siveness. *Am J Physiol* 1993; **264**: C1367–C1387.
6. Blaustein MP: Endogenous ouabain: role in the pathogenesis of hypertension. *Kidney Int* 1996; **49**: 1748–1753.
7. Nelson LD, Unlap MT, Lewis JL, Bell PD: Renal arteriolar Na^+/Ca^{2+} exchange in salt-sensitive hypertension. *Am J Physiol* 1999; **276**: F567–F573.
8. Nelson LD, Mashburn NA, Bell PD: Altered sodium-calcium exchange in afferent arterioles of the spontaneously hypertensive rat. *Kidney Int* 1996; **50**: 1889–1896.
9. Unlap MT, Peti-Peterdi J, Bell PD: Cloning of mesangial cell Na^+/Ca^{2+} exchangers from Dahl/Rapp salt-sensitive/resistant rats. *Am J Physiol Renal Physiol* 2000; **279**: F177–F184.
10. Ashida T, Kuramochi M, Omae T: Increased sodium-calcium exchange in arterial smooth muscle of spontaneously hypertensive rats. *Hypertension* 1989; **13**: 890–895.
11. Ashida T, Kawano Y, Yoshimi H, Kuramochi M, Omae T: Effects of dietary salt on sodium-calcium exchange and ATP-driven calcium pump in arterial smooth muscle of Dahl rats. *J Hypertens* 1992; **10**: 1335–1341.
12. Kraev A, Chumakov I, Carafoli E: The organization of the human gene *NCX1* encoding the sodium-calcium exchanger. *Genomics* 1996; **37**: 105–112.
13. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 1994; **269**: 5145–5149.
14. Barnes KV, Cheng G, Dawson MM, Menick DR: Cloning of cardiac, kidney, and brain promoters of the feline *NCX1* gene. *J Biol Chem* 1997; **272**: 11510–11517.
15. Lee SL, Yu AS, Lytton J: Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 1994; **269**: 14849–14852.
16. Nicholas SB, Yang W, Lee SL, Zhu H, Philipson KD, Lytton J: Alternative promoters and cardiac muscle cell-specific expression of the Na^+/Ca^{2+} exchanger gene. *Am J Physiol* 1998; **274**: H217–H232.
17. Scheller T, Kraev A, Skinner S, Carafoli E: Cloning of the multipartite promoter of the sodium-calcium exchanger gene *NCX1* and characterization of its activity in vascular smooth muscle cells. *J Biol Chem* 1998; **273**: 7643–7649.
18. Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: The sixth report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997; **157**: 2413–2446.
19. Kamide K, Tanaka C, Takiuchi S, *et al*: Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004; 333–338.
20. Tanaka C, Kamide K, Takiuchi S, *et al*: An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003; **26**: 301–306.
21. Inamoto N, Katsuya T, Kokubo Y, *et al*: Association of methylenetetrahydrofolate reductase gene polymorphism with carotid atherosclerosis depending on smoking status in a Japanese general population. *Stroke* 2003; **34**: 1628–1633.
22. Hwang EF, Williams I, Kovacs G, *et al*: Impaired ability of the Na^+/Ca^{2+} exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium. *Am J Physiol Renal Physiol* 2003; **284**: F1023–F1031.
23. Meade TW, Imeson JD, Gordon D, Peart WS: The epidemiology of plasma renin. *Clin Sci (Lond)* 1983; **64**: 273–280.
24. Alderman MH, Madhavan S, Cohen H, Sealey JE, Laragh JH: Low urinary sodium is associated with greater risk of myocardial infarction among treated hypertensive men. *Hypertension* 1995; **25**: 1144–1152.
25. Furman I, Cook O, Kasir J, Low W, Rahamimoff H: The putative amino-terminal signal peptide of the cloned rat brain Na^+-Ca^{2+} exchanger gene (RBE-1) is not mandatory for functional expression. *J Biol Chem* 1995; **270**: 19120–19127.
26. Loo TW, Ho C, Clarke DM: Expression of a functionally active human renal sodium-calcium exchanger lacking a signal sequence. *J Biol Chem* 1995; **270**: 19345–19350.

J Hum Genet (2004) 49:433–439
DOI 10.1007/s10038-004-0172-1

ORIGINAL ARTICLE

Keisuke Shioji · Toshifumi Mannami · Yoshihiro Kokubo
Yoichi Goto · Hiroshi Nonogi · Naoharu Iwai

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

Received: 16 February 2004 / Accepted: 10 May 2004 / Published online: 17 July 2004
© The Japan Society of Human Genetics and Springer-Verlag 2004

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

This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

K. Shioji · N. Iwai
Department of Epidemiology, Research Institute, National Cardiovascular Center, Suita, Japan

K. Shioji (✉)
Department of Cardiology, Kishiwada City Hospital, 1001 Gakuhara-cho, Kishiwada, Osaka 596-8501, Japan
E-mail: shiojikeisuke@kpe.biglobe.ne.jp
Tel.: +81-724-451000
Fax: +81-724-418809

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

T. Mannami · Y. Kokubo
Department of Preventive Cardiology, National Cardiovascular Center, Suita, Japan

Y. Goto · H. Nonogi
Division of Cardiology, National Cardiovascular Center, Suita, Japan

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

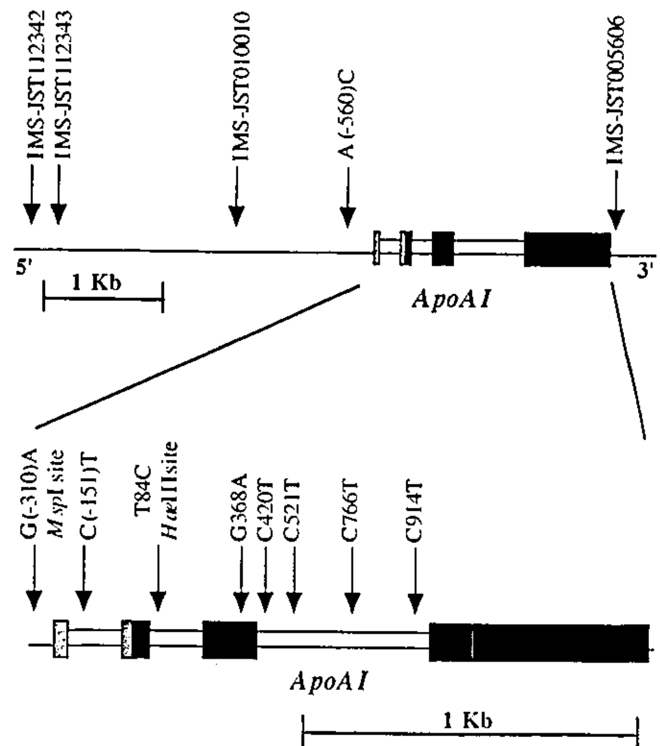

Fig. 1 Schema of the *ApoA1* gene and the positions of the determined polymorphisms. *Gray and black boxes* indicate the 5′-untranslated and coding regions, respectively

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 SNPAlyze 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	–	CCTAGGGAGCCAACCATCGGGGGGC[T/C]TTCTCCCTAAATCCCCGTGGCCCAC
G368A	–	0.063	Ala → Thr	CTATGTGTCCCAGTTTGAAGGCTCC[G/A]CCTTGGGAAAACAGCTAAAGTAAGG
C420T	2070655	0.375	–	CCCAGCCTGGGGTTGAGGGCAGGGG[C/T]AGGGGGCAGAGGCCTGTGGGATGAT
C521T	5072	0.387	–	CCACAGATGGTCTGGATGGAGAAAC[T/C]GGAATGGGATCTCCAGGCAGGGTCA
C766T	–	0.452	–	TTTGGAGACCAACGTAACTGGGCAC[T/C]AGTCCCAGCTCTGTCTCCTTTTTAG
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

Table 3 Linkage disequilibrium among the polymorphisms in *ApoA1*. R^2 values are shown, R^2 values described are based on the genotypes of 36 subjects used for sequence analyses. All values refer to the variant allele indicated in the table

	A(−560)C	G(−310)A	C(−151)T	T84C	G368A	C420T	C521T	C766T	C914T
A(−560)C		0.016	1	0.277	0.006	0.051	0.044	0.084	1
G(−310)A			0.016	0.057	0.004	0.111	0.121	0.158	0.016
C(−151)T				0.277	0.006	0.051	0.044	0.084	1
T84C					0.020	0.184	0.184	0.002	0.277
G368A						0.040	0.044	0.057	0.006
C420T							1	0.767	0.051
C521T								0.767	0.044
C766T									0.084
C914T									

Table 4 Lipid levels among the *ApoA1* T84C genotypes (Suita population). We excluded subjects who were receiving hypolipidemic medication. Values are mean ± SEM, Res. TC, Res. HDL-C, Res. LDL-C, and Res. TG were adjusted for gender, age, BMI, smoking (cigarettes/day), and alcohol consumption (ethanol ml/week). *P* value was calculated by ANOVA. *P* values were corrected (P_c) by multiplying by 8 (eight polymorphisms, Bonferroni). *BMI* body mass index, *%HT* percentage of subjects with hypertension, *%DM* percentage of subjects with diabetes mellitus, *%HLP* percentage of subjects with hyperlipidemia, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglyceride, *Res. TC* residuals of TC; *Res. HDL-C* residuals of HDL-C, *Res. LDL-C* residuals of LDL-C, *Res. TG* residuals of TG; *%drinking* percentage of subjects with a drinking habit; *%smoking* percentage of subjects with a smoking habit

ApoA1 T84C genotype	TT	TC	CC	*P* value	P_c value
Number (males/females)	469/487	279/310	48/42	0.5378	
Age (years)	63.8 ± 0.4	63.9 ± 0.5	65.5 ± 1.2	0.3890	
BMI (kg/m^2)	22.7 ± 0.1	22.6 ± 0.1	22.4 ± 0.3	0.7253	
%HT	37.6	37.2	48.9	0.0977	
%DM	6.5	7.3	10	0.4527	
%HLP	41.6	42.8	33.3	0.2307	
TC (mmol/l)	5.32 ± 0.03	5.39 ± 0.03	5.27 ± 0.09	0.2325	1
HDL-C (mmol/l)	1.54 ± 0.01	1.59 ± 0.02	1.68 ± 0.04	0.0005	0.0040
LDL-C (mmol/l)[a]	3.24 ± 0.02	3.29 ± 0.03	3.16 ± 0.08	0.2357	1
TG (mmol/l)[b]	1.26 ± 0.03	1.15 ± 0.04	0.95 ± 0.09	< 0.0001	0.0008
Res. TC (mmol/l)	−0.02 ± 0.03	0.03 ± 0.03	−0.05 ± 0.08	0.3332	1
Res. HDL-C (mmol/l)	−0.03 ± 0.01	0.02 ± 0.01	0.12 ± 0.04	0.0002	0.0016
Res. LDL-C (mmol/l)[a]	−0.01 ± 0.02	0.03 ± 0.03	−0.07 ± 0.08	0.3235	1
Res. TG (mmol/l)[b]	0.05 ± 0.03	−0.05 ± 0.03	−0.24 ± 0.09	< 0.0001	0.0008
%Drinking	47.7	47.9	55.6	0.3550	
%Smoking	18.3	20.0	13.3	0.2688	

[a]The formula for calculating LDL-C is described in "Subjects and methods", and we excluded subjects whose HDL-C or TG levels were ≥2.6 mM or 4.53 mM, respectively (TT, n(male/female) = 457/478: TC, n = 274/301: CC, n = 48/41)
[b]Test performed on log-transformed values

ACC, Ala → Thr) at codon 61 in exon 4 (Matsunaga et al. 1991).

Association study of *ApoA1* (Suita population)

The T84C polymorphism had the greatest effect on the levels of HDL-C and TG, but not the levels of TC and LDL-C, among the eight polymorphisms (sample power = 0.96, αvalue = 0.05, two-tailed, Table 4). The IMS-JST112342 and IMS-JST112343 polymorphisms were associated with the levels of HDL-C and TG (residuals of HDL-C, $P = 0.0059$, $P_c = 0.0472$, each: residuals of TG, $P = 0.0002$, $P_c = 0.0016$, each). The other polymorphisms were not associated with HDL-C or TG levels. The IMS-JST112342 polymorphism was in almost complete linkage with the IMS-JST112343 polymorphism ($r^2 = 0.98157$, D' value = 1, $P < 0.0001$). The IMS-JST112342 and IMS-JST112343 polymorphisms were in tight linkage with the T84C polymorphism ($r^2 = 0.41365$, D' value = 0.71155, $P < 0.0001$, each). Accordingly, the effects of the IMS-JST112342 and IMS-JST112343 polymorphisms may be mainly explained by their linkage with the T84C polymorphism. We previously reported that the *ApoE* genotype and the *ABCA1* G(−273)C effect the HDL-C level (Shioji et al. 2004b). Accordingly, we performed the multiple logistic analysis, which included gender, age, body mass index (BMI), smoking, alcohol consumption, *ApoE* genotype, *ABCA1* G(−273)C, and *ApoA1* T84C. As shown in Table 5, the multiple logistic analysis indicated that *ApoE* genotype, *ApoA1* T84C, and *ABCA1* G(−273)C were independent factors significantly associated with the HDL-C level. No significant deviation from the Hardy-Weinberg equilibrium was observed in the T84C polymorphism ($P = 0.8075$). Thus, we selected the T84C polymorphism for the following association study.

Association among *ApoA1* T84C and lipid profile (the MI group)

To confirm the association between the *ApoA1* T84C polymorphism and the levels of HDL-C and TG, we

Table 5 Sum of square and F value of high-density lipoprotein cholesterol (HDL-C) from multiple logistic analyses. *BMI* body mass index, *ABCA1* ATP-binding cassette transporter A1 gene

Source	Sum of squares	F value	Probability > F
BMI	31,815	171.5	<0.0001
Gender	18,881	101.7	<0.0001
Alcohol consumption (ethanol ml/week)	13,588	73.2	<0.0001
ApoE genotype	4,360	11.7	<0.0001
ApoA1 T84C	2,981	8.0	0.0003
Smoking (cigarettes/day)	1,972	10.6	0.0011
Age	1,761	9.5	0.0021
ABCA1 G(−273)C	1,475	4.0	0.0190

Table 6 Lipid levels among the *ApoA1* T84C genotypes [miocardial infarction (MI) group]. Values are expressed as the mean ± SEM. P value was calculated by ANOVA. *BMI* body mass index, *%HT* percentage of subjects with hypertension, *%DM* percentage of subjects with diabetes mellitus, *%HLP* percentage of subjects with hyperlipidemia, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglyceride

ApoA1 T84C genotype	TT	TC	CC	P value
Number (males/females)	326/61	204/27	17/2	0.3264
Age (years)	62.1±0.5	62.4±0.7	60.2±2.4	0.6632
BMI (kg/m^2)	23.7±0.2	23.7±0.2	24.7±0.7	0.3780
%HT	55.8	55.2	42.1	0.5076
%DM	47.1	37.2	31.6	0.0439
%HLP	55.5	60.2	52.6	0.4844
TC (mmol/l)	5.18±0.06	5.30±0.07	5.21±0.24	0.2752[a]
HDL-C (mmol/l)	1.09±0.02	1.11±0.03	1.35±0.08	0.0050[a]
LDL-C (mmol/l)	3.34±0.06	3.47±0.07	3.57±0.25	0.2252[a,b]
TG (mmol/l)	1.48±0.05	1.53±0.06	1.21±0.21	0.2872[c]

[a]Test performed on residual values adjusted for gender, age, and BMI
[b]The formula for calculating LDL-C is described in "Subjects and methods", and we excluded subjects whose HDL-C or TG levels were ≥2.6 mM or 4.53 mM, respectively [TT, n(male/female) = 322/61: TC, n = 202/27: CC, n = 16/2]
[c]Test performed on log-transformed residual values adjusted for gender, age, and BMI

determined the genotypes in the MI group. The T84C polymorphism was associated with the HDL-C level but not the TG level (Table 6). The T84C polymorphism also affected the prevalence of diabetes mellitus (DM, P = 0.0439). No significant deviation from the Hardy-Weinberg equilibrium was observed in the MI group (P = 0.2403). Thus, a positive association was observed between the T84C polymorphism and the HDL-C level in two groups: the Suita population and the MI group.

Association between *ApoA1* T84C and incidence of MI

We next evaluated whether the *ApoA1* T84C polymorphism was associated with the incidence of MI. Since the MI group and the Suita population were not matched

Table 7 Association between the *ApoA1* T84C polymorphism and the incidence of myocardial infarction (MI). All subjects are male. Values are expressed as the mean ± SEM. *Control* Suita subjects without ischemic heart disease, *MI* patients with myocardial infarction, *BMI* body mass index, *%HT* percentage of subjects with hypertension, *%DM* percentage of subjects with diabetes mellitus, *%HLP* percentage of subjects with hyperlipidemia, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglyceride

	Control	MI group	P value
Number	806	547	
Age (years)	65.8±0.4	60.8±0.4	
BMI (kg/m^2)	23.3±0.1	23.8±0.1	0.0003[a]
%HT	44.7	54.3	0.0003[a]
%DM	11.1	41.6	<0.0001[a]
%HLP	40.6	57.9	<0.0001[a]
TC (mmol/l)	5.14±0.03	5.16±0.04	0.3168[b]
HDL-C (mmol/l)	1.43±0.01	1.08±0.02	<0.0001[b]
LDL-C (mmol/l)	3.10±0.03	3.34±0.04	<0.0001[b,c]
TG (mmol/l)	1.40±0.04	1.54±0.05	0.0641[d]
ApoA1 T84C TT/TC/CC	477/280/49 59.2%/34.7%/6.1%	326/204/17 59.6%/37.3%/3.1%	0.0326[a]

[a]The distributions in the Suita population and patients with MI were compared by the chi-square test
[b]Student's t-test was performed on residual values adjusted for age and BMI
[c]The formula for calculating LDL-C is described in "Subjects and methods", and we excluded subjects whose HDL-C or TG levels were ≥2.6 mM or 4.53 mM, respectively (Control, n = 794: MI group, n = 403)
[d]Student's t test was performed on log-transformed residual values adjusted for age and BMI

for gender, we investigated only males. The T84C polymorphism was significantly associated with the incidence of MI (Table 7). 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, sample power = 0.75 (α value = 0.05, two-tailed)]. Accordingly, subjects with the CC genotype had higher levels of HDL-C and were less susceptible to MI. However, multiple logistic analysis, which included hypertension (HT), DM, hyperlipidemia (HLP), smoking, and the T84C polymorphism, indicated that smoking (P < 0.0001), DM (P < 0.0001), HLP (P = 0.0003), and HT (P = 0.0339) were predictors of incidence of MI but that the T84C polymorphism was not a predictor (P = 0.0175).

Discussion

In the present study, we conducted a sequence analysis and detected nine polymorphisms in *ApoA1*. We evaluated the effects of eight polymorphisms, including four selected from the JSNP database, on the lipid profile using a large cohort representing the general population in Japan. We next confirmed the effects of the *ApoA1*

T84C polymorphism on the HDL-C level in the MI group. Finally, we found a positive association between the *ApoA1* T84C polymorphism and the incidence of MI. However, this polymorphism was not an independent predictor when we performed the multiple logistic analysis, which included the established risk factors of smoking, DM, HLP, and HT.

The present study can be distinguished by three main features: an association study using a large cohort study in the general population (the Suita population), confirmation of the association using another set of subjects (the MI group), and the detection of a new protective marker for MI in the *ApoA1* gene.

As described previously (Zaman et al. 1997), the *ApoA1 Msp*I and *Sst*I polymorphisms were not associated with the levels of total cholesterol and HDL-C in the Shibata study, which represented the Japanese rural population. In the present study, we also did not observe an association between the *ApoA1Msp*I [G(−310)A] polymorphism and HDL-C or TG levels. Since the *ApoA1Msp*I polymorphism has only weak linkage with the *ApoA1* T84C polymorphism ($r^2 = 0.0567$, $P = 0.0568$), the *ApoA1* T84C polymorphism may have the greatest effect on the HDL-C level in the Japanese population.

The present study provides evidence that the *ApoA1* T84C polymorphism is associated with the incidence of MI. The *ApoA1* T84C polymorphism may act in a proatherogenic or antiatherogenic fashion via the modulation of the HDL-C level because the *ApoA1* polymorphisms have effects on the HDL-C level but not the LDL-C level. Yamada et al. reported that *ApoA1* polymorphisms were weakly associated with the incidence of MI (Yamada et al. 2002), but they did not investigate the *ApoA1* T84C polymorphism. Since the *ApoA1* T84C polymorphism may have the greatest effect on the HDL-C level in the Japanese population, it is possible that the *ApoA1* T84C polymorphism may also most strongly influence the risk of MI in the Japanese population. Since the sample power was 0.75 (α value = 0.05, two-tailed), this study has adequate statistical power. However, the present association ($P = 0.0145$) was marginal. After adjustment of risk factors—smoking, DM, HLP, and HT—the significant association between the *ApoA1* T84C polymorphism and the incidence of MI was not observed. Contradictory results often occur in association studies due to ethnic differences or variations, including covariates such as gender and environmental factors. 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 (Lohmueller et al. 2003). Accordingly, it will be necessary to verify the association between the *ApoA1* T84C polymorphism and the incidence of MI using another set of subjects.

The *ApoA1* T84C polymorphism, an *Hae*III restriction site, has recently been reported, and the frequency of the T allele of *ApoA1* T84C in familial combined hyperlipidemia probands has been reported to be higher than in their spouses (Groenendijk et al. 2001b). It has been reported that individuals homozygous for the T84C allele had higher TG and ApoC-III levels but not higher ApoA1 levels (Groenendijk et al. 2001b). Thus, it may be possible that this polymorphism could be in tight linkage with unknown polymorphisms located in exon or promoter regions in *ApoA1* or another genes.

We tried haplotype and diplotype analyses in the *ApoA1* gene. Since all of the polymorphisms were located on one haplotype block and the *ApoA1* T84C polymorphism strongly influenced the levels of HDL and TG, we found no useful haplotype combination that was more influential than the *ApoA1* T84C polymorphism.

The *ApoA1* T84C polymorphism was associated with the TG level in the Suita population but not in the MI group. One possible reason for the different results between the Suita population and the MI group may be that a substantial proportion of the MI group had dyslipidemia and had been treated with hypolipidemic drugs. We could not retrospectively research who was treated with hypolipidemic drugs and what kind of hypolipidemic drug was used in patients with MI, because we made DNA sample anonymous. Accordingly, we did not have the ability to investigate the relationship between *ApoA1* T84C and the lipid levels according to the drugs used drugs, such as statin or fibrate. Thus, another study is needed to confirm the genotype/drug interaction.

Our results indicated that three polymorphisms, *ApoE* genotype, *ApoA1* T84C, and *ABCA1* G(−273)C, were independently associated with the HDL-C level. Multiple components have been proposed to regulate the HDL-C level, including cholesterol ester transfer protein (CETP) (Barter et al. 2003), phospholipid transfer protein (Huuskonen et al. 2001), hepatic lipase (Deeb et al. 2003), lecithin cholesterol acyltransferase (Zhang et al. 2004), scavenger receptor class B type 1 (Hsu et al. 2003), endothelial lipase (Ma et al. 2003), ABC transporters, apolipoproteins, and several transcriptional factors. In Japanese, several polymorphisms such as *CETP* (Inazu et al. 1990) and *ApoE* (Zaman et al. 1997) were reported to be associated with the HDL-C level. Accordingly, a prospective study should be required to establish the contribution of the *ApoA1* T84C polymorphism on the HDL-C level and the incidence of MI.

In conclusion, the present results suggest that the *ApoA1* T84C polymorphism significantly affects the HDL-C level in the general Japanese population and that this polymorphism may be a new risk marker for MI in Japanese.

Acknowledgements This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan. We are deeply grateful to Dr. Soichiro Kitamura, President of the National Cardiovascular Center, for his support of our research. We would like to thank Dr. Mitsuo Matsuda for stimulating

discussions. We would also like to thank Dr. Otosaburo Hishikawa, Dr. Katsuyuki Kawanishi, and Mr. Shigeru Kobayashi for their continuous support of our population survey in Suita City. We also thank the members of the Satsuki-Junyukai.

References

Barter PJ, Brewer HB Jr, Chapman MJ, Hennekens CH, Rader DJ, Tall AR (2003) Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. Arterioscler Thromb Vasc Biol 23:160–167

Deeb SS, Zambon A, Carr MC, Ayyobi AF, Brunzell JD (2003) Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. J Lipid Res 44:1279–1286

Groenendijk M, Cantor RM, de Bruin TW, Dallinga-Thie GM (2001a) The apoAI-CIII-AIV gene cluster. Atherosclerosis 157:1–11

Groenendijk M, Cantor RM, De Bruin TWA, Dallinga-Thie GM (2001b) New genetic variants in the apoA-I and apoC-III genes and familial combined hyperlipidemia. J Lipid Res 42:188–194

Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y (2002) JSNP: a database of common gene variations in the Japanese population. Nucleic Acids Res 30:158–162

Hsu LA, Ko YL, Wu S, Teng MS, Peng TY, Chen CF, Chen CF, Lee YS (2003) Association between a novel 11-base pair deletion mutation in the promoter region of the scavenger receptor class B type I gene and plasma HDL cholesterol levels in Taiwanese Chinese. Arterioscler Thromb Vasc Biol 23:1869–1874

Huuskonen J, Olkkonen VM, Jauhiainen M, Ehnholm C (2001) The impact of phospholipid transfer protein (PLTP) on HDL metabolism. Atherosclerosis 155:269–281

Inazu A, Brown ML, Hesler CB, Agellon LB, Koizumi J, Takata K, Maruhama Y, Mabuchi H, Tall AR (1990) Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. N Engl J Med 323:1234–1238

Jeenah M, Kessling A, Miller N, Humphries S (1990) G to A substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI and high density lipoprotein cholesterol concentrations. Mol Biol Med 7:233–241

Kannel WB, Castelli WP, Gordon T (1979) Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. Ann Intern Med 90:85–91

Karathanasis SK, Norum RA, Zannis VI, Breslow JL (1983) An inherited polymorphism in the human apolipoprotein A-I gene locus related to the development of atherosclerosis. Nature 301:718–720

Lefevre M, Ginsberg HN, Kris-Etherton PM, Elmer PJ, Stewart PW, Ershow A, Pearson TA, Roheim PS, Ramakrishnan R, Derr J, Gordon DJ, Reed R (1997) ApoE genotype does not predict lipid response to changes in dietary saturated fatty acids in a heterogeneous normolipidemic population. The DELTA Research Group. Dietary effects on lipoproteins and thrombogenic activity. Arterioscler Thromb Vasc Biol 17:2914–2923

Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN (2003) Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. Nat Genet 33:177–182

Ma K, Cilingiroglu M, Otvos JD, Ballantyne CM, Marian AJ, Chan L (2003) Endothelial lipase is a major genetic determinant for high-density lipoprotein concentration, structure, and metabolism. Proc Natl Acad Sci USA 100:2748–2753

Maciejko JJ, Holmes DR, Kottke BA, Zinsmeister AR, Dinh DM, Mao SJT (1983) Apolipoprotein A-I as a marker of angiographically assessed coronary-artery disease. N Engl J Med 309:385–389

Mannami T, Konishi M, Baba S, Nishi N, Terao A (1997) Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study. Stroke 28:518–525

Marshall HW, Morrison LC, Wu LL, Anderson JL, Corneli PS, Stauffer DM, Allen A, Karagounis LA, Ward RH (1994) Apolipoprotein polymorphisms fail to define risk of coronary artery disease. Circulation 89:567–577

Matsunaga T, Hiasa Y, Yanagi H, Maeda T, Hattori N, Yamakawa K, Yamanouchi Y, Tanaka I, Obara T, Hamaguchi H (1991) Apolipoprotein A-I deficiency due to a codon 84 nonsense mutation of the apolipoprotein A-I gene. Proc Natl Acad Sci USA 88:2793–2797

Miller GJ, Miller NE (1975) Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. Lancet 1:16–19

Ordovas JM, Schaefer EJ, Salem D, Ward RH, Glueck CJ, Vergani C, Wilson PWF, Karathanasis SK (1986) Apolipoprotein A-I gene polymorphism associated with premature coronary artery disease and familial hypoalphalipoproteinemia. N Engl J Med 314:671–677

Ordovas JM, Civeira F, Genest J Jr, Craig S, Robbins AH, Meade T, Pocovi M, Frossard PM, Masharani U, Wilson PWF, Salem DN, Ward RH, Schaefer EJ (1991) Restriction fragment length polymorphisms of the apolipoprotein A-I, C-III, A-IV gene locus. Atherosclerosis 87:75–86

Pagani F, Sidoli A, Giudici GA, Barenghi L, Vergani C, Baralle FE (1990) Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. J Lipid Res 31:1371–1377

Reguero JR, Cubero GI, Batalla A, Alvarez V, Hevia S, Cortina A, Coto E (1998) Apolipoprotein A1 gene polymorphisms and risk of early coronary disease. Cardiology 90:231–235

Shioji K, Mannami T, Kokubo Y, Inamoto N, Takagi S, Goto Y, Nonogi H, Iwai N (2004a) Genetic variants in PCSK9 affect the cholesterol level in Japanese. J Hum Genet 49:109–114

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 (2004b) 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

Takagi S, Iwai N, Yamauchi R, Kojima S, Yasuno S, Baba T, Terashima M, Tsutsumi Y, Suzuki S, Morii I, Hanai S, Ono K, Baba S, Tomoike H, Kawamura A, Miyazaki S, Nonogi H, Goto Y (2002) Aldehyde dehydrogenase 2 gene is a risk factor for myocardial infarction in Japanese men. Hypertens Res 25:677–681

Talmud PJ, Ye S, Humphries SE (1994) Polymorphism in the promoter region of the apolipoprotein AI gene associated with differences in apolipoprotein AI levels: the European atherosclerosis research study. Genet Epidemiol 11:265–280

Tuteja R, Tuteja N, Melo C, Casari G, Baralle FE (1992) Transcription efficiency of human apolipoprotein A-I promoter varies with naturally occurring A to G transition. FEBS Lett 304:98–101

Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M, Yokota M (2002) Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med 347:1916–1923

Zaman MM, Ikemoto S, Yoshiike N, Date C, Yokoyama T, Tanaka H (1997) Association of apolipoprotein genetic polymorphisms with plasma cholesterol in a Japanese rural population. The Shibata Study. Arterioscler Thromb Vasc Biol 17:3495–3504

Zhang K, Zhang S, Zheng K, Hou Y, Liao L, He Y, Zhang L, Nebert DW, Shi J, Su Z, Xiao C (2004) Novel P143L polymorphism of the LCAT gene is associated with dyslipidemia in Chinese patients who have coronary athrosclerotic heart disease. Biochem Biophys Res Commun 318:4–10

Circ J 2005; **69:** 138–142

Association Analysis Between Hypertension and *CYBA*, *CLCNKB*, and *KCNMB1* Functional Polymorphisms in the Japanese Population

—— The Suita Study ——

Yoshihiro Kokubo, MD; Naoharu Iwai, MD; Naomi Tago, BS;
Nozomu Inamoto, MD; Akira Okayama, MD; Hideyuki Yamawaki, PhD;
Hiroaki Naraba, PhD; Hitonobu Tomoike, MD

Background Reproducibility of results is important for the validity of genetic association studies. Recently, 3 functional polymorphisms, G(–930)A in *CYBA*, T481S in *CLCNKB*, and E65K in *KCNMB1*, were reported to be associated with blood pressure (BP) status and the aim of this study was to confirm those findings using a large cohort representing the general Japanese population.

Methods and Results The study population consisted of 3,652 subjects recruited from the Suita study as representive of the general population in Japan. The genotypes of the 3 polymorphisms were determined by the TaqMan method. Logistic analysis indicated that the *CYBA*/G(–930)A polymorphism was associated with hypertension in male subjects. In the male population, the odds ratio of the GG genotype over GA+AA was 1.27 (95% confidence interval 1.01–1.57, p=0.034). Moreover, residuals of systolic and diastolic BP values were significantly higher in subjects with the GG genotype than in those with the GA or AA genotype (p=0.0007). However, such significant effects of the genotype on BP status were not observed in the female population. The significance of the *CLCNKB*/T481S and *KCNMB1*/E65K polymorphisms were not replicated in the present study.

Conclusion The significance of the G(–930)A polymorphism of *CYBA* was confirmed in the present study with adequate statistical power, which strengthens the hypothesis that this polymorphism is important in the pathogenesis of hypertension and confers susceptibility. (*Circ J* 2005; **69:** 138–142)

Key Words: Epidemiology; Genetic association study; Hypertension

Essential hypertension (HT) is a multifactorial disorder influenced by both genetic and environmental factors. Over the past few years, a large number of genetic polymorphisms of candidate genes have been tested for their association with HT, but with controversial results, probably because of inadequate sample size, ethnic differences, and/or population stratification. The practical implications of an association study strongly depend on the reproducibility of the findings[1]

Recently, 3 specific functional polymorphisms have been reported to be associated with HT: the G(–930)A polymorphism of *CYBA* (p22phox)[2] the E65K polymorphism of *KCNMB1*[3] and the T481S polymorphism of *CLCNKB*[4,5]

CYBA (p22phox) is a major component of NAD(P)H oxidase, and the NAD(P)H oxidase system is considered to be the most important source of superoxide anion in vascular tissues[6,7] The superoxide anion has been suspected of involvement in the pathogenesis of HT through the inactivation of NO produced by NO synthetase (*NOS3*) in the vascular endothelium. Moreno et al reported that the G allele of *CYBA* had higher promoter activity and is associated with HT[2]

(Received October 22, 2004; revised manuscript received November 19, 2004; accepted December 1, 2004)

National Cardiovascular Center, Suita, Osaka, Japan
Mailing address: Naoharu Iwai, MD, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Blood pressure (BP) depends on the resistance of resistant vessels, and a key element of arterial tone is the large-conductance Ca^{2+} and voltage-dependent K^+ (BK) channel. The BK channel is formed by an ion-conducting α subunit and a regulatory β_1 subunit (*KCNMB1*). An activating gain-of-function mutation, the K65 allele, is reported to be associated with a low prevalence of diastolic HT[3]

The chloride channel ClC-Kb is expressed in the distal nephron and is known to be responsible for classic Bartter syndrome. The S481 allele has been reported to confer a strong gain-of-function effect that leads to enhanced NaCl re-absorption, and as expected, susceptibility to HT, in a Caucasian population[5]

All 3 polymorphisms have functional significance and are thus intriguing candidate genes. However, the sample sizes of the studies to date have been relatively small, or the descriptions of the epidemiological aspects rather weak. The purpose of the present study was to replicate findings of the previous association studies in a large epidemiological cohort representing the general population in Japan and to evaluate the possible importance of these polymorphisms.

Methods

Study Population

The selection criteria and design of the Suita Study have been previously described[8–10] The present study was

Table 1 TaqMan Probes and Primers for *CYBA*, *CLCNKB*, and *KCNMB1*

	Probe		Primer	
	VIC	FAM	F	R
CYBA G (–930) A	CCAGCATTACTGCCTC	CAGCATTGCTGCCTC	GCCCCGGTGGCCAT	GAAACAGAAAAACGGCGGAG
CLCNKB T481S	TGACCCACACCATCTC	TGACCCACTCCATCTC	TGCAGCCTTCTCAGGGGC	ACCTCGAAGGCCAGCAGC
KCNMB1 K65E	CTTCAGCTTCTCCTGGT	TTCAGCTCCTCCTGGT	CGGCAGCTGACACGTTGA	CCAAGTGCCACCTGATTGAGA

Table 2 Characteristics of the Study Population

	Male	Female	p value	Hypertension	Normotension	p value
N	1,706	1,946		1,522	2,130	
M/F				776/746	930/1,200	<0.0001
Age (years)	66.12 (0.27)	63.47 (0.25)	<0.0001	68.81 (0.28)	61.77 (0.23)	<0.0001
Body mass index	23.32 (0.08)	22.38 (0.07)	<0.0001	23.56 (0.08)	22.29 (0.07)	<0.0001
HTN (%)	45.49	38.34	<0.0001			
AHT (%)	28.19	22.61	<0.0001	60.5	0	<0.0001
CVA (%)	4.1	1.85	<0.0001	5.12	1.31	<0.0001
MI (%)	2.29	0.57	<0.0001	2.23	0.75	0.0001
Drinking (%)	66.88	27.34	<0.0001	48.88	43.62	0.0070
Smoking (%)	30	5.96	<0.0001	14.19	19.34	<0.0001

Data are mean (standard error). Differences between the 2 groups (male vs female, hypertensives vs normotensives) were calculated by t-test or χ^2 analysis.
HTN, hypertension; AHT, antihypertensive medication; CVA, cerebrovascular accident; MI, myocardial infarction; drinking, alcohol drinking habbit; smoking, cigarette smoking habit.

Table 3 Genotype Distribution of *CYBA*/G(–930)A, *CLCNKB*/T481S, and *KCNMB1*/E65K in Hypertensive Subjects

	Major	Hetero	Minor	p value
CYBA				
G>A				
M	258/523 (49.3)	378/836 (45.2)	139/344 (40.4)	0.0344 (0.0393)
F	223/573 (38.9)	371/963 (38.5)	146/401 (36.4)	0.6990 (0.7189)
T	481/1,096 (43.9)	749/1,799 (41.6)	285/745 (38.3)	0.0548 (0.0886)
CLCNKB				
T>S				
M	747/1,644 (45.4)	26/54 (48.1)		0.6943 (0.6076)
F	720/1,879 (38.3)	20/58 (34.5)		0.5512 (0.3490)
T	1,467/3,523 (41.6)	46/112 (41.1)		0.9042 (0.7529)
KCNMB1				
E>K				
M	603/1,332 (45.3)	162/349 (46.4)	10/20 (50.0)	0.8577 (0.8967)
F	582/1,560 (37.3)	147/348 (42.2)	11/24 (45.8)	0.1757 (0.2646)
T	1,185/2,892 (41.0)	309/697 (44.3)	21/44 (47.7)	0.1966 (0.3478)

M, male subjects; F, female subjects; T, total (male + female) subjects. Major, Hetero, and Minor indicate major genotype, heterozygous genotype, and minor genotype, respectively.
The [number of hypertensive subjects/number of normotensive + hypertensive subjects] and (% of hypertensive subjects) are indicated. p values are calculated by χ^2 analyses using genotype as an independent variable; values in parentheses are p values obtained by multiple logistic analysis with the genotype (GG, GA and AA) as independent variable and age and BMI as covariates.

approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The genotypes were determined in 3,652 subjects recruited from the Suita Study between April 2002 to February 2004. All subjects provided written informed consent.

DNA Studies

The polymorphisms were determined by the TaqMan system as described previously![11] The primers and probes are summarized in Table 1.

Statistical Analysis

Values are expressed as the mean ± SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute, Inc, Cary, NC, USA). Multiple logistic analyses (presence of HT) were performed with age and body mass index (BMI) as covariates. Subjects were categorized as hypertensive (HTN) when they had a systolic BP (SBP) of 140 mmHg or higher and/or a diastolic BP (DBP) of 90 mmHg or higher. Subjects who were currently taking antihypertensive medication were also categorized as HTN. The effects of the polymorphisms on BP values were evaluated by excluding subjects who were taking antihypertensive medications, since HTN that has excellent BP control by medication may exhibit normal values. Residuals of the BP values were calculated by adjusting for age and BMI (Residuals represent the difference between the actual BP value for each observation and the value predicted on the basis of age and BMI). Differences among the groups were calculated by one-way AVOVA. The differences in frequencies were calculated by χ^2 analysis. The

Table 4 Blood Pressure Values According to the *CYBA*/G(−930)A, *CLCNKB*/T481S, and *KCNMB1*/E65K Genotype

	Major	*Hetero*	*Minor*	*p value*
Residuals of SBP				
CYBA				
G>A				
M	2.4 (0.9) [380]	−0.4 (0.7) [593]	−2.7 (1.1) [250]	0.0007
F	0.3 (0.8) [455]	0.0 (0.6) [728]	−0.4 (0.9) [319]	0.8440
T	1.3 (0.6) [835]	−0.3 (0.5) [1,321]	−1.3 (0.7) [569]	0.0108
CLCNKB				
T>S				
M	0.0 (0.5) [1,183]	−0.1 (2.8) [37]		0.9454
F	0.0 (0.4) [1,453]	−0.2 (2.4) [49]		0.9228
T	0.0 (0.3) [2,636]	0.0 (1.8) [86]		0.9732
KCNMB 1				
E>K				
M	−0.2 (0.5) [950]	1.1 (1.1) [256]	−0.6 (4.5) [14]	0.4955
F	−0.3 (0.5) [1,216]	1.4 (1.0) [265]	0.0 (4.2) [16]	0.3677
T	−0.3 (0.4) [2,166]	1.3 (0.7) [521]	−0.4 (3.1) [30]	0.1817
Residuals of DBP				
CYBA				
G>A				
M	1.0 (0.5) [380]	0.0 (0.4) [593]	−1.5 (0.6) [250]	0.0072
F	0.4 (0.4) [455]	−0.1 (0.3) [728]	−0.2 (0.5) [319]	0.6300
T	0.7 (0.3) [835]	−0.1 (0.3) [1,321]	−0.7 (0.4) [569]	0.0170
CLCNKB				
T>S				
M	0.0 (0.5) [1,183]	−0.1 (2.8) [37]		0.9454
F	0.0 (0.2) [1,453]	−0.4 (1.3) [49]		0.7633
T	0.0 (0.2) [2,636]	−0.3 (1.0) [86]		0.7624
KCNMB 1				
E>K				
M	0.1 (0.3) [950]	−0.3 (0.6) [256]	2.0 (2.6) [14]	0.6760
F	0.0 (0.3) [1,216]	−0.2 (0.6) [265]	1.1 (2.3) [16]	0.8420
T	0.0 (0.2) [2,166]	−0.1 (0.4) [521]	1.4 (1.7) [30]	0.6885
Hypertension				
CYBA				
G>A				
M	115/265	135/458	45/205	0.0014
F	105/350	136/592	64/255	0.2681
T	220/615	271/1,050	109/460	0.0014
CLCNKB				
T>S				
M	286/897	9/28		0.9206
F	294/1,159	11/38		0.8566
T	580/2,056	20/66		0.9912
KCNMB 1				
E>K				
M	222/726	68/186	4/10	0.4581
F	234/973	63/200	3/13	0.2889
T	456/1,699	131/386	7/23	0.1359

DBP, diastolic blood pressure; SBP, systolic blood pressure. Other abbreviations as in Table 3.
Residuals of systolic and diastolic blood pressure values were calculated by adjusting for age and BMI. Values (mmHg) are shown as mean (SEM). [Number]indicates the number of subjects in each group.
Subjects who were taking antihypertensive medication were excluded from this analysis. Prevalences of hypertension according to the genotypes in this study population (exclusing those with antihypertensive medication) are also shown at the bottom. p values are calculated by adjusting for age and BMI.

sample power was calculated using the Sample Power statistical package (SPSS Inc, Chicago, IL, USA).

Results

The characteristics of the study population are given in Table 2. The effects of the 3 polymorphisms on HT and BP are shown in Tables 3 and 4.

CYBA/G(−930)A

Multiple logistic analysis with age and BMI as covariates indicated that the GG (vs GA+AA) genotype was associated with HT, with an odds ratio of 1.27 (95% confidence interval 1.01–1.57, p=0.0340), in the male population. The genotype frequencies of the GG, GA, and AA genotypes were 0.307, 0.491, and 0.202 (Table 3). Based on these frequencies and the sample size (male n=1,703), the sample power of this statistic was calculated to be 0.75 (α=0.05, two-tailed). In this sample power calculation, subjects with the GA or AA genotype were categorized into one group. In males, residuals of the SBP and DBP values were also significantly higher in subjects with the GG genotype than in those with the GA or AA genotype (Table 4). The difference between the residuals of SBP for the GG and AA genotypes was 5.1 mmHg. Consistency between the analysis of the categorical data and the analysis of the numerical

data might strengthen the hypothesis that the *CYBA* promoter variant contributes to HT in men. Such significant effects of the genotype on BP status were not observed in the female population, although a similar non-significant trend was observed.

CLCNKB/T481S

We did not find any significant association between the *CLCNKB*/T481S polymorphism and HT, SBP, or DBP in male or female subjects (Tables 3,4).

KCNMB1/E65K

We did not find any significant association between the *KCNMB1*/E65K polymorphism and and HT, SBP, or DBP in male or female subjects (Tables 3,4). Fernandez-Fernandez et al reported that the genotype frequency (KK+KE) decreased with increasing DBP values[3] so on that basis we categorized subjects without HT medication into 4 groups based on the DBP values (Group-I: <79 mmHg; 80≤ Group-II <90 mmHg; 90≤ Group-III <99 mmHg; Group-IV: ≥100 mmHg). The respective genotype (KK+KE) frequencies were 20.4% (n=1,746), 20.3% (n=715), 19.4% (n=201), and 17.8% (n=39). No significant difference in the genotype frequency was observed among the 4 groups.

Discussion

Over the past decade, many genetic association studies have been performed with inconsistent results, and we are now recognizing that the odds of common HT alleles are less than expected[1,10] Thus, any single study that considers just a few thousand subjects may not be large enough to reach concrete conclusions and should be viewed as providing tentative results only.

In the present study, the genotype frequency of the heterozygote of the T481S polymorphism was just 0.03, and none of the subjects was homozygous for the 481S allele. This heterozygous genotype was expected to be associated with HT through enhanced sodium chloride reabsorption, but given its small frequency, odds of more than 1.76 would be required (α=0.05, two-tailed) to observe an association with a sample size of 3,652 subjects. Thus, a practical implication of the present study is that the odds of the heterozygous genotype of the T481S polymorphism, if any, should be less than 1.76. It is possible that the effects of this activating polymorphism could be more clearly observed under salt-loading conditions or in subjects homozygous for the mutation. An even larger study population with information on salt intake might be required to evaluate the significance of this polymorphism.

That situation is also true for the E65K polymorphism of *KCNMB1*. The K65 allele, an activating mutation of *KCNBM1*, was expected to be associated with a lower prevalence of HT. The frequency of the EK+KK genotype was 0.20, and therefore odds of less than 0.79 would be required (α=0.05, two-tailed) to observe an association with a sample size of 3,652. Thus, the present study indicated that the contribution of the E65K polymorphism, if any, is very slight, with an odds ratio of more than 0.79.

Our failure to replicate the possible involvement of the *CLCNKB*/T481S and *KCNMB1*/E65K polymorphisms in BP regulation might be ascribed to ethnic differences, which include not only genetic but also environmental differences. A genetic variation may be differentially expressed under different conditions.

The G(–930) allele of *CYBA* has been reported to have higher promoter activity, and may be associated with higher production of superoxide anion in vascular tissues[2] The superoxide anion has suspected involvement in the pathogenesis of HT by inactivating NO produced by NO synthetase (*NOS3*) in the vascular endothelium[6,7] The discrepancy between males and females in terms of the effects of the G(–930)A polymorphism on BP might be interpreted from the perspective of estrogen. Estrogen stimulates the production of NO in vascular tissues[12–14] and the effect of NO produced by estrogen on BP control was observed in mice deficient in the estrogen β-receptor[15] It is possible that the NO-inactivating-property of the G(–930) allele may be overcome in females because of higher levels of NO produced in the vascular tissues by estrogen.

Gender differences in superoxide generation in microvessels have been reported in the spontaneously hypertensive rat, and have been attributed to AT-1-dependent overexpression of the components of NAD(P)H oxidase[16] It is also possible that sexual dimorphism in the effects of the G(–930)A polymorphism on BP may be related to different expression of the CYBA protein.

The present study results indicate that, of the 3 polymorphisms investigated, the G(–930)A polymorphism of *CYBA* seems to be the most promising genetic variant conferring susceptibility to HT in males. From a clinical viewpoint, it might be interesting to investigate whether HTN with the GG genotype are more responsive to nitrate derivatives, bearing in mind our earlier concern about the size of any particular study in relation to its results.

Acknowledgments

We express our highest gratitude to the following people for their continuous support of our population survey: Dr Otosaburo Hishikawa, President; Dr Katsuyuki Kawanishu, Committee in Chief for the city health check-up service; other members of the Suita City Medical Association; and Mr Shigeru Kobayashi, Director of the City Health Center. We also express our great thanks to the members of our attendants' society (Satsuki-Junyu-kai) for their cooperation and assistance with our survey of risk factors and preventive activity on cardiovascular diseases. We also thank Professor Soichiro Kitamura, President of the National Cardiovascular Center, for considering our research work.

This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan (MPJ-3), and by grant-in-aid from the Salt Science Research Foundation, No.04C5.

References

1. Harrap SB. Private genes, public health. *Lancet* 1997; **349:** 1338–1339.
2. Moreno MU, Jose GS, Orbe J, Paramo JA, Beloqui O, Diez J, et al. Preliminary characterisation of the promoter of the human p22phox gene: Identification of a new polymorphism associated with hypertension. *FEBS Lett* 2003; **542:** 27–31.
3. Fernandez-Fernandez JM, Tomas M, Vazquez E, Orio P, Latorre R, Senti M, et al. Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J Clin Invest* 2004; **113:** 1032–1039.
4. Jeck N, Waldegger P, Doroszewicz J, Seyberth H, Waldegger S. A common sequence variation of the CLCNKB gene strongly activates ClC-Kb chloride channel activity. *Kidney Int* 2004; **65:** 190–197.
5. Jeck N, Waldegger S, Lampert A, Boehmer C, Waldegger P, Lang PA, et al. Activating mutation of the renal epithelial chloride channel ClC-Kb predisposing to hypertension. *Hypertension* 2004; **43:** 1175–1181.
6. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986; **320:** 454–456.
7. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of NAD(P)H oxidase and endothelial

nitric oxide synthase. *Circulation* 2002; **105:** 1656–1662.
8. Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: The Suita study. *Stroke* 1997; **28:** 518–525.
9. Iwai N, Tago N, Yasui N, Kokubo Y, Inamoto N, Tomoike H, et al. Genetic analysis of 22 candidate genes for hypertension in the Japanese population. *J Hypertens* 2004; **22:** 1119–1126.
10. Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, et al. Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105:** 41–47.
11. Shioji K, Kokubo Y, Mannami T, Inamoto N, Morisaki H, Mino Y, et al. Association between hypertension and the alpha-adducin, beta1-adrenoreceptor, and G-protein beta3 subunit genes in the Japanese population: The Suita study. *Hypertens Res* 2004; **27:** 31–37.
12. Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci USA* 1994; **91:** 5212–5216.
13. Goetz RM, Morano I, Calovini T, Studer R, Holtz J. Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. *Biochem Biophys Res Commun* 1994; **205:** 905–910.
14. Huang A, Sun D, Koller A, Kaley G. Gender difference in flow-induced dilation and regulation of shear stress: Role of estrogen and nitric oxide. *Am J Physiol Regul Integr Comp Physiol* 1998; **275:** R1571–R1577.
15. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, et al. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. *Science* 2002; **295:** 505–508.
16. Dantas APV, Franco MDCP, Silva-Antonialli MM, Tostes RCA, Fortes ZB, Nigro D, et al. Gender differences in superoxide generation in microvessels of hypertensive rats: Role of NAD(P)H-oxidase. *Cardiovasc Res* 2004; **61:** 22–29.