

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 MspI* and *SstI* 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 *ApoA1MspI* [G(-310)A] polymorphism and HDL-C or TG levels. Since the *ApoA1MspI* 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 ( $\alpha$  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 *HaeIII* 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 I (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.

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

## — The Suita Study —

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**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 representative 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

**E**ssential 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!

Recently, 3 specific functional polymorphisms have been reported to be associated with HT: the G(–930)A polymorphism of *CYBA* (p22<sup>phox</sup>),<sup>2</sup> the E65K polymorphism of *KCNMB1*,<sup>3</sup> and the T481S polymorphism of *CLCNKB*.<sup>4,5</sup>

*CYBA* (p22<sup>phox</sup>) 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.<sup>6,7</sup> 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.<sup>2</sup>

Blood pressure (BP) depends on the resistance of resistant vessels, and a key element of arterial tone is the large-conductance Ca<sup>2+</sup> and voltage-dependent K<sup>+</sup> (BK) channel. The BK channel is formed by an ion-conducting  $\alpha$  subunit and a regulatory  $\beta_1$  subunit (*KCNMB1*). An activating gain-of-function mutation, the K65 allele, is reported to be associated with a low prevalence of diastolic HT.<sup>3</sup>

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.<sup>5</sup>

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.<sup>8–10</sup> The present study was

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

	Probe		Primer	
	VIC	FAM	F	R
<i>CYBA</i> G (-930) A	CCAGCATTACTGCCTC	CAGCATTGCTGCCTC	GCCCCGGTGGCCAT	GAAACAGAAAAACGGCGGAG
<i>CLCNKB</i> T481S	TGACCCACACCATCTC	TGACCCACTCCATCTC	TGCAGCCTTCTCAGGGGC	ACCTCGAAGGCCAGCAGC
<i>KCNMB1</i> 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  $\chi^2$  analysis.

HTN, hypertension; AHT, antihypertensive medication; CVA, cerebrovascular accident; MI, myocardial infarction; drinking, alcohol drinking habit; 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
<i>CYBA</i>				
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)
<i>CLCNKB</i>				
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)
<i>KCNMB1</i>				
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  $\chi^2$  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.<sup>11</sup> The primers and probes are summarized in Table 1.

#### Statistical Analysis

Values are expressed as the mean  $\pm$  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 ANOVA. The differences in frequencies were calculated by  $\chi^2$  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
<i>Residuals of SBP</i>				
<i>CYBA</i>				
<i>G&gt;A</i>				
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
<i>CLCNKB</i>				
<i>T&gt;S</i>				
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
<i>KCNMB 1</i>				
<i>E&gt;K</i>				
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
<i>Residuals of DBP</i>				
<i>CYBA</i>				
<i>G&gt;A</i>				
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
<i>CLCNKB</i>				
<i>T&gt;S</i>				
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
<i>KCNMB 1</i>				
<i>E&gt;K</i>				
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
<i>Hypertension</i>				
<i>CYBA</i>				
<i>G&gt;A</i>				
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
<i>CLCNKB</i>				
<i>T&gt;S</i>				
M	286/897	9/28		0.9206
F	294/1,159	11/38		0.8566
T	580/2,056	20/66		0.9912
<i>KCNMB 1</i>				
<i>E&gt;K</i>				
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 (excluding 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 ( $\alpha=0.05$ , 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 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,<sup>3</sup> 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.<sup>1,10</sup> 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 ( $\alpha=0.05$ , 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 *KCNMB1*, 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 ( $\alpha=0.05$ , 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.<sup>2</sup> The superoxide anion has suspected involvement in the pathogenesis of HT by inactivating NO produced by NO synthetase (*NOS3*) in the vascular endothelium.<sup>6,7</sup> 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,<sup>2-14</sup> and the effect of NO produced by estrogen on BP control was observed in mice deficient in the estrogen  $\beta$ -receptor.<sup>15</sup> 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.<sup>6</sup> 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.

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