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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Lesion Severity and Hypercholesterolemia Determine Long-Term Prognosis of Vasospastic Angina Treated With Calcium Channel Antagonists

Masakazu Yamagishi, MD; Kenichi Ito, MD; Hiroshi Tsutsui, MD; Shunichi Miyazaki, MD; Yoichi Goto, MD; Noritoshi Nagaya, MD; Tetsuya Sumiyoshi, MD*; Kenichi Fukami, MD**; Kazuo Haze, MD†; Masafumi Kitakaze, MD; Hiroshi Nonogi, MD; Hitonobu Tomoike, MD

Although patients with medically treated vasospastic angina have a good outcome, few data exist regarding the role of underlying lesion severity associated with or without hyperlipidemia in the prognosis. Therefore, the aim of the present study was to assess the relationship between the long-term outcome of vasospastic angina and the factors influencing its prognosis. A total of 256 patients (219 men, 37 women; mean age, 54.1±9.2) who had coronary spasm with or without underlying lesions and were being treated with calcium channel antagonists were enrolled and followed for 13.6±3.7 years. Cardiac events consisted of cardiac death and ischemic events, which included acute myocardial infarction and unstable angina. Cox analysis selected coronary artery stenosis (CAS, ≥50%) and risk factors such as age, hypertension, diabetes mellitus, low-density lipoprotein-cholesterol (LDL-C), sex and smoking. There were 19 cases of cardiac death (7.4%) and 58 of ischemic events (22.7%) during the follow-up period. The presence of significant CAS was an independent predictor of event-free survival (hazard ratio (HR)=2.84, 95% confidence interval (CI)=1.79–4.52, p<0.0001). In 193 patients without significant CAS, there were 10 cases of cardiac death (5.2%, p<0.05) and 34 of ischemic events (17.6%, p<0.01). In that group, high LDL-C was the independent predictor of event-free survival (HR=3.89, 95% CI=1.20–12.6, p=0.02). Kaplan-Meier survival analysis revealed significantly lower event-free survival in patients with than in those without lesions (p<0.0001 by log-rank test). These results demonstrate that the most important factor for long-term prognosis of vasospastic angina treated with calcium channel antagonists is significant CAS. High LDL-C, which might alter the underlying coronary endothelial function and/or accelerate atherosclerotic lesions, could also contribute to the occurrence of cardiac events, particularly in patients without significant CAS. (*Circ J* 2003; **67**: 1029–1035)

Key Words: Calcium channel antagonist; Coronary artery disease; Long-term prognosis; Vasospasm

Coronary spasm provokes the myocardial ischemia associated with angina pectoris, acute myocardial infarction and sudden death[1,2] Calcium channel antagonists, nitrates or a combination of both drugs have been used effectively to prevent coronary spasm[3–6] Indeed, the overall cardiac mortality is relatively low and the prognosis seems to be good for medically treated patients with coronary spasm[7–9] Coronary spasm frequently occurs in minimally narrowed coronary segments;[10,11] suggesting a pathophysiologic correlation between coronary spasm and atherosclerosis, which has been demonstrated in an experimental animal model[12] Several studies have examined the relationship between the clinical characteristics and prognosis of this type of angina, and demonstrated that preexisting atherosclerosis could be an important risk factor for cardiac death and myocardial infarction during the relatively early phase[5–9] However, there is little information regarding the long-term prognosis of medically treated coronary spasm with or without atherosclerotic risks, such as underlying coronary artery stenosis (CAS) and intrinsic hyperlipidemia. Such data should be important for not only preventing vasospasm, but also preventing the development of atherosclerosis. The purpose of this study was, first, to determine the event-free survival rate in patients with angiographically documented coronary spasm, and second, to identify the clinical predictors of cardiac events, particularly in patients with and without significant CAS.

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*Division of Cardiovascular Medicine, National Cardiovascular Center, Suita, *Division of Cardiology, Sakakibara Heart Institute, Tokyo, **Division of Cardiology, Iwate Medical College, Morioka and †Division of Cardiology, Osaka City General Hospital Medical Center, Osaka, Japan*

A part of this work was presented at American Heart Association Scientific Sessions in Anaheim, 2001.

Mailing address: Masakazu Yamagishi, MD, PhD, FACC, Division of Cardiovascular Medicine, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail: myamagi@hsp.ncvc.go.jp

Methods

Patient Population

Of 2,740 patients who underwent diagnostic coronary angiography for suspected ischemic heart disease between 1977 and 1987, 284 consecutive patients with coronary spasm were enrolled and followed. During the follow-up, 21 patients went missing. Patient enrollment was completed in 1987 when 3-hydroxy-3-methyleglutaryl coenzyme A (HMG-CoA) reductase inhibitor, which might have altered the long-term prognosis[13] was not generally available. Therefore, no patient had been given HMG-CoA reductase

Table 1 Baseline Clinical and Angiographic Characteristics

	Total (n=256)	Without CAS (n=193)	With CAS (n=63)	p value
Age (years)	54.1±9.2	54.4±8.0	55.0±10.3	NS
Male sex (%)	219 (85.5)	163 (84.5)	56 (88.9)	NS
Coronary risk factors				
Hypertension (%)	115 (44.9)	91 (47.2)	24 (38.0)	NS
High LDL-C (%)	61 (23.8)	39 (20.2)	22 (34.9)	NS
Diabetes mellitus (%)	7 (2.7)	3 (1.6)	4 (6.3)	NS
Smoking (%)	174 (66.8)	129 (66.8)	45 (71.4)	NS
Arrhythmia during angina (%)	10 (3.9)	10 (5.2)	0 (0)	NS
Multivessel spasm (%)	18 (7.0)	17 (8.8)	1 (1.6)	NS

CAS, coronary artery stenosis; LDL-C, low-density lipoprotein-cholesterol.

Table 2 Incidence of Cardiac Events During Follow-up Period

Cardiac event	Total (n=256)	Without CAS (n=193)	With CAS (n=63)	p value
Cardiac death (%)	19 (7.4)	10 (5.2)	9 (14.3)	0.017
Sudden death (%)	8 (3.1)	3 (1.6)	5 (7.9)	0.024
Myocardial infarction (%)	3 (1.1)	2 (1.0)	1 (1.6)	NS
Heart failure (%)	5 (2.0)	4 (2.1)	1 (1.6)	NS
Others (%)	3 (1.2)	1 (0.5)	2 (3.2)	NS
Ischemic event (%)	58 (22.7)	34 (17.6)	24 (38.1)	<0.01
Nonfatal myocardial infarction (%)	15 (5.9)	8 (4.1)	7 (11.1)	0.041
Unstable angina (%)	29 (11.3)	21 (10.9)	8 (12.7)	NS
PTCA (%)	12 (4.7)	5 (2.6)	7 (11.1)	0.011
CABG (%)	2 (0.8)	0 (0)	2 (3.2)	NS
Time of event from registration (years)	4.9±4.7	4.6±4.3	5.3±5.3	NS

CABG, coronary artery bypass grafting; CAS, coronary artery stenosis; PTCA, percutaneous transluminal coronary angioplasty.

inhibitor at the time follow-up began, but 7 patients were unexpectedly given this drug during the follow-up period, particularly after 1990. Therefore, these 28 patients were excluded, and a total of 256 patients were analyzed in this study. There were 219 men and 37 women aged 54.1±9.2 years. All the patients had chest pain at rest and/or on exertion. ECG changes such as ST elevation or depression during chest pain attacks were demonstrated in 154 patients.

Cardiac Catheterization Procedures

Written informed consent was obtained from all patients for cardiac catheterization and the provocation of coronary spasm. All patients were fasted and received 3,000–5,000 U heparin intravenously before the procedure. Calcium channel antagonists, nitrates, β-blockers and other anti-anginal drugs were discontinued at least 9 h before procedures, because these drugs could alter the basal coronary tone associated with the occurrence of spasm.

After control coronary angiography, the provocative tests were performed. We initially used intravenous ergonovine to induce spasm[14] but since 1986, the intracoronary ergonovine test has been used for safety reasons[15] When coronary spasm was not provoked, ergonovine maleate was administered until the total dose reached 0.4 mg in the intravenous test, or 0.04 mg in the intracoronary test. Under these conditions, the standard 12-lead ECG was continuously monitored to record ST change (≥0.1 mV) and associated arrhythmias. When chest pain or significant ST-segment changes were observed, selective coronary angiography was immediately performed. Coronary spasm was considered positive when there was luminal narrowing ≥75%; however, diffuse spasm ≥75% without signs of myocardial ischemia was not considered positive, because it is difficult to differentiate real spasm from a pharmacological reaction[16] Multi-vessel spasm was defined as vasospasm in more than one major coronary artery.

After provocation, 0.25–0.5 mg (0.5 mg/ml) nitroglycerin was given to obtain maximal coronary dilation, and coronary angiography was again performed in multiple projections to evaluate the extent and severity of CAS. Left ventriculography was performed to calculate the left ventricular ejection fraction by the area–length method. Coronary angiography and left ventriculography were evaluated by at least 2 angiographers who were independent of each other. Significant CAS was defined as lumen stenosis ≥50%. In the case of repeated angiography, progression of lesion severity was defined as an increase of ≥20% in a preexisting stenosis of ≥50%, and an increase of ≥30% in a stenosis <50%, or any increase in lesion severity that resulted in total coronary occlusion. New stenoses were defined as stenoses ≥20% that developed at a site that was previously angiographically normal[17]

Assessment of Coronary Risk Factors

The coronary risk factors at baseline were hypertension, diabetes mellitus, high low-density lipoprotein-cholesterol (LDL-C) and history of smoking. Blood sugar, total cholesterol, LDL-C, high-density lipoprotein-cholesterol, and triglycerides were measured on the morning of angiography while the patient was still in a fasting state. In patients who could be followed up for more than 6 months, these variables were re-examined as frequently as possible during the follow-up period. In patients in whom LDL-C had not been measured and the triglyceride concentration was <300 mg/dl, the LDL-C concentration was calculated using the following equation: LDL-C=(total cholesterol–

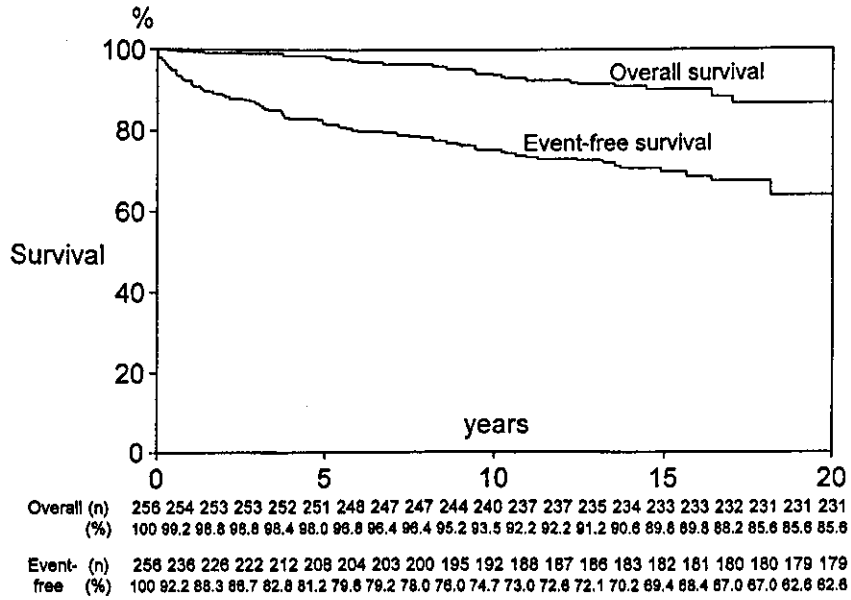

Fig 1. Kaplan-Meier analysis of overall mortality and event-free survival rate for entire patient group. Vertical and horizontal axes represent % survival rate and time from registration, respectively.

HDL-C−1/5 triglycerides). Hypertension was defined as systolic blood pressure >140 mmHg, diabetes mellitus was defined as fasting glucose >140 mg/dl, and high LDL-C was defined as serum LDL-C concentration >140 mg/dl. A smoking history was obtained from all patients: smokers were defined as patients who had smoked more than 20 cigarettes per day for >20 years. We also evaluated the contribution of the patients' sex and age to the prognosis.

Long-Term Follow-up

The cardiac events included cardiac death and ischemic events. Cardiac death consisted of sudden death, which was defined as death within 1 h after collapse, and death associated with acute myocardial infarction, heart failure, or other cardiovascular disease. Ischemic events consisted mainly of non-fatal myocardial infarction, unstable angina and the need for percutaneous transluminal angioplasty (PTCA) or coronary artery bypass surgery (CABG). Unstable angina was defined as worsening of chest symptoms,[8] and except for emergency procedures the indication for PTCA and CABG was considered by at least 2 cardiologists independent of this study.

Myocardial infarction was diagnosed on the basis of the development of a new Q wave in the ECG and elevation of serum concentrations of cardiac enzymes. Unstable angina was defined as repeated anginal attacks at rest. Non-fatal myocardial infarction was defined as survival of the patient who could then be discharged from hospital. In this study, if a patient died of cardiac death and had suffered an ischemic event before death, the cardiac event was not defined as cardiac death, but as an ischemic event. Accordingly, a cardiac event in this study meant the first cardiac event that occurred during follow-up.

All patients were given at least either dihydropyridine or diltiazem hydrochloride. Drug therapy was started immediately after angiographic diagnosis and continued by the individual physicians until the end-point. The end-points of follow-up were the first cardiac event and non-cardiac death without a cardiac event. The follow-up period of each patient was calculated from the date of the initial diagnostic angiography. Those who did not visit for follow-up examination and checking the status of medical compliance were followed by telephone interviews with the patient or the family, from whom the status of smoking was carefully elicited. When anginal attacks became frequent or were not relieved by medical treatment, the patient was admitted and coronary angiography was performed again to determine alternative methods of management.

Table 3 Predictors of Cardiac Events in All Patients During Follow-up Period

	Hazard ratio	*95% CI*	*p value*
CAS	*2.84*	*1.79–4.52*	*<0.0001*
High LDL-C	*2.21*	*0.79–6.21*	*0.079*
Diabetes mellitus	*1.72*	*0.94–3.16*	*0.16*
Hypertension	*1.21*	*0.71–2.00*	*0.45*
Sex	*1.18*	*0.59–2.37*	*0.65*
Age	*0.99*	*0.97–1.03*	*0.89*
Smoking	*0.99*	*0.58–1.68*	*0.96*

CAS, coronary artery stenosis; CI, confidence interval; LDL-C, low-density lipoprotein-cholesterol.

Statistical Analysis

All values are expressed as mean±SD. Continuous variables were compared by unpaired Students' t-test. Categorical variables were compared by chi-square test (with Fisher's exact test, as appropriate for smaller sample size). Multivariate analysis with Cox proportional-hazards regression analysis was used to assess the independent significance of prognostic factors for cardiac events. Event-free survival rate was calculated using the Kaplan-Meier method, and differences between survival curves were assessed by the log-rank test.[9] Values of $p<0.05$ were considered significant. Analyses were performed using StatView 4.5 statistical software (Abacus Concepts, Berkeley, CA, USA).

Results

Baseline Data Collection (Table 1)

Coronary spasm was angiographically demonstrated during spontaneous angina in 40 patients and in 216 patients by provocation tests. Spasm in more than one

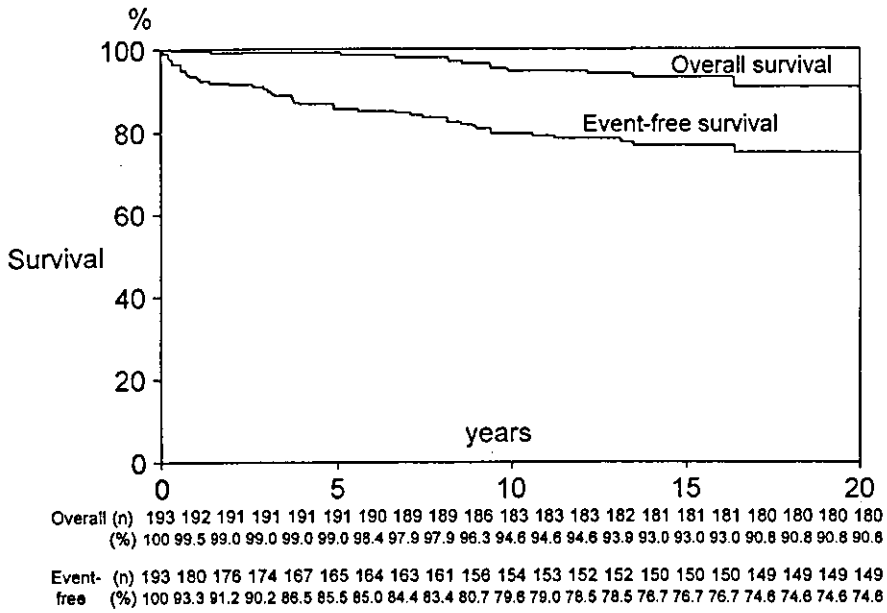

Fig 2. Kaplan-Meier analysis of overall mortality and event-free survival in patients without significant coronary artery stenosis. Vertical and horizontal axes represent % survival rate and time from registration, respectively.

Table 4 Predictors of Cardiac Events in Patients Without Significant CAS During Follow-up Period

	Hazard ratio	*95% CI*	*p value*
High LDL-C	*3.89*	*1.20–12.6*	*0.02*
Sex	*1.51*	*0.64–3.59*	*0.35*
Smoking	*1.14*	*0.57–2.28*	*0.71*
Age	*0.99*	*0.95–1.03*	*0.51*
Hypertension	*0.95*	*0.50–1.82*	*0.87*
Diabetes mellitus	*0.97*	*0.13–7.41*	*0.98*

CAS, coronary artery stenosis; CI, confidence interval; LDL-C, low-density lipoprotein-cholesterol.

major coronary artery was demonstrated in 18 patients. Of the 256 patients, 63 had significant CAS; 53 with single-vessel disease, 8 with double-vessel disease, and 2 with triple-vessel disease. Among the remaining 193 patients who did not have significant CAS, 99 exhibited normal coronary angiography after nitroglycerin administration.

ECG during anginal attacks showed ST elevation in 123 patients, depression in 51 patients and no significant ST change in 82 patients. Ventricular tachycardia was recorded in 6 patients, and second- or third-degree atrioventricular block in 4 patients during provoked coronary spasm. Left ventriculography was normal in 200 patients; segmental wall motion abnormalities were observed in 31 patients. Left ventriculography was not performed in 25 patients because previous echocardiography had shown normal wall motion and cardiac systolic function. Ninety-nine patients were treated with a calcium channel antagonist such as dihydropyridine (30–60 mg/day) or diltiazem hydrochloride (90–120 mg/day), and 157 patients with a combination of calcium channel antagonist and nitrate (40–80 mg/day). In addition to these medications, β-blockers were used in 9 patients. The dose of each drug was calculated to prevent the occurrence of angina during daily activities.

Incidence of Cardiac Events During Follow-up (Table 2)

The mean duration of follow-up was 13.6±3.7 years (range 0.3–20 years). The first cardiac event occurred at 4.9±4.7 years from registration. Of the 46 patients who died during follow-up, 19 died of cardiac death (8 sudden death, 3 myocardial infarction, 5 unexpected heart failure, 3 unknown cause). The other 27 patients died from non-cardiac causes. Ischemic events occurred in 58 patients (non-fatal acute myocardial infarction in 15, unstable angina in 29, PTCA in 12, CABG in 2). The coronary lesions causing the ischemic events were confirmed to coincide with the previous sites of vasospasm in 30 patients and could not be confirmed in the remaining 28 patients.

The overall survival rate was 99.2% at 1 year, 98.0% at 5 years, 93.5% at 10 years and 85.6% at 20 years. Under these conditions, the event-free survival rate was 92.2%, 81.2%, 74.7%, and 62.6%, respectively (Fig 1). When all of the 7 variables were analyzed, the presence of CAS was shown to be an independent predictor of cardiac events (hazard ratio (HR)=2.84, 95% confidence interval (CI)= 1.79–4.52, p<0.0001). There was no difference in survival rate between the patients with CAS in the left coronary and right coronary arteries. High LDL-C and diabetes mellitus had relatively high hazard ratios (2.21 and 1.72, respectively) without statistical significance. Other factors were not significant predictors of cardiac events (Table 3).

In patients without CAS, Kaplan-Meier survival analysis revealed an event-free survival rate of 85.5% at 5 years, 79.6% at 10 years, 76.7% at 15 years and 74.6% at 20 years (Fig 2). Under these conditions, high LDL-C was the only independent predictor of event-free survival (HR=3.89, 95% CI=1.20–12.6, p=0.02, Table 4). As for the relationship between ST deviation and occurrence of cardiac events, cardiac events occurred in 56 of 174 patients (32.2%) with ST deviation, elevation or depression, during vasospasm and in 21 of 82 patients (25.6%, NS) without ST deviation. In 99 patients with normal angiography after nitroglycerin, the incidence of cardiac events and the event-free survival rate were not different from those of the remaining 94 patients with minimal and not significant lesion (p=0.074).

Association Between CAS and Cardiac Events

Although there were no differences in the baseline clinical characteristics between patients with and without CAS

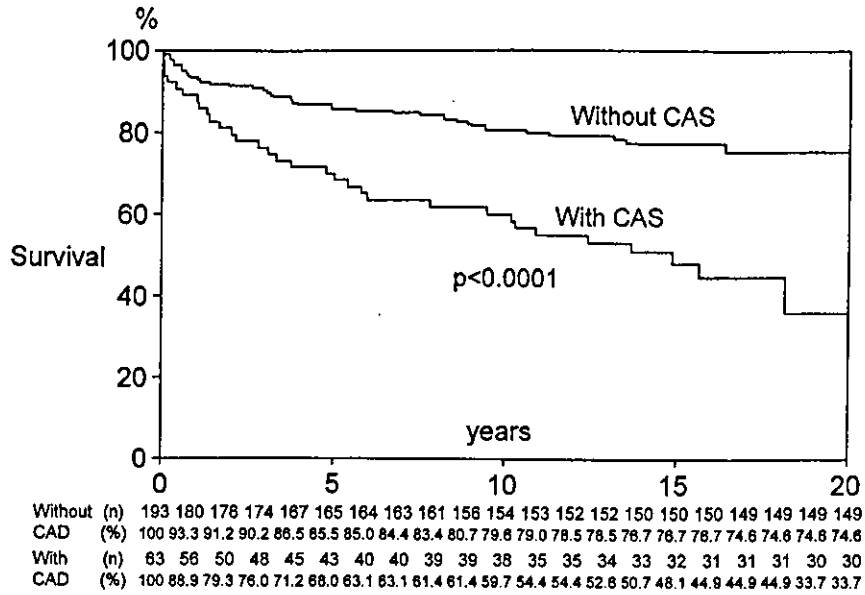

Fig 3. Comparison of event-free survival curves between patients with and without significant coronary artery stenosis (CAS). Vertical and horizontal axes represent % survival rate and time from registration, respectively.

(Table 1), the overall rate of cardiac events was significantly lower in patients without CAS (22.8%) than in those with CAS (52.4%, p<0.01). As for cardiac deaths, 9 of 63 patients with CAS died and only 10 of 193 patients without CAS, yielding a significant difference in cardiac mortality between patients with and without CAS (p=0.017). Also, there was a significant difference in the incidence of sudden death between these subgroups (p=0.024).

Eight of 15 patients with non-fatal myocardial infarction did not have CAS whereas the remaining 7 patients did. Thus, the incidence of non-fatal myocardial infarction in patients without CAS was significantly lower than that in patients with CAS (p=0.041). However, there was no significant difference in the time interval between registration and the first cardiac events in patients without (4.6±4.3 years) and with (5.3±5.3 years) CAS. There was a marked difference in the event-free survival rate between the subgroups with and without CAS (p<0.0001, Fig 3).

Coronary angiography was repeated in 45 of the 256 patients. Progression of coronary atherosclerosis was observed in 10 patients with CAS (45.5%) and in 13 patients without CAS (56.5%, NS). It was interesting that progression of coronary atherosclerosis was more frequently observed at previous sites of spasm (17 patients or 37.8%) than at non-spastic sites (7 patients or 15.5%, p=0.016).

Discussion

Prognostic Value of CAS and High LDL-C

One of the important findings of the present study is that the presence of significant CAS was an independent predictor of event-free survival, as well as the simple survival, in patients with vasospastic angina treated with calcium channel antagonists. The local effect of more complete occlusion during coronary spasm may predispose to malignant arrhythmia or severe myocardial ischemia, although there was no difference in the incidence of cardiac events, including sudden death, in patients with and without ST deviation that may represent the severity of transient myocardial ischemia. Treatment with calcium channel antagonists could reduce this transient ischemia in both patients with and without significant CAS.

Coronary spasm is a possible mechanism for the progression of atherosclerosis![17,20,21] Indeed, in the present study repeated coronary angiography revealed that approximately 40% of patients had progression of coronary atherosclerosis at the sites of previously demonstrated vasospasm. We speculate that coronary spasm accelerates the progression of atherosclerosis, resulting in sudden death and other cardiac events. Under these conditions, elevation of the LDL-C concentration might play an important role in aggravating atherosclerosis through the disintegration of endothelial function![22] However, high LDL-C was second to CAS in terms of hazard ratio in all the present patients. It is possible that a relatively close relation of high LDL-C to significant CAS might make it difficult to differentiate LDL-C as an independent factor in the presence of CAS. Actually, this was shown to be the most important independent factor for cardiac events in patients without CAS. Based on an animal model, an important early step in the development of atherosclerotic lesions is endothelial dysfunction associated with high cholesterol, leading to abnormal vessel tonus characterized by paradoxical constriction to physiologic and pathologic stimuli![23] Vasospasm may be an extreme of this phenomenon and related to lesion development. From this point of view, it is interesting to speculate that high LDL-C may be related to impairment of coronary endothelial dysfunction![24] although recent work does not support this hypothesis![25]

Previous studies suggest that the occurrence of coronary spasm is independent of plasma cholesterol concentrations; in the present study, two-thirds of the patients had normal cholesterol levels. However, under treatment with calcium channel antagonists, which effectively prevent spasm, conventional risk factors such as hypercholesterolemia might be important for the development of atherosclerotic lesions associated with cardiac events. Also it was unclear in the previous studies whether or not the LDL-C concentration was high, even though the total cholesterol concentration was within normal range.

The finding that the incidence of sudden death was lower than in previous studies may be explained by the small number of patients with life-threatening arrhythmias in the present patient group; Millar et al reported that nearly 80%

of patients who died from sudden death had a history of ventricular tachyarrhythmias[26] Even so, 8 patients died suddenly during the follow-up period. It is unclear whether the presence of CAS is a major determinant of sudden death, because sudden death also occurred frequently in patients without CAS[27] Although a previous study suggested that spasm in multiple vessels could be an important determinant of prognosis,[28] the present study could not confirm this finding because of the small number of patients with multi-vessel spasm. One of the reasons why there were relatively few instances of multi-vessel spasm was the different way of provoking spasm. Selective intracoronary injection of ergonovine might not have induced multivessel spasm in some patients in the present study.

Prognostic Value of Other Risk Factors

Except for high LDL-C, there was no statistically significant difference between the other coronary risk factors and cardiac events, although smoking is considered to be one of the major risk factors for the occurrence of vasospastic angina[29] The patients with a history of smoking were carefully questioned about whether they had discontinued smoking during the follow-up period, but it is difficult to confirm the actual status of smoking just by questionnaires at the out-patient clinic. Discontinuation or, at least, a reduction in smoking might result in statistical insignificance of the history of smoking as a risk factor for cardiac events and sudden death.

Coronary spasm without CAS has a fairly good long-term clinical outcome, but even so, high LDL-C was an independent prognostic factor in this subgroup. This suggests that for patients with coronary spasm, manipulation of the therapeutic regimen to prevent not only the occurrence of vasospasm, but also the progression of atherosclerosis should be considered. Calcium channel antagonists such as nifedipine or nicardipine do not reduce cardiac events[30,31] and therefore, a combination with other agents, such as an HMG-CoA reductase inhibitor, that may stabilize the atherosclerotic plaque would further reduce cardiac events in vasospastic angina[13] although the present study excluded patients in whom HMG-CoA reductase inhibitors were given.

The finding that other traditional risk factors were not significant for long-term prognosis can be explained by the specific situation of vasospastic angina. Indeed, previous studies demonstrated that coronary spasm could occur in the absence of hypertension and diabetes mellitus[29] Magnesium deficiency, which was not determined in the present study, might be another risk factor for prognosis of coronary spasm, because 50% of patients with recent myocardial infarction have been shown to have coronary spasm associated with it[32]

Study Limitations

We previously reported that even in the absence of angiographic coronary disease, atherosclerosis can be demonstrated by intravascular ultrasound at the sites of spasm[33,34] Therefore, we could not precisely evaluate the progression or regression of coronary disease in all patients. Prospective follow-up of patients using ultrasound may resolve this problem. Whether patients consistently complied with medication during the follow-up period is important in considering the long-term prognosis. However, the mid-term prognosis of the present study was similar to those of others,[8,9] which suggests that there was acceptable drug compliance during the follow-up period. The use of different kinds of calcium channel antagonists for treatment may have had a small effect on the long-term prognosis, although dihydropyridines and diltiazem have a similar effect on coronary spasm in the relatively acute phase.[3-6]

Although it is clinically interesting to consider the relationship between the frequency of anginal attacks and long-term prognosis, we could not determine the significance, because of difficulty in quantitatively estimating the frequency. For the same reason, it was also difficult to correlate the presence of CAS with anginal frequency.

To further understand the role of high LDL-C in the long-term prognosis of vasospastic angina, the medical regimen of HMG-CoA reductase inhibitors in addition to calcium channel antagonists should be challenged. It is also necessary to compare the long-term prognosis between CAS patients with and without spasm. However, practically, it is difficult to enroll stable patients without coronary spasm, because PTCA or CABG rather than medical treatment is now the primary treatment.[35]

Finally, the present study dealt only with Japanese patients with coronary spasm. However, despite the racial difference in coronary vasomotor reactivity,[36] the clinical outcome of the present patients is similar to the results of studies in Europe and North America. Therefore, the present data provide important information on the long-term prognosis of medically treated coronary spasm patients with or without CAS.

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Original Article

Association of Genetic Polymorphisms of Sodium-Calcium Exchanger 1 Gene, *NCX1*, with Hypertension in a Japanese General Population

Yoshihiro KOKUBO*[1], Nozomu INAMOTO*[1], Hitonobu TOMOIKE*[1], Kei KAMIDE*[2], Shin TAKIUCHI*[2], Yuhei KAWANO*[2], Chihiro TANAKA*[3], Yuki KATANOSAKA*[3], Shigeo WAKABAYASHI*[3], Munekazu SHIGEKAWA*[3], Otosaburo HISHIKAWA*[4], and Toshiyuki MIYATA*[3]

The Na^+/Ca^{2+} exchanger (NCX) is a membrane protein involved in calcium homeostasis, catalyzing the exchange of one Ca^{2+} ion for three Na^+ ions across the cell membrane. The Na^+/Ca^{2+} exchange has been suggested to play a role in the pathogenesis of hypertension. Therefore, we examined whether genetic variations in *NCX1* were associated with hypertension. Among 15 polymorphisms identified in 96 hypertensive subjects by sequencing the entire exon and promoter regions of *NCX1*, 7 representative polymorphisms with a minor allele frequency of greater than 4% were genotyped in 1,865 individuals, of whom 787 were hypertensive and 1,072 were normotensive. These subjects were residents of Suita City and were randomly selected as a population for the Suita cohort study. Multivariate logistic regression analysis performed after adjusting for age, body mass index, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that the −23200T>C and −23181T>C polymorphisms in the 5′ upstream region of exon 1c were significantly associated with hypertension in men (−23200T>C: CC *vs.* TC+TT: odds ratio=0.61; 95% confidence intervals: 0.39 to 0.97; p=0.04) and in women (−23181T>C: CC *vs.* TC+TT: odds ratio=1.45; 95% confidence intervals: 1.04 to 2.02; p=0.03), respectively. Thus, our study suggests that *NCX1* is one of the genes related to susceptibility to essential hypertension in the Japanese general population.
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***Key Words*: NCX1, Na^+/Ca^{2+} exchanger, gene variants, hypertension**

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is an important membrane protein involved in calcium homeostasis in various cell types and catalyzes the electrogenic exchange of one Ca^{2+} ion for three Na^+ ions across the plasma membrane (*1–3*). The Na^+/Ca^{2+} exchange has been well demonstrated to play a role in the pathogenesis of hypertension. Blaustein *et al.* suggested that excessive Na^+ retention may secrete an ouabain-like substance that increases the cytosolic Na^+ concentration by inhibiting the plasmalemmal Na^+-pump, which increases the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by reducing Ca^{2+}-extrusion *via* Na^+/Ca^{2+} exchange (*4–6*). The increase in arteri-

From the *[1]Division of Preventive Cardiology, *[2]Division of Hypertension and Nephrology, and *[3]Research Institute, National Cardiovascular Center, Suita, Japan, and *[4]Suita City Medical Association, Suita, Japan.

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Address for Reprints: Yoshihiro Kokubo, M.D., Ph.D., Division of Preventive Cardiology, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita 565-8565, Japan. E-mail: ykokubo@hsp.ncvc.go.jp

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Table 1. Basic Characteristics of Subjects in Suita, a Japanese Urban Population, 2002

	Men (n=858)	Women (n=1,007)
Age (year)	66.3±11.1*	63.3±11.0*
Systolic blood pressure (mmHg)	131.9±19.5*	128.0±19.6*
Diastolic blood pressure (mmHg)	79.7±10.7*	76.6±10.7*
Body mass index (kg/m^2)	23.3±3.0*	22.3±3.2*
Total cholesterol (mmol/l)	5.10±0.78	5.57±0.79*
HDL-cholesterol (mmol/l)	1.42±0.36	1.67±0.40*
Current smokers (%)	30.1†	6.3†
Current drinkers (%)	67.0†	29.3†
Present illness (%)		
Hypertension	47.4†	38.2
Hyperlipidemia	27.4	55.2†
Diabetes mellitus	12.6†	5.2

Values are mean±SD or percentage. Hypertension indicates systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or antihypertensive medication; hyperlipidemia, total cholesterol ≥5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥7.0 mmol/l (126 mg/dl) or non-fasting plasma glucose ≥11.1 mmol/l (200 mg/dl) or HbA1c ≥6.5% or antidiabetic medication. * $p<0.05$ between women and men by Student's t-test. † $p<0.05$ between women and men by χ^2 test. HDL, high-density lipoprotein.

al tone caused by high $[Ca^{2+}]_i$ would thus result in an elevation of blood pressure. Indeed, several previous studies have reported that Na^+/Ca^{2+} exchange activity was altered in the renal arterioles or arterial smooth muscle of spontaneous or salt-sensitive hypertensive rats (*7–11*). However, it is unknown whether such a mechanism relates to the occurrence of essential hypertension.

Of three isoforms (NCX1–3) derived from different genes, NCX1 is predominantly expressed in the heart, neurons and renal tubules, but is expressed at lower levels in other tissues, including the smooth muscle, skeletal muscle, lung and spleen (*1–3*). The *NCX1* gene (*SLC8A1*) is located on human chromosome 2p22.1 and includes 12 exons (*12*). There are at least 12 splice variants generated in different combinations from six exons in a tissue-specific manner (*13*). In addition, five exons encode 5′-untranslated sequences that are under the control of three tissue-specific promoters (*14–17*).

This study was undertaken to identify genetic variations in *NCX1* in a group of hypertensive subjects, and to examine the association of these variations with the presence of hypertension in a general population. In contrast to other association studies, which often focus on a limited number of polymorphisms in a gene, our study evaluated the full array of coding- and promoter-sequence polymorphisms in *NCX1*.

Methods

Subjects of the Suita Population Study

The subjects of the Suita study consisted of 14,200 men and women (30 to 79 years of age), who had been randomly selected from the municipal population registry and stratified by in consideration of gender and age (stratified in 10-year intervals). They were all invited, by letter, to receive medical and behavioral examinations every 2 years at the Division of Preventive Cardiology, National Cardiovascular Center, Japan. DNA from the leukocytes was collected from participants who visited the National Cardiovascular Center between May 2002 and February 2003. All of the participants were Japanese. Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. In this study, the genotypes of 1,865 samples were determined. The characteristics of 1,865 participants (858 men, 1,007 women) are shown in Table 1. Routine blood examinations that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and glucose levels were performed. A physician or nurse interviewed each patient in regard to smoking and drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke.

Blood pressure was measured in a sitting position after at least 10 min of rest. Systolic and diastolic blood pressures (SBP/DBP) were taken as the means of two measurements recorded more than 3 min apart by well-trained doctors. Hypertension was defined as SBP of ≥140 mmHg, DBP of ≥90 mmHg, or the current use of antihypertensive medication (*18*). Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/l (126 mg/dl), non-fasting plasma glucose ≥11.1 mmol/l (200 mg/dl), current use of antidiabetic medication, or HbA1c ≥6.5%. Hyperlipidemia was defined as total cholesterol ≥5.68 mmol/l (220 mg/dl) or current use of antihyperlipidemia medication. Body mass index (BMI)

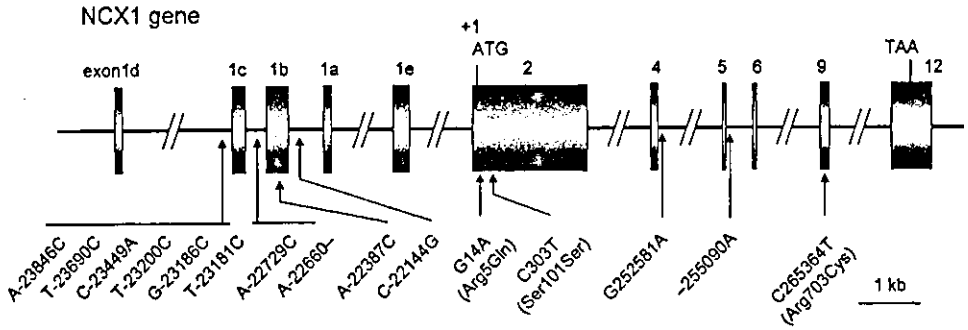

Fig. 1. *Genome structure of human* NCX1. *The* NCX1 *gene consists of sixteen exons, five (exons 1a–1e) of which direct tissue-specific transcription and eleven (exons 2–12) of which encode the open reading frame (17). The five tissue-specific transcription exons (exons 1a–1e) and the exons in which the SNPs were identified are depicted. The nucleotide changes and amino acid substitutions are also shown. The A of the ATG of the initiator Met codon is denoted nucleotide +1.*

was calculated as weight (in kg) divided by height (in m) squared.

Direct Sequencing for Single Nucleotide Polymorphism (SNP) Discovery and Genotyping of Polymorphisms

For DNA sequencing, 96 patients with essential hypertension were recruited from the Division of Hypertension and Nephrology, National Cardiovascular Center, Japan. The method of direct sequencing was described previously (*19*). Fifteen polymorphisms were identified by sequencing and 7 representative polymorphisms with a minor allele frequency of greater than 4% were genotyped by the TaqMan-polymerase chain reaction (PCR) system (*20*). Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Statistical Analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis.

Logistic regression analyses were used to examine the association between the genotypes and blood pressure in each sex with consideration for potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with 95% confidence intervals. The relationship between genotype and risk of hypertension was expressed in terms of the odds ratios adjusted for possible confounding effects including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SAS statistical software (release 8.2; SAS Institute, Cary, USA) was used for statistical analyses (*21*).

Results

Basic Characteristics of Subjects in the Suita Study

The characteristics of the 1,865 participants (858 men, 1,007 women) are summarized in Table 1. Age, SBP, DBP, BMI, percentage of current smokers, percentage of current drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, HDL-cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men.

Polymorphisms of *NCX1*

The *NCX1* gene has a complicated genome structure containing five alternative 5′ exons producing separate tissue-specific promoters and six exons encoding open reading frames (Fig. 1). We sequenced the entire exon and promoter regions of *NCX1* from 96 patients (182 alleles) with hypertension, and identified 15 polymorphisms (Table 2, Fig. 1). We identified two missense mutations, Arg5Gln in exon 2 and Arg703Cys in exon 9, in *NCX1* (Table 2). Each of the missense mutations was identified in one out of 96 individuals, indicating that their allele frequencies were rare. Two SNPs, −23200T>C and −23186G>C, were in linkage disequilibrium. Seven representative polymorphisms with a minor allele frequency of greater than 4% were genotyped for the association study.

Susceptible SNPs Related to Hypertension

Seven polymorphisms in *NCX1* were genotyped in 1,865 individuals, of whom 787 were hypertensive and 1,072 were normotensive. The primers and probes of the TagMan-PCR system and the genotyping results are summarized in Table

Table 2. List of 15 Polymorphisms and Their Allele Frequencies in the *NCX1* Gene Identified by Direct Sequencing

Allele 1/Allele 2 SNPs	TaqMan typing	Amino acid change	Region	Allele 1 Homo	Hetero	Allele 2 Homo	Total	Allele frequency Allele 1	Allele frequency Allele 2	Flanking sequence
−23846A>C			intron 1d	94	1	0	95	0.995	0.005	tcacactgcctt[a/c]aattcagggact
−23690T>C	typing		intron 1d	62	31	2	95	0.816	0.184	aaatttaactta[t/c]agcaaggaaaga
−23449C>A	typing		intron 1d	85	9	1	95	0.942	0.058	catactcacatt[c/a]atgtttgaggag
−23200T>C*	typing		intron 1d	0	9	86	95	0.047	0.953	attccgcccccct[t/c]tttgttgcggag
−23186G>C*			intron 1d	0	9	86	95	0.047	0.953	ttgttgcggagg[g/c]aaactgaggttc
−23181T>C	typing		intron 1d	18	57	20	95	0.489	0.511	gcggaggcaaac[t/c]gaggttcctgga
−22729A>C	typing		intron 1c	71	23	1	95	0.868	0.132	taattatgagga[a/c]agtgatttattg
−22660delA			intron 1c	94	1	0	95	0.995	0.005	gattgctgcatt[a/−]ggtttttccca
−22387A>C		5′ UTR	exon 1b	93	3	0	96	0.984	0.016	attaaaaaaaaa[a/c]tcattgatatat
−22144C>G	typing		intron 1b	84	9	2	95	0.932	0.068	gcgcggccacaa[c/g]gcactgcggggc
14G>A		Arg5Gln	exon 2	95	1	0	96	0.995	0.005	tgtacaacatgc[g/a]gcgattaagtct
303C>T		Ser101Ser	exon 2	95	1	0	96	0.995	0.005	tcggttcatgtc[c/t]tctatagaagtc
252581G>A	typing		intron 4	45	40	11	96	0.677	0.323	tcttctctctcc[g/a]tgtctccctact
255089–255090insA			intron 5	94	1	0	95	0.995	0.005	tcaggtgataca[−/a]gtagctctgtga
265364C>T		Arg703Cys	exon 9	95	1	0	96	0.995	0.005	gcagaaatgggg[c/t]gccccatcctgg

The A of the ATG of the initiator Met codon is denoted nucleotide +1. * The apparent linkage disequilibrium ($r^2 \geq 0.5$). NCX1, Na^+/Ca^{2+} exchanger; SNP, single nucleotide polymorphism.

3. Multivariate logistic regression analysis after adjusting for confounding risk variables such as age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking, revealed that two polymorphisms, −23200T>C and −23181T>C, in the 5′ upstream region of exon 1c were significantly associated with hypertension in men (−23200T>C: CC *vs.* TC+TT: odds ratio=0.61; 95% confidence interval: 0.39 to 0.97; $p=0.04$) and in women (−23181T>C: CC *vs.* TC+TT: odds ratio=1.45; 95% confidence interval: 1.04 to 2.02; $p=0.03$), respectively (Table 4). When normotension was defined as SBP ≤120 mmHg, DBP ≤80 mmHg, and the absence of anti-hypertensive medication, and hypertension was defined as SBP ≥160 mmHg, DBP ≥100 mmHg, or the current use of antihypertensive medication, −23200T>C polymorphism was significantly associated with hypertension in men (CC *vs.* TC+TT: odds ratio=0.42; 95% confidence interval: 0.20 to 0.92; $p=0.03$) after adjusting for the confounding factors described above.

Discussion

In this study, we sequenced the exon and promoter regions of *NCX1* and identified 15 polymorphisms. Seven representative polymorphisms were genotyped from 1,865 subjects to examine the association of hypertension with *NCX1*. After adjustment for various confounding factors, we identified that the −23200T>C polymorphism in the 5′ upstream region of exon 1c was significantly associated with hypertension in men and the −23181T>C polymorphism in the 5′ upstream region of exon 1c was significantly associated with hypertension in women.

The *NCX1* gene has at least 12 splice variants generated in different combinations from six exons in a tissue-specific manner (*13*). In addition, three exons encode 5′-untranslated sequences that are under the control of three tissue-specific promoters (*14–16*). Exon 1c is a part of the "heart" specific transcript (*17*) and its upstream region is not likely a promoter. Therefore, the −23200T>C and −23181T>C polymorphisms present in the upstream region of exon 1c are not likely to be directly involved in transcription of *NCX1*. Rather, these polymorphisms may be in linkage disequilibrium with other polymorphisms in the region that were not examined by sequencing in this study.

In this study, the −23200T>C polymorphism in men and −23181T>C polymorphism in women were identified as SNPs conferring susceptibility for hypertension. It is well known that the greater incidence of hypertension and coronary artery disease in men is, in part, related to gender differences in possible vascular protective effects of the female sex hormones estrogen and progesterone. Furthermore, NCX1 might be related to salt-sensitive hypertension (*22*). Since there is a gender difference in salt-sensitivity and plasma renin activity (*23*, *24*), −23200T>C and −23181T>C in *NCX1* may be linked with unidentified causative genetic variations that would be influenced by the female sex hormones and/or salt-sensitivity.

In this study, we identified two missense mutations, Arg5Gln in exon 2 and Arg703Cys in exon 9, in *NCX1*. Arg5 is located within the signal peptide sequence consisting of the first N-terminal 35 amino acids of NCX1, which are removed during biosynthesis (*1*). We expressed a mutant canine NCX1 with the Arg5Gln substitution in the fibroblastic

Table 3. Genotyping Conditions and Results of *NCX1* Polymorphisms in 1,818 Individuals by TaqMan-PCR Method

SNP	Primer	Probe	Genotypes results
−23690T>C	CTCTCCCCACAGGTCATTCTG	Fam-ATTTAACTTATAGCAAGGAA-MGB	(TT/TC/CC)
	GCAGGAATCGTTCTTGCCTAA	Vic-TTAACTTACAGCAAGGAA-MGB	=(1,140/590/88)
−23449C>A	GAATCTGCAATCCCCATGTGAT	Fam-CTCACATTCATGTTTGAG-MGB	(CC/CA/AA)
	AGAACCACTGCTCTAGGCCAAT	Vic-ACTCACATTAATGTTTGAGG-MGB	=(1,542/261/15)
−23200T>C	TTCTGAGGTGCAAGGAGGGTT	Fam-CCCCCTTTTTGTTGC-MGB	(TT/TC/CC)
	GGCAGTCACCACGACTGATAGA	Vic-CCCCCTCTTTGTTG-MGB	=(4/196/1,618)
−23181T>C	GGCAGTCACCACGACTGATAGA	Fam-TCCAGGAACCTCAGTTT-MGB	(TT/TC/CC)
	AGGCTATTTCTTCCATTCCGC	Vic-CCAGGAACCTCGGTTT-MGB	=(503/869/446)
−22729A>C	GCCTGGTGCAGTGTTCCTTTA	Fam-ATTATGAGGAAAGTGATTTA-MGB	(AA/AC/CC)
	GCCCTTTCCAAGAGAAGCATTA	Vic-TATGAGGACAGTGATTTA-MGB	=(1,369/406/43)
−22144C>G	AAAAGAAAAGTTGCAGCGCCT	Fam-CCACAACGCACTGC-MGB	(CC/CG/GG)
	TTTTTCGATTTCCTGCCGG	Vic-CACAAGGCACTGCG-MGB	=(1,687/131/0)
252581G>A	AAACAAAGACATACCAGCGAGAAA	Fam-CTCTCTCCGTGTCTC-MGB	(GG/GA/AA)
	AAATTGCTAAAGCTTCAAAGGCA	Vic-TCTCTCCATGTCTCC-MGB	=(823/798/197)

PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Table 4. Odds Ratio of −23200T>C Polymorphism in Men and −23181T>C Polymorphism in Women*

Gender	SNP	OR (95% CI)		*p*	OR (95% CI)		*p*
Men	−23200T>C	CC	1 (reference)		CC+TC	1 (reference)	
		TC+TT	0.61 (0.39–0.97)	0.04	TT	—	—
Women	−23181T>C	CC	1 (reference)		CC+TC	1 (reference)	
		TC+TT	1.45 (1.04–2.02)	0.03	TT	1.39 (1.00–1.92)	0.05

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence intervals.

cell line CCL39, and found that this mutant NCX1 was properly targeted into the plasma membrane and exhibited the normal Na^+/Ca^{2+} exchange activity (unpublished observations), consistent with previous reports stating that signal sequence is not essential for functional expression of the NCX1 protein (*25*, *26*). On the other hand, Arg703 is located within the large cytoplasmic loop connecting the transmembrane segments 5 and 6, which are not essential for the functional expression of the NCX1 protein (*1*). Thus, the two rare mutations identified in this study would not grossly impair the function of NCX1.

In summary, we showed that the SNPs −23200T>C and −23181T>C in *NCX1* were associated with hypertension. The pathophysiological functional behaviors of these polymorphisms remain to be clarified. In future studies, it will be necessary to clarify the function of these polymorphisms or to identify the causative polymorphisms that are in linkage disequilibrium with these polymorphisms.

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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

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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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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

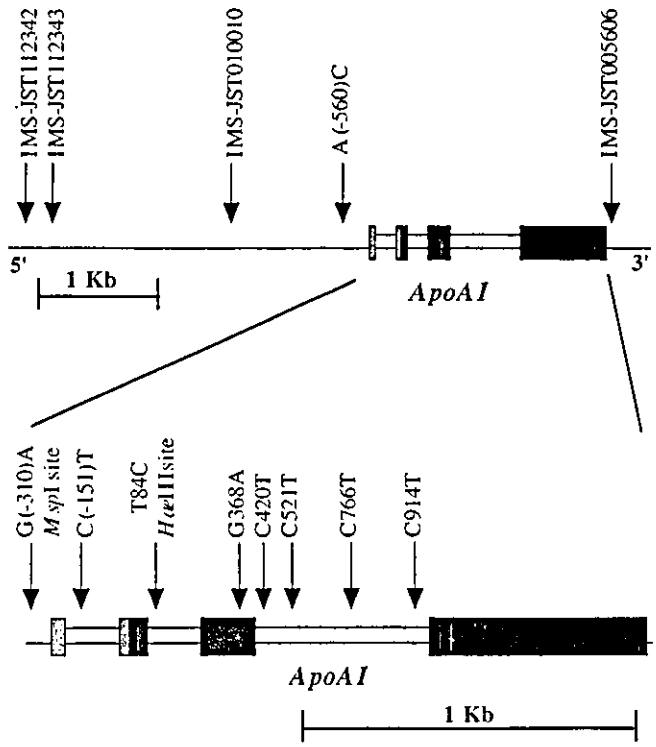

Fig. 1 Schema of the *ApoA1* gene and the positions of the determined polymorphisms. *Gray and black boxes* indicate the 5'-untranslated and coding regions, respectively

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 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	–	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	–	CTCCGCGGACAGGGTGTCACCCAGGG[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	326/204/17	0.0326[a]
	59.2%/34.7%/6.1%	59.6%/37.3%/3.1%	

[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*