表4. 4項目のメタボリック・シンドロームに対する年齢調整オッズ比と95%信頼区間。

男性	年齢(歳)	肯定的な回答の割合(%)	年齢調整オッズ比	(95%信頼区間)	p値
	30-49	27	3.70	(2.51 - 5.44)	<0.0001
1. 食べる量	50-69	16	1.90	(1.58 - 2.29)	<0.0001
	70-89	10	2.00	(1.53 - 2.61)	<0.0001
	30-49	54	1.63	(1.11 - 2.39)	0.0118
18. 早食い	50-69	39	1.69	(1.45 - 1.98)	<0.0001
	70-89	25	1.72	(1.39 - 2.12)	<0.0001
	30-49	34	1.58	(1.08 - 2.32)	0.0189
29. 睡眠不規則	50-69	15	1.27	(1.03 - 1.57)	0.0229
	70-89	14	1.38	(1.05 - 1.80)	0.0189
	30-49	54	1.53	(1.05 - 2.24)	0.0285
24. 歩行時間	50-69	56	1.37	(1.17 - 1.61)	0.0001
	70-89	69	1.56	(1.24 - 1.97)	0.0001
女性					
	30-49	15	3.72	(2.49 - 5.55)	<0.0001
1. 食べる量	50-69	15	2.54	(2.18 - 2.97)	<0.0001
	70-89	11	2.20	(1.75 - 2.77)	<0.0001
	30-49	37	2.02	(1.37 - 3.00)	0.0004
18. 早食い	50-69	35	1.89	(1.65 - 2.17)	<0.0001
	70-89	23	1.61	(1.33 - 1.96)	<0.0001
	30-49	21	1.80	(1.18 - 2.73)	0.0061
29. 睡眠不規則	50-69	18	1.55	(1.31 - 1.82)	<0.0001
	70-89	18	1.27	(1.03 - 1.57)	0.0253
	30-49	38	1.41	(0.95 - 2.09)	0.0913
24. 歩行時間	50-69	40	1.48	(1.28 - 1.71)	<0.0001
	70-89	60	1.34	(1.11 - 1.63)	0.0029

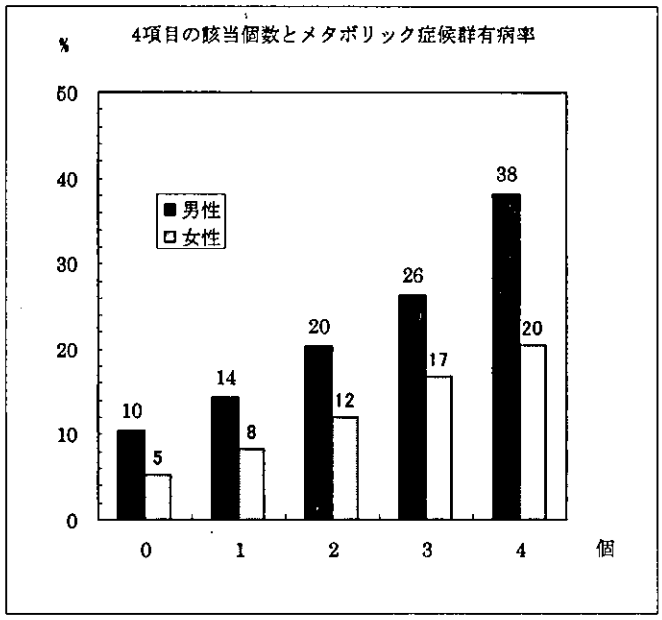

図 3. 「食べる量が多い」「早食いである」「睡眠時間が不規則である」「立位・歩行時間が1日3時間未満である」の4項目で該当する個数別によるメタボリック症候群有病率を示した。

E. 健康危険情報：

特になし

F. 研究発表：

1．論文発表

現在執筆中

2．学会発表

なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表(平成 16 年度)

主任研究者:友池仁暢

発表者氏名	論 文 タ イ ト ル 名	発 表 誌 名	巻号	ページ	出版年
Iwanaga Y, Ono K, Takagi S, Terashima M, Tsutsumi Y, Mannami T, Yasui N, Goto Y, Nonogi H, Iwai N	Association analysis between polymorphisms of the lymphotoxin-alfa gene and myocardial infarction in s Japanese population	Atherosclerosis	172	197-198	2004
Shioji K, Kokubo Y, Goto Y, Nonogi H, Iwai N,	An association analysis between genetic polymorphisms of matrix metalloproteinase-3 and methylenetetrahydrofolate reductase and myocardial infarction in Japanese	J Thromb Haemostasis	2	527-528	2004
Shioji K, Mannami T, Kokubo Y, Inamoto N, Takagi S, Goto Y, Nonogi H, Iwai N	Genetic variants in PCSK9 affect the cholesterol level in Japanese	J Hum Genet	49	109-114	2004
Shioji K, Nishioka J, Naraba H, Kokubo Y, Mannami T, Inamoto N, Kamide K, Takiuchi S, Yoshii M, Miwa Y, Kawano Y, Miyata T, Miyazaki S, Goto Y, Nonogi H, Tago N, Iwai N	A promoter variant of the ATP-binding cassette transporter A1 gene alters the HDL cholesterol level in the general Japanese population	J Hum Genet	49	141-147	2004
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Kokubo Y, Inamoto N, Tomoike H, Kamide K, Takiuchi S, Kawano Y, Tanaka C, Katanosaka Y, Wakabayashi S, Shigekawa M, Hishikawa O, Miyata T	Association of genetic polymorphisms of sodium-calcium exchanger 1 gene, NCX1, with hypertension in a Japanese general population.	Hypertens Res	27	697-702	2004
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Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H, Naraba H, Tomoike H	Association analysis between hypertension and CYBA, CLCNKB, and KCNMB1 functional polymorphisms in the Japanese population-The Suita Study-	Circ. J.	69	138-142	2005

Ⅳ. 研究成果の刊行物・別刷

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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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5 September 2003

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)

[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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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

-1600	AAGGGGCCATGCCACCCAGA	GTTATGAGTACCTGGGACTC	CAGAATTCCTTGCCTGGTGG	CCTCCACATGCACTTCCAGG	GCCTGCTTGGGCCTCTTCTA
-1500	TGGGTCTGTCCTGAGTGTTG G(-1498)C	ATAGAACCACTGATGTGAGT	ACCTGGGCTTGAGCCGTGGC	CTGGAGATCCTGTTGACTGT T(-1423)C	AGCATGGAGGGGGCTTGTGC
-1400	AGCTGAATGTCTGTATGCAG T(-1387)C	GTGGTGGGAGTTCTGGAATA	TGATGGAGCTGGAGGTGGGA	AGAGAAGTAGGCTTGGGGCA	GCTCTCTCATGCCACCTCAT
-1300	TCTGGCCAAAACTCAGGTCA	AACTGTGAAGAGTCTAAATG	TGAATCTGCCCTTCAAGGTG	GCTACAAAGGTATCTTTGTC	AAGGTAGGAGACCTTGTGGC
-1200	CTCCACGTGCACTTCCAGGG	CCTGCTTGGGCCTCTTCTAC	GGGTCTGTCCTGAGTCTTCT	ATGAATCTGCCCTTCAGGGC	AGATTCATATTTAGACTCTT
-1100	CACAGTTTGACCTGAGTTTT	GGCCAGAATAAGGTGACATT	TAGTTTGTTGGCTTGATGGA	TGACTTAAATATTTAGACAT	ATGGTGTGTAGGCCTGCATT AT(-1019)(-)
-1000	CCTACTCTTGCCTTTTTTTT (-980)T(10)/T(9)/T(8)	TGCCCCTCCAGTGTTTTGGG	TAGTTTTGCTCCCCTACAGC	CAAAGGCAAACAGATAAGTT G(-926)T	GGAGGTCTGGAGTGGCTACA
-900	TAATTTTACACGACTGCAAT	TCTCTGGCTGCACTTCACAA	ATGTATACAAACTAAATACA	AGTCCTGTGTTTTTATCACA	GGGAGGCTGATCAATATAAT
-800	GAAATTAAAAGGGGGCTGGT C(-790)A	CCATATTGTTCTGTGTTTTT	GTTTGTTT_GTTTCTTTTTTT GTTTTGTTT(-752)(-) (I/D #1)	GTTTTTGTGGCCTCCTTCCT	CTCAATTTATGAAGAGAAGC
-700	AGTAAGATGTTCCTCTCGGG	TCCTCTGAGGGACCTGGGGA	GCTCAGGCTGGGAATCTCCA	AGGCAGTAGGTCGCCTATCA	AAAATCAAAGTCCAGGTTTG
-600	TGGGGGGAAAACAAAAGCAG	CCCATTACCCAGAGGACTGT	CCGCCTTCCCCTCACCCCAG C(-559)T	CCTAGGCCTTTGAAAGGAAA	CAAAAGACAAGACAAAATGA
-500	TTGGCGTCCTGAGGGAGATT	CAGCCTAGAGCTCTCTCTCC	CCCAATCCCTCCCTCCGGCT	GAGGAAACTAACAAAGGAAA	AAAAAATTGCGGAAAGCAGG G(-402)C
-400	ATTTAGAGGAAGCAAATTCC	ACTGGTGCCCTTGGCTGCCG	GGAACGTGGACTAGAGAGTC	TGCGGCGCAGCCCCGAGCCC	AGCGCTTCCCGCGCGTCTTA
-300	GGCCGGCGGGCCCGGGCGGG C(-297)T	GGAAGGGGACGCAGACCGCG G(-273)C	GACCCTAAGACACCTGCTGT	ACCCTCCACCCCCA_CCCCAC TGGGC(-226)(-) (I/D #2)	CCACCTCCCCCCAACTCCCT
-200	AGATGTGTCGTGGGCGGCTG	AACGTCGCCCGTTTAAGGGG	CGGGCCCCGGCTCCACGTGC	TTTCTGCTGAGTGACTGAAC	TACATAAACAGAGGCCGGGA
-100	ACGGGGCGGGGAGGAGGGAG G(-99)C	AGCACAGGCTTTGACCGATA	GTAACCTCTGCGCTCGGTGC	AGCCGAATCTATAAAAGGAA	CTAGTCCCGGCAAAAACCCC C(-14)T
1	*GTAATTGCGAGCGAGAGTGA*	*GTGGGGCCGGGACCCGCAGA*	*GCCGAGCCGACCTTCTCTC* C52A	*CCGGGCTGCGGCAGGGCAGG*	*GCGGGGAGCTCCGCGCACCA*
101	*ACAGAGCCGGTTCTCAGGGC*	*GCTTTGCTCCTTGTTTTTTC*	*CCCGGTTCTGTTTTCTCCCC*	*TTCTCCGGAAGGCTTGTCAA*	*GGGGTAGGAGAAAGAGACGC*
201	*AAACACAAAAGTGGAAAACA*	*GGTAAGAGGCTCTCCAGTGA*	*CTTACTTGGGCGTTATTGTT*	*TTGTTTCGAGGCCAAGGAGG*	*CTTCGGGAAGTGCTCGGTTT*
301	CGGGGACTTTGATCCGGAGC T313C	CCCACATCCCCACCACTTGC	AACTCAGATGGGACCGGAGG	CGGTGTTAAATGGGGAGACG G38UT	ATGTCCTAGTACGAGCTCTG
401	GTGACCCCAGGACTCTGCGC	TGCTGCGCTTGGGGCTTGCC	CGACGGTGGAGACCGGGGAG	CATCTCTGGGCGTGGAGACC	CGGGCGCAGTACCCCGGGCT

Fig. 1 Nucleotide sequence of the 5′-flanking region and exon 1 of *ABCA1*. The nucleotide sequence in *italics* indicates exon 1

(Wang et al. 2000; Clee et al. 2001; Lutucuta et al. 2001; Harada et al. 2003). However, few of these findings have been replicated, and there are inconsistencies among previous association studies. Accordingly, the associations between *ABCA1* variants and HDL-C are still controversial (Singaraja et al. 2003). One possible reason for these differences may be that the sample sizes in these studies were relatively small and lacked statistical power. Thus, to evaluate the effect of polymorphisms in *ABCA1* on the HDL-C level, we conducted an association study using a large cohort (the Suita population, $n = 1{,}880$), representing the general population in Japan.

Materials and methods

Subjects

The Suita population The selection criteria and design of the Suita Study have been described previously (Mannami et al. 1997; Shioji et al. 2004). The genotypes were determined in 1,880 consecutive subjects who visited the National Cardiovascular Center between April 2002 and February 2003 (867 male subjects, 1,013 female subjects).

The hypertension group The hypertension (HTN) group consisted of 743 Japanese subjects (422 men and 321 women), aged 18–91 years [65.2 ± 0.4 (mean ± SEM)], who were enrolled in the Division of Hypertension and Nephrology at the National Cardiovascular Center between May 2001 and April 2003.

The myocardial infarction group The selection criteria and design of the myocardial infarction (MI) group have been described previously (Takagi et al. 2002). This group consisted of 706 patients with MI (598 men and 108 women, aged 61.3 ± 0.4 years) who were enrolled in the Division of Cardiology at National Cardiovascular Center between May 2001 and April 2003. In the present study, we investigated only males ($n = 598$).

Written informed consent was obtained from every subject after a full explanation of the study, which 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.

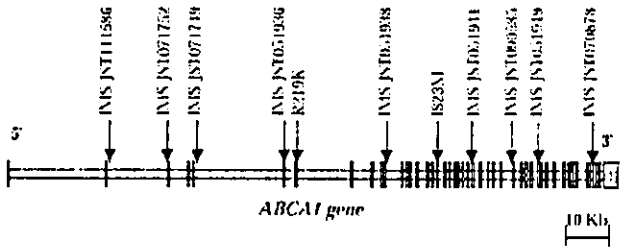

Fig. 2 Schema of *ABCA1* and the position of the determined polymorphisms. *Grayboxes* indicate exonic regions

DNA studies

The regions of the promoter and exon 1 in *ABCA1* were sequenced for polymorphisms in 24 subjects (Fig. 1). The primer sequences are available on request. For exonic regions (Fig. 2), we selected nine SNPs for genotyping from the public database (JSNP, http://snp.ims.u-tokyo.ac.jp) (Iida et al. 2001; Hirakawa et al. 2002). Well-known common variants, *ABCA1* R219K and I823M, were also selected (Wang et al. 2000; Clee et al. 2001; Harada et al. 2003).

The preliminary study revealed that *JST-IMS005607* had the greatest effect on the HDL-C level among seven SNPs on the *ApoA1* region, including the promoter region (up to −3Kb).

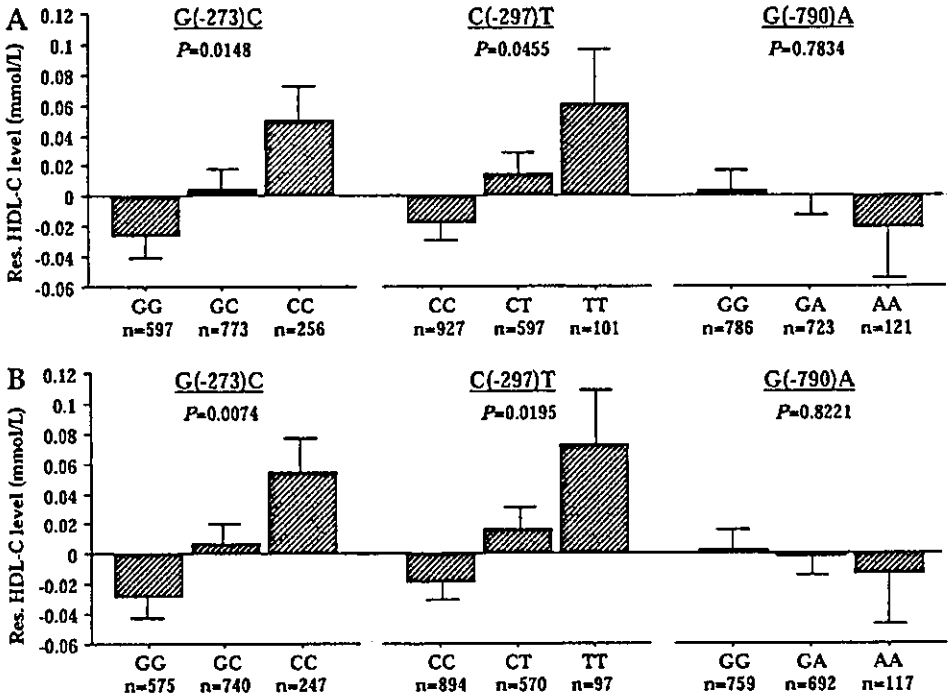

Fig. 3A, B Residual HDL cholesterol levels among the *ABCA1 G(−273)C*, *C(−297)T*, and *G(−790)A* genotypes. A Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, and alcohol consumption. B Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, alcohol consumption, *ApoE* genotype, and *ApoA1* genotype (*JST-IMS005603*)

Thus, we selected *JST-IMS005607* for adjusting HDL-C. The genotyping of *ApoE* was performed according to a previous report (Katsuya et al. 2002). *ApoE* polymorphisms were categorized into three genotypes: *E2* ($\epsilon2/\epsilon2+\epsilon2/\epsilon3+\epsilon2/\epsilon4$ subjects), *E3* ($\epsilon3/\epsilon3$ subjects), *E4*($\epsilon3/\epsilon4+\epsilon4/\epsilon4$ subjects) (Lefevre et al. 1997). All polymorphisms were determined by the TaqMan System.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). For triglyceride values, a logarithmic transformation was applied for the statistical test, but untransformed values are shown in the Tables 1 and 2. All statistical analyses were performed with the JMP statistical package (SAS Institute). Values of $P<0.05$ were considered to indicate statistical significance. Multiple linear regression and multiple logistic analyses were performed with other covariates. The residual HDL-C level was calculated by adjusting for sex, age, and body-mass index (BMI), smoking (cigarettes/day) and consumption of alcohol (ethanol, ml/week). For analyses of the effects of the *ABCA1* genotype (in the Suita population), the residual HDL-C level was calculated by adjusting not only for the above five factors, but also for the *ApoA1*(*JST-IMS005603*), and the *ApoE* (*E2*, *E3*, and *E4*) genotypes. Differences in numerical data among the groups were evaluated by one-way analysis of variance (ANOVA). Hardy-Weinberg equilibrium was calculated by a chi-square test (Table 3). To measure linkage disequilibrium (LD) between SNPs, D' and r^2 values were analyzed using the SNPAlyze statistical package (Dynacom).

Results

Polymorphisms of the 5′-flanking region and exon 1 of the *ABCA1* gene

We found 14 polymorphisms in the promoter region, 1 polymorphism in exon 1 (5′-untranslated region), and 2 polymorphisms in intron 1 (Fig. 1).

LD was evaluated by calculating r^2 values (Table 1). We regarded $r^2>0.5$ as tight linkage. The minor allele frequency of the *T*(−1423)*C* and *G52A* polymorphisms was low (4% each), and these SNPs were neglected in further analyses. The frequencies of *T*(10), *T*(9), and *T*(8) were 4, 92, and 4%, respectively, in the (−980)*T*(10)/*T*(9)/*T*(8) polymorphism, and this polymorphism was also neglected because this is not suitable for TaqMan genotyping. Accordingly, we selected three polymorphisms, *G(−790)A*, *C(−297)T*, and *G(−273)C*, for the following association study.

Association study of *ApoA1* and *ApoE*

To observe the effect of *ABCA1*polymorphisms on the HDL-C level more clearly, the HDL-C level should be adjusted by various well-known influential factors.

The *ApoA1 IMS-JST005603* polymorphism was associated with the levels of HDL-C and triglyceride [HDL-C: TT 1.54 ± 0.001 mmol/l, TC 1.59 ± 0.02, CC 1.68 ± 0.04, $P=0.0002$ (residual); triglyceride: TT 1.26 ± 0.03 mmol/l, TC 1.15 ± 0.04, CC 0.95 ± 0.09, $P<0.0001$ (residual)]. *IMS-JST005603* corresponds to the *Hae*III (*C317T*) polymorphism described in a previous paper (Groenendijk et al. 2001b).

The *ApoE* polymorphism was also strongly associated with the levels of total cholesterol and HDL-C [total cholesterol: *E2* 5.13 ± 0.06 mmol/l, *E3* 5.37 ± 0.02, *E4* 5.41 ± 0.05, $P=0.0002$ (residual); HDL-C: E2 1s.67 ± 0.03 mmol/l, *E3* 1.56 ± 0.01, *E4* 1.52 ± 0.02, $P<0.0001$ (residual)].

Accordingly, we evaluated the effect of the *ABCA1* polymorphisms on the HDL-C level adjusted for the

Table 1 Linkage disequilibrium between SNPs in the 5′-flanking region and exon 1 of the *ABCA1* gene. *I/D#1 GTTTTGTTTT(−752)*

Genotype	*G(−1498)C*	*T(−1423)C*	*T(−1387)C*	*AT(−1019)(−)*	*G(−926)T*	*G(−790)A*	I/D#1	*C(−559)T*
G(−1498)C		0.01976	0.41818***	1***	0.41818***	0.00047	0.67347***	0.22034**
T(−1423)C			0.04726	0.01976	0.04726	0.01003	0	0.06087
T(−1387)C				0.41818***	1***	0.00111	0.67347***	0.65714***
AT(−1019)(−)					0.41818***	0.00047	0.67347***	0.22034**
G(−926)T						0.00111	0.67347***	0.65714***
G(−790)A							0.14667*	0.16483**
I/D#1								1***
C(−559)T								
G(−402)C								
C(−297)T								
G(−273)C								
I/D#2								
G(−99)C								
C(−14)T								
C52A								
T313C								
G380T								

R^2 values are shown in the *upper right*, and *bolded values* indicate $r^2 > 0.5$. Absolute D'-values are shown in the *lower left*, and *bolded*
Significance levels: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

ApoA1 IMS-JST005603 and *ApoE*polymorphisms in addition to standard factors, including sex, age, BMI, smoking, and consumption of alcohol.

Association study of *ABCA1* (Suita population)

The association between the *G(−273)C* polymorphism and the lipid level in the Suita population is presented in Table 2. The genotype frequency of the *G(−273)C* polymorphism in the Suita population was not deviated from the Hardy-Weinberg equilibrium. The HDL-C level adjusted for age, sex, BMI, smoking, and consumption of alcohol was significantly associated with the *G(−273)C* polymorphism ($P=0.0148$). The *G(−273)C* polymorphism was even more tightly associated with the HDL-C level when adjusted for the *ApoE* and *ApoA1(IMS-JST005603)* genotypes in addition to the standard factors ($P=0.0074$). The *C(−297)T* polymorphism was also associated with the HDL-C level ($P=0.0455$ adjusted for age, sex, BMI, smoking, and consumption of alcohol; $P=0.0195$ when also adjusted for the *ApoE* and *ApoA1* genotypes). The effect of the *C(−297)T* polymorphism on the HDL-C level may be, at least in part, explained by its linkage with the *G(−273)C* polymorphism ($r^2=0.46667$, D' value $=1$, $P<0.0001$). *G(−790)A* was not associated with the lipid levels. Among the polymorphisms selected from JSNPs, including R219K and I823M, only the *IMS-JST071749* polymorphism was associated with the HDL-C level ($P=0.0060$ adjusted for age, sex, BMI, smoking, and consumption of alcohol; $P=0.0093$ when also adjusted for the *ApoE* and *ApoA1 (IMS-JST005603)* genotypes). The *R219K* and *I823M* polymorphisms were not associated with the HDL-C level [$P=0.3877$ (*R219K*) and $P=0.2286$ (*I823M*) adjusted for age, sex, BMI, smoking and consumption of alcohol; $P=0.1926$ (*R219K*) and $P=0.1209$ (*I823M*) when also adjusted for the *ApoE* and *ApoA1* genotypes].

Association study of *ABCA1* (HTN group)

To reconfirm the association between the *G(−273)C*, *C(−297)T*, and *IMS-JST071749* polymorphisms and the HDL-C level, we determined the genotypes in the HTN group. As shown in Table 3, the *G(−273)C* polymorphism was associated with the residual HDL-C level ($P=0.0310$). The genotype frequency of the *G(−273)C* polymorphism in the HTN group was in accordance with Hardy-Weinberg equilibrium and did not differ from that of the Suita population ($P=0.2953$). The *C(−297)T*($P=0.1829$) and *IMS-JST071749*($P=0.4130$) polymorphisms were not associated with the residual HDL-C level. Thus, a positive association was observed between *G(−273)C* and the HDL-C level in two groups: the Suita population and the HTN group.

Association between *ABCA1 G(−273)C* and incidence of MI

We next evaluated whether the *ABCA1 G(−273)C* polymorphism was associated with the incidence of MI. The HDL-C level in the male MI group (1.09 ± 0.01, $P<0.0001$) was significantly lower than that in the male Suita subjects (1.44 ± 0.02). The effects of this genotype on the HDL-C level were not observed in this group, probably because a substantial proportion of this group had dyslipidemia and had been treated with hypolipidemic drugs.

No significant association was observed between the *ABCA1 G(−273)C* polymorphism and the incidence of MI [the MI group: *GG* $n=212$ (38.6%), *GC* $n=289$ (45.2%), *CC* $n=130$ (16.2%); the Suita population: *GG* $n=309$ (35.5%), *GC* $n=362$ (48.3%), *CC* $n=130$ (16.2%), $P=0.4443$].

(−), *I/D#2T GGGG(−226)(−)*

G(−402)C	*C(−297)T*	*G(−273)C*	I/D#2	*G(−99)C*	*C(−14)T*	*C52A*	*T313C*	*G380T*
0.22034**	0.73333***	0.22034**	0.73333***	0.29781***	0.55012***	0.01524	0.55012***	0.52781***
0.06087	0.01449	0.06087	0.01449	0.06636	0.10559*	0.21726***	0.10559*	0.11538*
0.65714***	0.30667***	0.65714***	0.30667***	0.71214***	0.37882***	0.04726	0.37882***	0.36111***
0.22034**	0.73333***	0.22034**	0.73333***	0.29781***	0.55012***	0.01524	0.55012***	0.52781***
0.65714***	0.30667***	0.65714***	0.30667***	0.71214***	0.37882***	0.04726	0.37882***	0.36111***
0.16483**	0.07692	0.16483**	0.07692	0.15119**	0.09502*	0.01003	0.09502*	0.09582*
1***	1***	1***	1***	0.40741***	1***	0.06158	1***	1***
1***	0.46667***	1***	0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
	0.46667***	1***	0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
		0.46667***	1***	0.21839**	0.80952***	0	0.80952***	0.7978***
			0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
				0.21839**	0.80952***	0	0.80952***	0.7978***
					0.26978***	0.06636	0.26978***	0.25325***
						0.10559*	1***	1***
							0.10559*	0.11538*
								1***

values indicate $D' > 0.5$. All values refer to the variant allele indicated in the table

Table 2 Lipid levels in the *ABCA1 G(−273)C* genotypes (Suita population). Subjects who were receiving anti-hyperlipidemic medication were excluded. Values are mean ± SEM. *P*-values calculated by ANOVA

Factors	*GG*	*GC*	*CC*	*P*-value
n (male/female)	306/291	358/415	127/129	
Age (y)	64.1 ± 0.5	63.7 ± 0.4	63.9 ± 0.7	0.7934
BMI (kg/m^2)[a]	22.7 ± 0.1	22.4 ± 0.1	22.9 ± 0.2	0.0607
Smoking (cigarettes/day)	9.2 ± 0.5	8.5 ± 0.5	8.6 ± 0.8	0.5806
Alcohol consumption (ml/week)	85.7 ± 5.5	80.1 ± 4.9	71.3 ± 8.5	0.3597
Total cholesterol (mmol/l)	5.31 ± 0.03	5.36 ± 0.03	5.38 ± 0.05	0.3559
HDL[b]cholesterol (mmol/l)	1.53 ± 0.02	1.58 ± 0.01	1.60 ± 0.03	0.0258
Triglycerides (mmol/l)[c]	1.25 ± 0.04	1.15 ± 0.03	1.18 ± 0.05	0.2583
Residual HDL cholesterol (mmol/l)[d]	−0.03 ± 0.01	0.00 ± 0.01	0.05 ± 0.02	0.0148
Residual HDL cholesterol (mmol/l)[e]	−0.03 ± 0.01	0.01 ± 0.01	0.05 ± 0.02	0.0074

[a]Body-mass index
[b]High-density lipoprotein
[c]Test performed on log-transformed values
[d]Residual HDL cholesterol was adjusted for sex, age, body-mass index, smoking, and alcohol consumption
[e]Residual HDL cholesterol was adjusted for sex, age, BMI, smoking, alcohol consumption, *ApoE*genotype, and *ApoA1*genotype (*JST-IMS005603*)

Table 3 Lipid levels in the *ABCA1 G(−273)C*genotypes (hypertension group). Values are mean ± SEM. *P*-values calculated by ANOVA

Factors	*GG*	*GC*	*CC*	*P*-value
n (male/female)	165/128	196/141	58/47	
Age (y)	64.5 ± 0.6	65.6 ± 0.6	65.3 ± 1.1	0.4561
BMI (kg/m^2)	24.1 ± 0.3	23.8 ± 0.3	23.3 ± 0.4	0.2766
Smoking (cigarettes/day)	11.6 ± 0.9	10.9 ± 0.9	12.1 ± 1.6	0.7828
Drinking habit (I/II)[a]	117/170	154/180	41/60	0.3460
Total cholesterol (mmol/l)	5.18 ± 0.05	5.28 ± 0.05	5.33 ± 0.09	0.2316
HDL cholesterol (mmol/l)	1.31 ± 0.02	1.36 ± 0.02	1.44 ± 0.04	0.0259
Triglycerides (mmol/l)[b]	1.54 ± 0.07	1.52 ± 0.07	1.64 ± 0.12	0.9429
Residual HDL cholesterol (mmol/l)[c]	−0.04 ± 0.02	0.02 ± 0.02	0.07 ± 0.04	0.0310

[a]Drinking habit: *I* subjects with drinking habit, *II* subjects without drinking habit
[b]Test performed on log-transformed values
[c]Residual HDL cholesterol was adjusted for sex, age, BMI, smoking, and drinking habit

Discussion

In the present study, we evaluated the effects of polymorphisms in *ABCA1* on the HDL-C level using a large cohort representing the general population in Japan (the Suita Study). To evaluate the genetic influence of *ABCA1*polymorphisms on HDL-C level, the HDL-C level was adjusted not only for standard