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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A promoter variant of the heme oxygenase-1 gene may reduce the incidence of ischemic heart disease in Japanese

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Abstract

Heme oxygenase-1 (HO-1) has been suggested to have antiatherogenic properties. This study was designed to examine the relationship between the HO-1 gene (*HMOX1*) and ischemic heart disease. The study population consisted of 1972 control subjects and 597 subjects with ischemic heart disease (myocardial infarction (MI) $n = 393$, *HMOX1* $n = 204$). The control subjects were consecutively selected from the Suita study, an epidemiological cohort representing the general population in Japan. Patients with ischemic heart disease were recruited from the outpatient clinic of the National Cardiovascular Center (NCVC). We sequenced *HMOX1* and found a *T(−413)A* polymorphism in the promoter region. Multiple logistic analyses indicated that the *T(−413)A* (*TA* + *TT*/*AA*) polymorphism, sex, smoking habit, DM and BMI affected the occurrence of ischemic heart disease. The odds ratios of the *TA* + *TT* allele for MI and AP were 1.42 ($P = 0.0468$, 95% confidence interval: 0.01–0.35) and 1.86 ($P = 0.0096$, 95% confidence interval: 0.08–0.55), respectively. Luciferase reporter assay indicated that the *A* allele promoter had significantly higher activity than the *T* allele promoter. The *AA* genotype of *HMOX1* reduced the incidence of ischemic heart disease, possibly due to the high expression level of *HMOX1*.

Keywords: Heme oxygenase-1; Ischemic heart disease; Epidemiology; Genetics; Antioxidants

1. Introduction

Oxidative stress is believed to underlie the etiology of numerous human pathological conditions, including atherosclerosis, myocardial ischemia, and several neurodegenerative disorders [1]. Cellular antioxidants appear to be crucial for the reduction of oxidative stress and prevention of the associated pathology.

Heme oxygenases (HO), which are essential for heme degradation, have been shown to have antioxidative properties via the production of bile pigments, carbon monoxide and ferritin induction [2]. Three isoforms of HO have been identified so far. HO-2 and HO-3 are produced constitutively, whereas HO-1 is an inducible form. HO-1 expression is normally difficult to detect in cells other than macrophages, but it is markedly activated in virtually all cell types by initiators of stress such as hyperthermia [3], heme [4], oxidized lipoproteins [5], inflammatory cytokines [6], and hypoxia [7], and has been shown to be up-regulated in disease models such as atherosclerosis [8]. Recently, Ishikawa et al. demonstrated that HO-1 has antiatherogenic properties in Watanabe heritable hyperlipidemic rabbits and LDL-receptor knockout mice [9,10].

Therefore, it could be hypothesized that if the expression of HO-1 varies according to polymorphism in its promoter region, this may be associated with the development of oxidative stress-inducing diseases.

In the present study, we screened for sequence variations in the promoter region of *HMOX1* and evaluated the significance of polymorphisms in myocardial infarction (MI) and angina pectoris (AP).

2. Methods

2.1. Subjects

One thousand nine hundred and seventy two consecutive subjects without any cardiovascular complications were

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Table 1
Primers and probes for genotype determination

HMOX1	Sequence
G(−1135)A	
Sense	5′-TGACATTTTAGGGAGCTGGAGACA-3′
Antisense	5′-CCACCATGCCCAGCTAATTTA-3′
Probe for *G(−1135)*	5′-Fam-GAGACCCTGTCTCTACA-MGB-3′
Probe for *A(−1135)*	5′-Vic-AGACCCCGTCTCTACA-MGB-3′′
A(−413)T	
Sense	5′-TGACATTTTAGGGAGCTGGAGACA-3′
Antisense	5′-AGGCGTCCCAGAAGGTTCCA-3′
Probe for *A(−413)*	5′-Fam-CCCACCAGGCTATTGCTCTGAGCA-Tamra-3′
Probe for *T(−413)*	5′-Tet-CCCACCAGGCTTTTGCTCTGAGC-Tamra-3′

selected as controls from the Suita study. The selection criteria and design of the Suita study have been described previously [11]. Subjects with documented MI ($n = 393$) or AP ($n = 204$) were randomly recruited from the outpatient clinic of the National Cardiovascular Center (NCVC). Written informed consent was obtained from all of the subjects. The present study was approved by the Committee on Genetic Analysis and Genetic Therapy and the Ethics Committee of NCVC.

2.2. DNA studies

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

2.3. Expression study

To explore the regulatory effects of the *T(−413)A* polymorphism in the promoter region, we constructed HO-1 promoter/luciferase fusion genes. The promoter region between −1876 and +75 was amplified by PCR with a sense primer, P2-S (5′-CACCAGACCCAGACAGATTTACCTG-3′) and an antisense primer, P2-AS (5′-GTGCTGGGCTCGTTCGTGCTGGCTCC-3′) (Fig. 1) and subcloned into pGL2-Basic or pGL2-Enhancer DNA (Promega), which does not contain any promoter sequence. Site-specific mutation was made by a QuickChange site-directed mutagenesis kit (Stratagene). Transfection was performed in bovine aortic endothelial cells (BAECs) with PRL-CMV vector (Promega) as an internal standard. *Photinus* and *Renilla* luciferase activities were measured with a dual luciferase kit (PG-DUAL-SP, Toyo Ink Co.).

2.4. Statistical analysis

Values are expressed as mean ± standard error of the mean (S.E.M.). Deviation of the genotype distribution from Hardy–Weinberg equilibrium was analyzed by SNPAlyze software (DYNACOM Co. Ltd., Japan). All other statistical analyses were performed using the JMP statistical software packages (SAS Institute Inc., USA). Multiple logistic analyses were performed with other covariates. Differences in numerical data among the groups were analyzed by one-way/two-way ANOVA and the unpaired *t*-test. Differences in frequencies among the groups were tested by contingency table analysis.

3. Results

3.1. HMOX1 promoter polymorphisms

The nucleotide sequence of the 5′-flanking region and exon 1 of human *HMOX1* is shown in Fig. 1. We found *G(−1135)A* and *T(−413)A* polymorphisms. We analyzed the distribution of each genotype in cases and control. No significant deviation from Hardy–Weinberg equilibrium was observed (Table 2). We also confirmed the existence of a $(GT)_n$ repeat length polymorphism in the promoter region of HO-1. The $(GT)_n$ repeat length in the *HMOX1* ranged from 15 to 43. There were 22 genotypes in $(GT)_n$ repeat polymorphism with frequencies above 1% (Table 3). Haplotype frequencies were estimated from Table 3 and are shown in Table 4.

3.2. Association study

Table 5 shows characteristics of the study population. The frequency of the *AA* genotype in *T(−413)A* polymorphism was significantly higher in control subjects (21.3%) than in subjects with MI or AP (16.3 and 15.7%, $P = 0.0173$). There was no significant difference in the frequency of the *AA* genotype between MI and AP subjects ($P = 0.607$). No significant difference was observed in the frequency of the *G(−1135)A* genotype between controls and subjects with MI or AP ($P = 0.195$ and 0.209, respectively). Multiple logistic analyses indicated that the *T(−413)A* (*TA+TT/AA*) polymorphism (odds ratio, 1.42; 95% confidence interval,

Table 2
Deviation of each polymorphism from Hardy–Weinberg equilibrium

HMOX1	Group	χ^2	*P*
G(−1135)A	Control	0.630	0.427
	AP	2.596	0.107
	MI	0.157	0.692
A(−413)T	Control	0.020	0.888
	AP	2.936	0.087
	MI	0.015	0.903

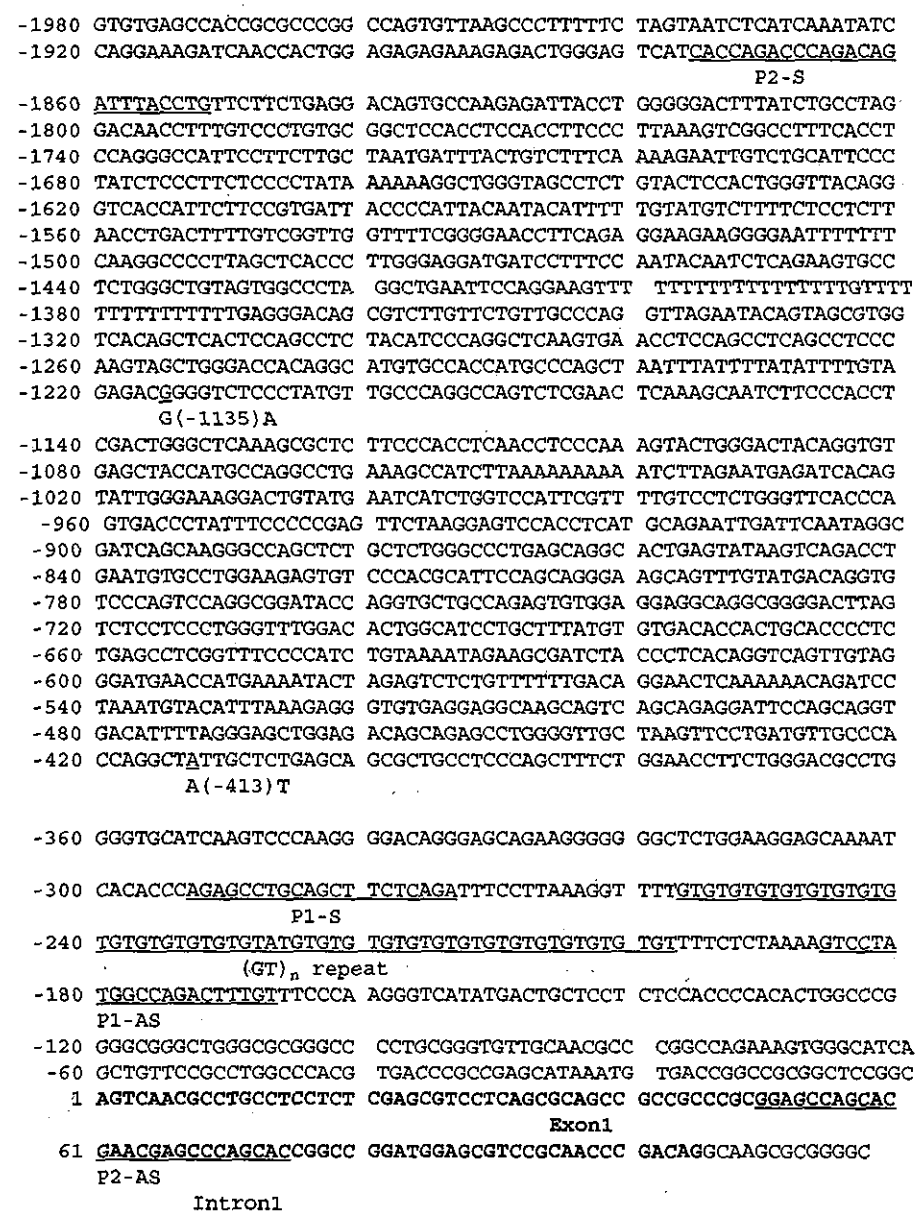

Fig. 1. Nucleotide sequence of the 5′-flanking region and exon 1 of the human *HMOX1* (Tyrrell et al., 1993; GenBank S58267). The fragment between P1-S and P1-AS was amplified by PCR and the $(GT)_n$ repeat length was determined. P2-S and P2-AS were used to construct *HMOX1* promoter/luciferase fusion genes.

0.006–0.352; $P = 0.0468$), sex ($P < 0.0001$), smoking habit ($P < 0.0001$), DM ($P < 0.0001$) and BMI ($P = 0.0049$) affected the occurrence of MI and the *T(−413)A* (*TA* + *TT*/*AA*) polymorphism (odds ratio, 1.86; 95% confidence interval, 0.008–0.554; $P = 0.0096$), sex ($P < 0.0001$), smoking habit ($P < 0.0001$), DM ($P < 0.0001$) and BMI ($P = 0.0045$) affected the occurrence of AP.

3.3. Functional significance of T(−413)A polymorphism

We next examined the functional significance of the *T(−413)A* polymorphism in vitro using BAECs. As shown in Fig. 2, the promoter activity of the *A(−413)*-$(GT)_{30}$ and *A(−413)*-$(GT)_{23}$ alleles was significantly higher than that of the *T(−413)*-$(GT)_{23}$ and *T(−413)*-$(GT)_{30}$ alleles in vitro ($P < 0.01$).

4. Discussion

In the present study, we found previously unidentified sequence variations in the promoter region of *HMOX1*. We then examined the relationship between these polymorphisms and the occurrence of ischemic heart disease. The *AA* genotype of *T(−413)A* polymorphism in the promoter region of the *HMOX1* was found to reduce the incidence

Table 3
Number of *A(−413)T* genotypes in each genotype of $(GT)_n$ repeat length polymorphism

$(GT)_n$ repeat length polymorphism	*AA*	*AT*	*TT*
(21, 30)	0	30	2
(22, 23)	0	1	26
(22, 30)	0	26	0
(23, 23)	0	8	107
(23, 24)	0	5	81
(23, 25)	2	7	65
(23, 30)	8	304	6
(23, 31)	2	47	1
(23, 33)	0	23	20
(23, 34)	2	3	39
(24, 25)	0	2	34
(24, 30)	2	92	2
(25, 30)	5	97	0
(25, 33)	0	16	9
(26, 30)	2	28	0
(30, 30)	229	13	4
(30, 31)	67	5	1
(30, 32)	28	5	0
(30, 33)	58	19	3
(30, 34)	2	63	1
(30, 36)	0	30	0

of MI and AP, possibly due to the high expression level of HO-1.

Recently, *HMOX1* promoter microsatellite polymorphism was reported to be associated with emphysema, restenosis after percutaneous transluminal angioplasty and coronary artery disease [12–14]. While these authors have categorized the length of $(GT)_n$ repeats into three classes, there is no rational explanation for such a classification. Yamada et al. constructed *HMOX1* promoter/luciferase fusion genes containing different numbers of $(GT)_n$ repeats (n = 16, 20, 29, or 38) and conducted a luciferase reporter assay [12]. Although they concluded that the longer promoters have lower activities, there is no difference in promoter activity between $(GT)_{29}$ and $(GT)_{38}$ and the differences in relative luciferase units among these four promoters are only about two-fold

Table 4
Estimated allele frequency

Number of $(GT)_n$ repeats	−413	Estimated allele frequency (%)
30	*A*	39.0
23	*T*	28.0
24	*T*	7.2
25	*T*	6.7
31	*A*	4.3
33	*A*	3.6
34	*T*	2.5
33	*T*	2.0
32	*A*	1.7
22	*T*	1.6
30	*T*	1.4

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

Table 5
Characteristics of the study population

	Control	MI	AP	*P*
n	1972	393	204	
Male (%)	47.9	83.2	83.3	<0.0001
Age (years)	59.9 (0.3)	58.4 (0.6)	59.7 (0.8)	0.066
BMI (kg/m^2)	22.7 (0.1)	23.6 (0.2)	23.8 (0.2)	<0.0001
DM (%)	4.7	43.8	43.4	<0.0001
HTN (%)	38.2	58.3	66.2	<0.0001
HDL (mg/dl)	58.5 (0.4)	43.8 (0.7)	44.1 (1.5)	<0.0001
LDL (mg/dl)	126.4 (0.7)	133.1 (1.8)	133.0 (3.3)	0.0004
TG (mg/dl)	127.0 (2.3)	132.5 (3.6)	164.6 (7.8)	<0.0001
Current smoking (%)	22.9	55.5	52.0	<0.0001
TT/TA/AA	622/930/420	121/208/64	71/101/32	0.0484
TT + TA/AA	1552/420	329/64	172/32	0.0173
TT/TA + AA	622/1350	121/272	71/133	0.586

Values are expressed as mean (S.E.M.). MI, subjects with myocardial infarction; AP, subjects with angina pectoris; BMI, body mass index; DM, diabetes mellitus; HTN, hypertension; TG, triglycerides.

in A549 cells. Moreover, the frequencies of these promoters with $(GT)_{16}$, $(GT)_{20}$, $(GT)_{29}$, or $(GT)_{38}$ are low and we do not know whether we can apply their conclusion to the activities of other common promoters. Furthermore, they did not take into account other polymorphisms and there is a possibility that the genotypes of *A(−413)T* were different among their promoter/luciferase genes. Therefore, it is difficult to explain their clinical results simply from the number of $(GT)_n$ repeats. As shown in Fig. 2, the promoter activity of the *A(−413)*-$(GT)_{30}$ and *A(−413)*-$(GT)_{23}$ alleles was significantly higher than that of the *T(−413)*-$(GT)_{23}$ and *T(−413)*-$(GT)_{30}$ alleles in vitro ($P < 0.01$).

Therefore, the *T(−413)A* genotype may be responsible for the promoter activity. Since *A(−413)*-$(GT)_{30}$ and *T(−413)*-$(GT)_{23}$ are the two major alleles and our promoter assay showed that the promoter activity of the *A(−413)*-$(GT)_{30}$ allele was significantly higher than that of the *T(−413)*-$(GT)_{23}$ allele, it should be sufficient to

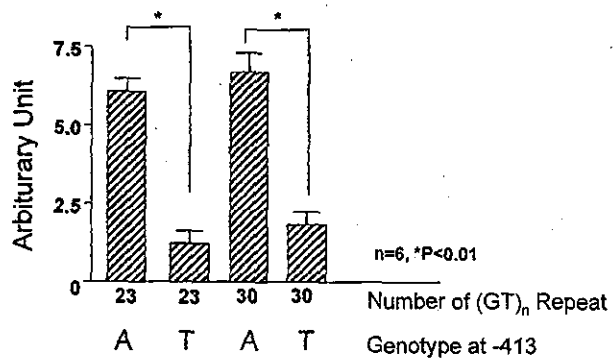

Fig. 2. Assessment of promoter activity. Transient transfection of *HMOX1* promoter/luciferase fusion genes was performed in BAECs. *Photinus* luciferase activity, which indicated promoter activity of the *HMOX1*, was divided by *Renilla* luciferase activity and expressed as relative luciferase units. The *A(−413)*-$(GT)_{30}$ and *A(−413)*-$(GT)_{23}$ alleles had significantly higher promoter activity than the *T(−413)*-$(GT)_{30}$ and *T(−413)*-$(GT)_{23}$ alleles.

determine the *T(−413)A* genotype to conclude whether there are functional alterations in *HMOX1*.

Ever since the biochemical isolation of the HO enzyme in 1968, much of the focus of HO research has been on the role of HO in heme metabolism. However, in recent years, as a result of the emerging role of HO in a variety of biological processes, there has been growing interest in the role of HO in maintaining cellular homeostasis. Indeed, many laboratories have demonstrated that the induction of endogenous HO-1 provides protection against oxidative stress in various models. Recently, Ishikawa et al. demonstrated that HO-1 has antiatherogenic properties in Watanabe heritable hyperlipidemic rabbits and LDL-receptor knockout mice [9,10].

Since biliverdin and bilirubin have been shown to scavenge reactive oxygen species and inhibit lipid peroxidation in vitro [15], they might reduce the inflammatory responses of vascular wall cells to oxidized LDL [16]. HO induction is also reported to enhance plasma nitrite and nitrate concentrations [10], which may be another mechanism by which HO protects against atherogenesis. Moreover, carbon monoxide, which is another product of HO, has been reported to have an inhibitory effect on cell cycle genes and platelet aggregation [17,18].

As mentioned above, there is considerable evidence that HO-1 plays a critical role in the adaptive response of the cardiovascular system to a variety of stresses. The present study indicates that the *AA* genotype of the *T(−413)A* polymorphism is less likely to be associated with MI and AP, probably due to its high *HMOX1* promoter activity.

Based on our findings, we believe that a strategy to enhance HO-1 activity could serve as a basis for a potential preventive therapeutic modality for patients at future risk for myocardial ischemia.

5. Study limitations

In this study, a luciferase reporter assay indicated that the *A* allele promoter had significantly higher activity than the *T* allele promoter. However, a promoter assay in vitro does not necessarily represent the expression level of the gene in vivo. Therefore, to confirm our results, further studies are needed that will examine a larger population or sample from different ethnic groups.

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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 G(-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 TGGGG(-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*	*GCCGAGCCGAC C CTTCTCTC* C52A	*CCGGGCTGCGGCAGGGCAGG*	*GCGGGGAGCTCCGCGCACCA*
101	*ACAGAGCCGGTTCTCAGGGC*	*GCTTTGCTCCTTGTTTTTTC*	*CCCGGTTCTGTTTTCTCCCC*	*TTCTCCGGAAGGCTTGTCAA*	*GGGGTAGGAGAAAGAGACGC*
201	*AAACACAAAAGTGGAAAACA*	*GGTAAGAGGCTCTCCAGTGA*	CTTACTTGGGCGTTATTGTT	TTGTTTCGAGGCCAAGGAGG	CTTCGGGAAGTGCTCGGTTT
301	CGGGGACTTTGATCCGGAGC T313C	CCCACATCCCCACCACTTGC	AACTCAGATGGGACCGGAGG	CGGTGTTAAATGGGGAGACG G380T	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 ($n = 598$).

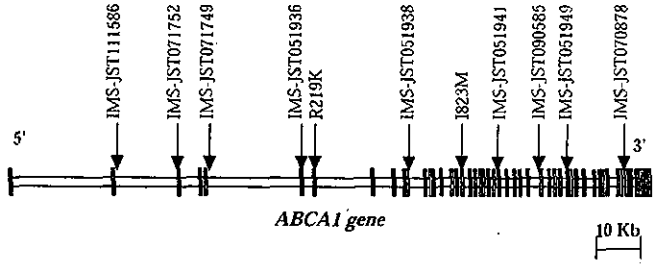

Fig. 2 Schema of *ABCA1* and the position of the determined polymorphisms. *Grayboxes* indicate exonic regions

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.

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).

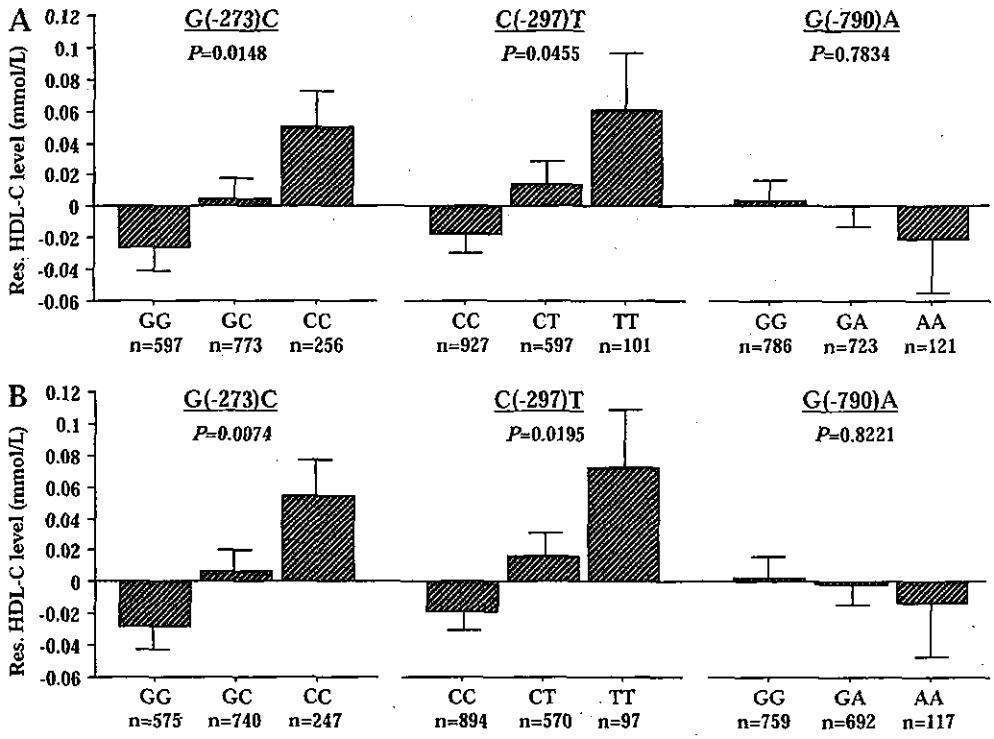

Fig. 3A, B Residual HDL cholesterol levels among the *ABCA1 G(−273)C, C(−297)T,* and *G(−790)A*genotypes. **A** Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, and alcohol consumption. **B** Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, 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-1MS005603*)

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

factors but also for other important genetic factors including the *ApoA1* and *ApoE* polymorphisms. Moreover, we reconfirmed the effects of *ABCA1 G(−273)C* polymorphism on HDL-C in the HTN group. We next investigated the association between the *ABCA1 G(−273)C* and the incidence of MI, but did not observe any association.

The present study is distinguished by three main features: (1) an association study using a large cohort study (the Suita population), (2) taking into account of the influence of the *ApoA1* and *ApoE* polymorphisms, and (3) a confirmation of the association using another set of subjects (the HTN group).

We found that three SNPs were associated with the HDL-C level in 14 SNPs of the *ABCA1*gene in the Suita population. However, if we applied Bonferroni's correction for multiple tests, three SNPs might not be considered significantly associated with the HDL-C level [*G(−273)C*, $P=0.1036$: *C(−297)T*, $P=0.273$: *IMS-JST071749*, $P=0.1302$, P values are corrected by multiplying with 14 (14 SNPs)]. Thus, we verified this positive association in another set of subjects (the HTN group). This association study revealed that *G(−273)C*, but not *C(−297)T* or *IMS-JST071749*, was associated with the HDL-C level. Thus, it is highly likely that *ABCA1 G(−273)C* was truly associated with the HDL-C level.

Since the *ABCA1 G(−273)C* polymorphism is in the promoter region, it is likely that this polymorphism may alter the expression level of ABCA1. However, this polymorphic site had no consensus sequence for transcriptional factors. The *TGGGG(−226)(−)* insertion-deletion polymorphism, which is one of the polymorphisms in LD with the *G(−273)C* polymorphism ($r^2=0.46667$), was in the middle of the consensus sequence of the ZNF202 binding site (GnT repeat)(Porsch-Ozcurumez et al. 2001). The insertion allele, which mainly corresponds to the *(−273)C* allele, should disrupt this binding site and may be associated with higher transcriptional activity of the *ABCA1* gene, which may lead to higher HDL cholesterol levels. However, the *C(−297)T*polymorphism, which was in more tight LD with the *TGGGG(−226)(−)* insertion-deletion polymorphism, appeared to have less effect on the HDL cholesterol level than the *G(−273)C* polymorphism. It remains to be determined whether this discrepancy merely reflects a statistical error or if the *G(−273)C* polymorphism might have additional functional significance. A more detailed promoter analysis will be needed to determine which polymorphisms are functionally important.

The present study revealed that the *ABCA1I823M* polymorphism was not associated with the HDL-C level, inconsistent with a previous report (Harada et al. 2003). This discrepancy may be due to the study design, since a small-scale association study has relatively weak statistical power. In the present study, the sample power was 0.77 for the distribution, sample size, frequencies of the alleles, and α value (0.05, two-tailed). The sample size in the previous study ($n=410$) does not seem to be sufficient to give adequate statistical power. Moreover, the frequency of the *I823* allele in the previous study (allele frequency 0.492) was different from that in the Suita population (0.36) and JSNP information (0.38). Thus, the subjects in the previous study did not seem to be representative of the general Japanese population, as noted by Harada et al. (2003).

Recently, the polymorphisms in the promoter region of *ABCA1*, which corresponds to *C(−559)T* in the present study and seems to be in tight linkage with *G(−273)C* ($r^2=1$, D'-value $=1$), was found to be modestly, but not significantly ($P=0.09$), associated with the HDL-C level using LCAS subjects (Lutucuta et al. 2001). The effect of the *ABCA1 G(−273)C* polymorphism on the HDL-C level was significant, but still relatively weak ($r^2=0.0050$). Accordingly, the sample size ($n=372$) in the previous study (Lutucuta et al. 2001) seems to have been too small to detect the effect of polymorphisms on the HDL-C level clearly.

While the *ABCA1 G(−273)C* polymorphism was associated with HDL-C level, it was not found to be associated with the incidence of MI. The *ApoE* polymorphism (*E2*, *E3*, and *E4*) had the greatest influence on the HDL-C level among the three polymorphisms, *ABCA1 G(−273)C* ($r^2=0.0050$), *ApoA1 JST-IMS005603* (0.0100), and *ApoE*(0.0118). However, the *ApoE* polymorphism was only weakly associated with the incidence of MI ($P=0.0840$). Thus, *ABCA1 G(−273)C* may have too weak an influence on the HDL-C level to alter the incidence of MI through a reduction of the HDL-C level. More large numbers of MI subjects might be necessary to detect the influence of the *ABCA1 G(−273)C* polymorphism on MI incidence.

In summary, the present study provides the first evidence that the common *ABCA1 G(−273)C* polymorphism in the promoter region is significantly associated with the level of HDL cholesterol in the Japanese.

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ORIGINAL ARTICLE

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An association analysis between *ApoA1* polymorphisms and the high-density lipoprotein (HDL) cholesterol level and myocardial infarction (MI) in Japanese

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Abstract Association studies were performed to confirm the effect of polymorphisms in apolipoprotein A1 (*ApoA1*) on the high-density lipoprotein cholesterol (HDL-C) level and the incidence of myocardial infarction (MI). A sequence analysis identified nine polymorphisms in *ApoA1*. After considering linkage disequilibrium, four polymorphisms in *ApoA1*and four polymorphisms in the 5′-flanking regions and 3′-flanking regions from the JSNP database were determined in 1,880 subjects recruited from the Suita study, which represents the general population in Japan. Of the eight polymorphisms tested, the *ApoA1* T84C polymorphism had the greatest effect on the levels of HDL-C ($P=0.0005$, $P_c=0.0040$ corrected by the Bonferroni method) and triglyceride ($P<0.0001$, $P_c=0.0008$). The *ApoA1 Msp*I polymorphism was not associated with HDL-C or triglyceride levels. We confirmed that the *ApoA1* T84C polymorphism was associated with the HDL-C level but not the triglyceride level in patients with MI ($n=637$). Moreover, this polymorphism was associated with the incidence of MI in male subjects ($P=0.0326$). A logistic analysis indicated that the frequency of MI in the CC genotype was lower than that in the CT+TT genotype ($P=0.0145$, OR = 0.4955, 95% CI: 0.2746–0.8525). The *ApoA1* T84C polymorphism is an important marker for the HDL-C level and may be a new risk marker for MI in Japanese.

Keywords *ApoA1* · Polymorphisms · HDL cholesterol · Myocardial infarction · Association study

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Introduction

Lipid profiles are well known to play a pivotal role in the progression of coronary artery disease (CAD): a decreased plasma concentration of high-density lipoprotein cholesterol (HDL-C) and an increased plasma concentration of low-density lipoprotein cholesterol (LDL-C) are associated with the development of CAD (Miller and Miller 1975; Kannel et al. 1979). Apolipoprotein A1 (ApoA1), a component of HDL-C, is a major participant in the regulation of reverse cholesterol transport from peripheral tissues to the liver, and this pathway is thought to help protect against atherosclerosis. In fact, epidemiological studies have reported that decreased plasma concentrations of both HDL-C and ApoA1 were associated with premature CAD (Maciejko et al. 1983).

Genetic factors have been reported to influence the distribution of lipids and lipoprotein levels, including the ApoA1 level (Groenendijk et al. 2001a). A rare variant nonsense mutation at codon 84 has been reported to result in ApoA1 deficiency (Matsunaga et al. 1991). Recent epidemiological studies have reported that common *ApoA1* polymorphisms influence the levels of HDL-C and triglycerides (TG) (Ordovas et al. 1986; Jeenah et al. 1990; Pagani et al. 1990; Talmud et al. 1994; Groenendijk et al. 2001b). In addition, several

researchers reported associations between *ApoA1* polymorphisms and CAD (Karathanasis et al. 1983; Ordovas et al. 1986; Reguero et al. 1998), whereas others found no positive association (Ordovas et al. 1991; Marshall et al. 1994; Yamada et al. 2002). One possible reason for the inconsistencies among previous association studies may be that almost all of these studies considered only a few restriction fragment-length polymorphisms instead of every polymorphism in the *ApoA1*gene. Thus, the polymorphism that has the greatest effect on the HDL-C level and the incidence of CAD may have been missed in previous studies.

To evaluate the effects of polymorphisms in *ApoA1* on lipid levels, we sequenced the *ApoA1* gene and conducted an association study using a large cohort (the Suita population $n=1,880$), representing the general population in Japan. In addition, we confirmed an association between *ApoA1* polymorphisms and lipid levels. Finally, we investigated the association between the *ApoA1* polymorphism and the incidence of myocardial infarction (MI) using patients with MI ($n=637$).

Subjects 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. 2004a). Genotypes were determined in 1,880 consecutive subjects who visited the National Cardiovascular Center between April 2002 and February 2003 (867 men, 1,013 women). The characteristics of this population are shown in Table 1.

Table 1 Characteristics of the Suita population. *P* value was calculated by the Student's *t* test. *BMI* body mass index, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglyceride, *%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	Male	Female	*P* value
n	867	1,013	
Age (year)	66.3 ± 0.4	63.3 ± 0.3	< 0.0001
BMI (kg/m^2)	23.2 ± 0.1	22.3 ± 0.1	< 0.0001
TC (mmol/l)	5.13 ± 0.03	5.58 ± 0.02	< 0.0001
HDL-C (mmol/l)	1.43 ± 0.01	1.68 ± 0.01	< 0.0001
TG (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

When the association between the *ApoA1* T84C polymorphism and the incidence of myocardial infarction was analyzed, subjects with ischemic heart disease were excluded.

The myocardial infarction (MI) group The selection criteria and design of the MI group have been described previously (Takagi et al. 2002). This group consisted of randomly selected inpatients and outpatients with documented MI ($n=637$, 547 men and 90 women) who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003 and met the following criteria: (1) chest pain of ≥30 min duration; (2) electrocardiographic ST segment elevation of ≥0.1 mV in two or more leads in the same vascular territory; and (3) subsequent elevation of creatine phosphokinase levels to more than twice the normal range.

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.

DNA studies

The promoter region (up to −1 kb) and all of the exonic regions in *ApoA1* were sequenced for polymorphisms

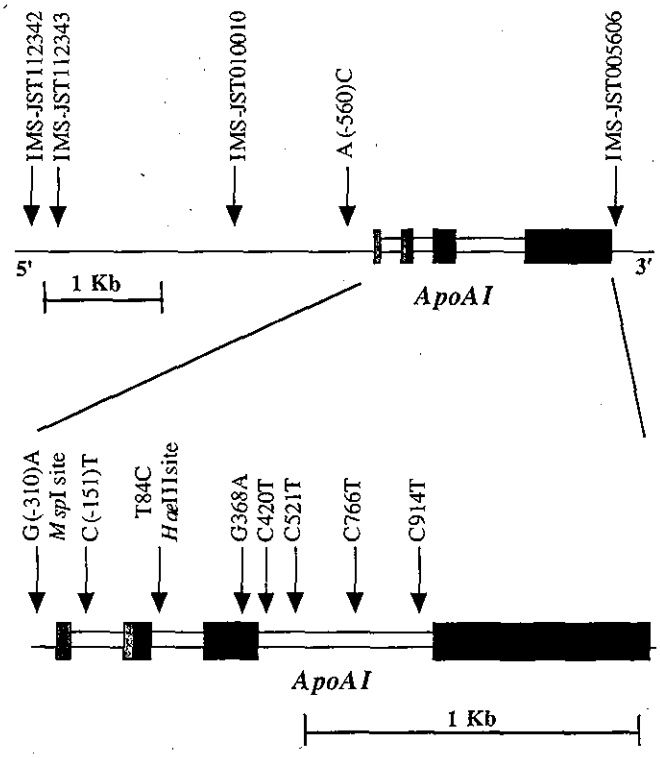

Fig. 1 Schema of the *ApoA1* gene and the positions of the determined polymorphisms. *Gray and black boxes* indicate the 5′-untranslated and coding regions, respectively

in 36 subjects (Fig. 1). For the 5′-flanking regions and 3′-flanking regions, we selected four polymorphisms for genotyping from a public database (JSNP, http://www.snp.ims.u-tokyo.ac.jp, Fig. 1) (Hirakawa et al. 2002). The *ApoE* and ATP-binding cassette transporter A1 (*ABCA1*) G(−273)C polymorphisms were also determined as previously described (Shioji et al. 2004b). *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; Shioji et al. 2004b). All polymorphisms were determined by the TaqMan system. The primer and probe sequences are available on request.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). For TG values, while a logarithmic transformation was applied for the statistical test, untransformed values are shown in the table. LDL-C was calculated by Friedewald's formula [(LDL-C) = (total cholesterol, TC)−(HDL-C)−(TG/5). We excluded those whose HDL-C or TG levels were ≥2.6 mM or 4.53 mM, respectively]. All statistical analyses were performed with the JMP statistical software package (SAS Institute, Inc.). 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 levels were calculated by adjusting for covariates. Differences in numerical data among the groups were evaluated by Student's *t* test or one-way analysis of variance (ANOVA). Hardy-Weinberg equilibrium was calculated by a chi-square test. To measure linkage disequilibrium (LD) between polymorphisms, D' and r^2 values were analyzed using the SNP-Alyze statistical software package (Dynacom, Inc.). In some settings, the P values were corrected (P_c) by multiplying by 8 (eight polymorphisms, Bonferroni).

Results

Polymorphisms of the promoter and exonic regions in *ApoA1*

We found two polymorphisms in the promoter region, one in intron 1, one in intron 2, one in exon 3, and four in intron 3 (Table 2 and Fig. 1).

LD was evaluated by calculating r^2 values (Table 3). We regarded $r^2 > 0.25$ as tight linkage. Accordingly, we selected four polymorphisms, G(−310)A, T84C, G368A, and C420T, for the following association study. The G(−310)A and T(84)C polymorphisms correspond to the *Msp*I (Pagani et al. 1990; Tuteja et al. 1992) and *Hae*III (Groenendijk et al. 2001b) polymorphisms, respectively. The G368A polymorphism was accompanied by a missense mutation (GCC →

Table 2 Polymorphisms in *ApoA1*. The nucleotide numbers of polymorphisms are given according to the number from ATG

SNP name	dbSNP No.	Minor allele frequency	Amino acid change	Sequence
Polymorphisms detected by sequence				
A(−560)C	–	0.078	–	GACACTCCCCTCCCGCCCCCACTGA[A/C]CCCTTGACCCCTGCCCTGCAGCCCC
G(−310)A	670	0.156	–	AGGACCAGTGAGCAGCAACAGGGCC[G/A]GGGCTGGGCTTATCAGCCTCCCAGC
C(−151)T	5069	0.078	–	TCAAGGTTCAGGCCTTGCCCCAGGC[C/T]GGGCCTCTGGGTACCTGAGGTCTTC
T84C	5070	0.234	–	CCTAGGGAGCCAACCATCGGGGGGGC[T/C]TTCTCCCTAAATCCCCGTGGCCCAC
G368A	–	0.063	Ala → Thr	CTATGTGTCCCAGTTTGAAGGCTCC[G/A]CCTTGGGAAAACAGCTAAAGTAAGG
C420T	2070655	0.375	–	CCCAGCCTGGGGTTGAGGGCAGGGG[C/T]AGGGGGGCAGAGGCCTGTGGGATGAT
C521T	5072	0.387	–	CCACAGATGGTCTGGATGGAGAAACT[C/T]GGAATGGGATCTCCAGGCAGGGTCA
C766T	–	0.452	–	TTTGGAGACCAACGTAACTGGGCAC[T/C]AGTCCCAGCTCTGTCTCCTTTTAG
C914T	5076	0.078	–	CTCCGCGGACAGGTGTCACCCAGGG[C/T]TCACCCCTGATAGGCTGGGGCGCTG
Polymorphisms from JSNP database				
IMS-JST010010		0.219	–	TTCTCCTGGAAGGCCCAGACCTCCC[C/T]CAGCAGGTTACTGATAGGACCTGAG
IMS-JST112343		0.279	–	CACTTTCACAACTTAGAATATCCCT[A/G]TAAGGCTGGAGGCCAGATTTTACCC
IMS-JST112342		0.274	–	CTTGCACCCTTGCGGAGCCTGCAGC[C/T]TTTGCAGTCTGATCAGGGACTTCTC
IMS-JST005606		0.108	–	CGTCGATCTTGGCCCTAAGACGTCC[A/T]GTCTGGGCACGGAGTTGTTGAGATC