389 allele had significantly higher diastolic pressures and higher heart rates than siblings carrying one or two copies of the G389 allele. However, McCaffery *et al.* reported that subjects carrying any *ADRB1*/G389 allele, but not the *ADRB1*/R389 allele, exhibited elevated systolic and diastolic blood pressure (*10*). The present study, which employed a dominant model, indicated that the GG genotype of the *ADRB1* gene tended to be associated with hypertension in male subjects. However, the frequency of the GG genotype was relatively low, and the sample power was weak (sample power: 0.69, α=0.05, two-tailed). A much larger number of subjects may be required to confirm the present association.

The *GNB3*/C825T polymorphism is associated with the occurrence of a splicing variant, *GNB3*-s (encoding Gβ3-s), in which nucleotides 498–620 of exon 9 are deleted. This in-frame deletion causes the loss of 41 amino acids and one WD repeat domain of the Gβ subunit (*11*). A significantly higher frequency of the *GNB3*/T825 allele has been reported in subjects with essential hypertension using unselected normotensive control subjects of European origin in three independent studies (*11–13*), but not in a fourth study (*14*). In blacks, the *GNB3*/T825 allele was reported to be a susceptibility factor for the development of hypertension (*17*). In a Japanese population, Izawa *et al.* demonstrated an association between hypertension and the *GNB3*/C825T polymorphism in male subjects (*15*). However, Ishikawa *et al.* reported that the *GNB3*/C825T polymorphism was associated with serum potassium and total cholesterol levels, but not with blood pressure (*16*). The present study revealed that the *GNB3*/T825 allele is not associated with either hypertensive status (*15*, *16*) or the total cholesterol level (all subjects, p= 0.9381), contrary to previous reports (*15*, *16*).

As stated above, the present results do not agree with those in previous reports. Almost all common variants may have only a modest effect on common diseases, and a single study may lack the statistical power to detect a real association (*26*, *27*). Recently, it was reported that a meta-analysis of genetic association studies may support the notion that common variants may contribute to a susceptibility to a multifactorial common disease (*27*). It is recommended that a single, nominally significant association should be viewed as tentative until it has been independently replicated at least once, and preferably twice (*27*). Accordingly, it will be necessary to verify the association between these three genes and hypertension using a larger number of subjects from the Suita cohort or another population.

In conclusion, the present tentative results suggest that the WW genotype of the *ADD1* gene may be involved in hypertension in female subjects. The GG genotype of the *ADRB1* gene may play a protective role against hypertension in male subjects.

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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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Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese

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The frequency of factor (F)V Leiden mutation is relatively high among individuals of Caucasian descent, being from 2 to 15% in the general population and up to 50% in selected patients with thromboembolism [1]. The risk of the first episodes of thromboembolism as estimated in a large case–control study is 7-fold for heterozygous FV Leiden carriers [2]. Although the frequency of deficiencies of natural anticoagulants, protein C or antithrombin in the general population is low, prospective studies indicate that low levels of protein C and antithrombin

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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).

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]TCGCCCGCTGCGCCCCAGATGAGGA
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)

	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]

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

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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ORIGINAL ARTICLE

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A promoter variant of the ATP-binding cassette transporter A1 gene alters the HDL cholesterol level in the general Japanese population

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Abstract To investigate the effects of polymorphisms in the ATP-binding cassette transporter A1 (*ABCA1*) gene on the high-density lipoprotein cholesterol (HDL-C) level and the incidence of myocardial infarction (MI), we performed association studies. Sequence analysis identified 14 polymorphisms in the promoter region of *ABCA1*. After considering linkage disequilibrium, three polymorphisms in the promoter region and 11 polymorphisms from the JSNP database were determined in 1,880 subjects recruited from the Suita Study, representing the general population in Japan. We evaluated the association between the *ABCA1* genotype and HDL-C level adjusted not only for standard factors, but also for genetic factors including *ApoA1* and *ApoE* genotypes. Of the 14 polymorphisms tested, the *G(−273)C* ($P=0.0074$), *C(−297)T* ($P=0.0195$), and *IMS-JST071749* ($P=0.0093$) polymorphisms were significantly associated with the HDL-C level in the Suita population. We could reconfirm that the *G(−273)C* genotype was influential in another set of subjects ($P=0.0310$, $n=743$). However, the distribution of the *ABCA1 G(−273)C* genotype in subjects with MI ($n=598$) was not different from that in the control population ($n=801$). These results indicate that *ABCA1 G(−273)C* has a significant effect on the HDL-C level in the general Japanese population, but not on the incidence of MI.

Keywords ABCA1 · Polymorphism · Association study · HDL cholesterol · Myocardial infarction

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Introduction

The high-density lipoprotein cholesterol (HDL-C) level is inversely correlated with the development of atherosclerosis and is inversely related to the incidence of coronary artery disease (Castelli et al. 1986) and ischemic stroke in the elderly (Sacco et al. 2001). The HDL-C level has been shown to be affected by both genetic and environmental factors, including obesity, smoking, and alcohol consumption. Among genetic factors, the apolipoprotein A1 (*ApoA1*) (Groenendijk et al. 2001a,b) and *ApoE* genotypes (Lefevre et al. 1997; Katsuya et al. 2002) are well known to influence the HDL-C level.

Genetic mutations in the ATP-binding cassette transporter A1 (*ABCA1*) gene have been shown to cause Tangier disease (TD) (Bodzioch et al. 1999; Brooks-Wilson et al. 1999; Rust et al. 1999) and familial HDL deficiency (Marcil et al. 1999). ABCA1 regulates cellular cholesterol efflux and facilitates lipid binding to ApoA1 (Wang and Tall 2003). Patients with TD show characteristic HDL deficiency, defective apolipoprotein-mediated phospholipid and cholesterol efflux from cells, and the accumulation of macrophage foam cells in various tissues, including arteries (Clifton-Bligh et al. 1972). Recent epidemiological studies have reported that *ABCA1* polymorphisms were associated with the HDL-C level