Table 1 Comparison of prevalence of plasminogen deficiency between diseased groups and age-matched and sex-matched controls

	Number of heterozygote (prevalence)	Odds ratio (95% CI) (vs. controls)	*P*-value
Patients with DVT ($n = 108$)	3 (2.78%)		
Controls ($n = 324$)	13 (4.01%)	0.65 (0.21–2.07)	0.62
Patients with cardioembolic stroke due to NVAF ($n = 110$)	6 (5.55%)		
Controls ($n = 330$)	13 (3.94%)	1.31 (0.57–3.03)	0.52

CI, confidence interval; DVT, deep vein thrombosis; NVAF, non-valvular atrial fibrillation.

Table 2 Characteristics of homozygous type I plasminogen deficiency identified in National Cardiovascular Center

Patient number	Age (sex)	Plg-act (%)	Plg-ag (%)	Age at onset for thrombosis	Diagnosis	Family study phenotypic*
1	84 (F)	16.0	146.5	70	Stroke	nt
2	85 (M)	14.0	97.7	74	Stroke, arteriosclerotic obliterance	nt
3	72 (M)	10.8	98.2	63	Acute myocardial infarction, double aortic arch	9/9
4	73 (F)	15.2	89.3	55	Myocardial infarction	nt
5	15 (M)	8.2	61.6	–	Tetralogy of Fallot	nt
6	53 (M)	5.5	95.0	–	Aortic regurgitation	3/3
7	78 (M)	8.3	83.1	–	Cerebral hemorrhage	2/2
8	69 (M)	14.1	103.0	–	Arteriosclerotic obliteranse, chronic renal failure	nt
9	6 (F)	10.5	102.3	–	Ventricular septal defect	2/2
10	71 (F)	4.8	85.8	66	Angina pectoris, atrial septal defect	nt
11	74 (M)	11.6	78.7	63	Acute myocardial infarction	nt
12	73 (F)	1.5	107.0	–	Mitral stenosis	nt
13	13 (F)	11.7	104.4	–	Atrial septal defect, ventricular septal defect	3/3
14	55 (M)	13.6	95.7	50	Angina pectoris	nt
15	53 (M)	6.7	84.3	–	Cerebral hemorrhage	nt
16	56 (M)	17.7	79.8	55	Acute myocardial infarction	nt
17	72 (F)	7.3	73.1	65	Mitral stenosis	nt
18	71 (M)	9.7	88.3	64	Angina pectoris	nt
19	65 (M)	8.4	78.0	65	Stroke	nt

Plg-act, plasminogen activity; Plg-ag, plasminogen antigen. *The number of homozygotes or heterozygotes in the pedigree in which plasminogen activity was studied is indicated in the denominator. Within the numerator the total number of tested family members. M, male; F, female; nt, not tested.

Relevance of plasminogen deficiency to deep vein thrombosis and cardioembolic stroke due to NVAF

To establish whether plasminogen deficiency is a risk factor for thrombotic disorders, we identified plasminogen deficiency in 3 patients with DVT ($n = 108$) and in 6 patients with cardioembolic stroke due to NVAF ($n = 110$) by using the cut-off ratio of 0.69. Table 1 shows the prevalence of the plasminogen deficiency between the patient group and the age- and sex-matched controls. The results indicate that the prevalence was not different, indicating that plasminogen deficiency is not a risk factor for these thrombotic disorders.

Clinical phenotype of homozygous plasminogen deficiency

In an independent study, we screened for plasminogen activity in patients admitted to our hospital over 8 years, and identified 19 patients with extremely low plasminogen activity. Table 2 shows the characteristics and disease phenotypes of these patients. Most of the patients showed around 10% plasminogen activity (10.3% in average), except for patient 12 with only 1.5% activity. All of the patients had a normal level of antigen, indicating type II plasminogen deficiency, probably carrying plasminogen Tochigi mutation. Some of the patients had arterial thrombotic complications such as stroke ($n = 3$), MI ($n = 4$), or angina pectoris ($n = 3$). However, the disease occurred at advanced age, suggesting that homozygous plasminogen deficiency would not be a primary cause of these thrombotic diseases. None of the patients showed DVT.

Discussion

Here, we reported age- and gender-related changes of plasminogen activity using a Japanese general population comprising 4517 adults aged 32–89 years. Changes of the plasminogen activity have been reported in a healthy Scottish population comprising 9811 adults aged 17–65 years [25]. Comparing these two studies, our population covered an elderly population and the Scotland study covered a younger population. Age-related changes of plasminogen activity in the age groups

overlapping between the two studies were well consistent. In addition, we observed a decrease of plasminogen activity in the elderly, between 70–79 years and 80–89 years (Fig. 1). The low plasminogen activity in the elderly is probably affected by liver's ability to generate protein, because a decrease of albumin level with age also was observed ($r = -0.35$, $P < 0.0001$).

In the present study, we found the prevalence of plasminogen deficiency to be 4.30% in the Japanese general population ($n = 4517$), the prevalences of type I and II heterozygous plasminogen deficiency being 0.42% and 3.87%, respectively. The prevalence of type II plasminogen deficiency in the present study agreed with previously reported results in Japanese [6–9]. We also obtained the prevalence of plasminogen deficiency in patients with DVT and in patients with cardioembolic stroke and found that those prevalences were not different from those obtained from age-matched and sex-matched control groups selected from the general population. Most of these patients were residents in the northern Osaka area where the cohort study took place. Therefore, our study indicated that heterozygous plasminogen deficiency is not a risk factor for thrombotic complications.

We identified 173 heterozygotes and two homozygotes in 4517 individuals. If we assume that all those with type II deficiency carried the plasminogen Tochigi mutation, the Ala→Thr substitution at position 601, we can calculate the allele frequency of plasminogen Tochigi to be 1.96% in the Japanese general population. This allele frequency is similar to those of factor V Leiden mutation (2–7%) [26] and prothrombin 20210 A mutation (0.35–2.0%) [27] found in the Caucasian general population. We also identified 19 heterozygotes of type I plasminogen deficiency. The prevalence of type I plasminogen deficiency thus obtained (0.42%) showed quite good agreement with the previously observed prevalence in the Scotland population (28/9,611, 0.29%) [18].

There are several reports addressing a phenotype of mice with homozygous type I plasminogen deficiency [28,29]. The plasminogen gene in these mice was abnormal so that no plasminogen activity was present in plasma, resulting in spontaneous fibrin deposition due to impaired thrombolysis. One of the useful features of engineered mice is that although a transgenic or knockout gene may have no phenotype, a phenotype may become apparent with a physiological or pathological challenge. For example, mice deficient in plasminogen exacerbated renal injury in experimental crescentic glomerulonephritis [30]. Those mice also abolished wound healing after myocardial infarction [31]. These studies suggest that even though individuals with plasminogen deficiency did not show venous thrombosis, they may express a certain phenotype after a challenge or insult. Therefore, careful continuous observation in individuals with plasminogen deficiency is required for assessment of relation of plasminogen deficiency with its phenotype.

In conclusion, the prevalence of heterozygous plasminogen deficiency is about 4% in Japanese, and plasminogen deficiency is not a primary cause of thrombosis. This conclusion was also supported by the phenotypes of 19 patients with homozygous type II plasminogen deficiency.

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Addendum of the roles of authors

Drs Okamoto, Sakata, Mannami, and Miyata were responsible for the study design, interpretation of the data and preparation of the article. Drs Baba, Katayama, Matsuo, Yasaka, Minematsu, and Tomoike were responsible for sample collection, steering and discussion.

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Association analyses between polymorphisms in the GJA4 gene cluster and myocardial infarction in Japanese

Dear Sir,

Connexin 37 (*GJA4*) is a major gap junction protein that is mainly expressed in vascular endothelial cells. Connexin 37 has been suggested to play a role in atherogenesis (1). Recently, the *C1019T* polymorphism in *GJA4* has been reported to be associated with myocardial infarction (MI) in a large-scale association study (2). However, this might be a result of linkage disequilibrium with some other truly important polymorphisms of the *GJA4* cluster. Therefore, we performed extensive association analyses between polymorphisms in the *GJA4* cluster and MI.

The *GJA4* cluster in chromosome 1p35 contains 2 related genes *(GJB3 and 5)* within a 40-kb region. Direct sequencing (36 randomly selected subjects) in this region revealed 20 polymorphisms, including the *C1019T* polymorphism that has been reported to be associated with MI (2). We genotyped all of the 20 polymorphisms by the TaqMan method. The study population consisted of 524 male patients (58 ± 10 yrs) with MI recruited from the National Cardiovascular Center, and 594 male controls (65 ± 11 yrs) consecutively recruited from the Suita Study (3).

Univariate analyses showed that the *GJB3 G1182C* (ddSNP:2236214, p=0.0040), *GJA4-1930C/T* (p=0.0162), and *I1297D* (ddSNP:3841825, p=0.0028), but not *C1019T* (p=0.1393), polymorphisms were significantly associated with MI. Logistic analysis indicated that the DD genotype of *I1297D* was more susceptible for MI than the II+ID genotype (p=0.0005, Odds=1.728, 95%CI; 1.270-2.348). No significant deviation from Hardy-Weinberg equilibrium was observed for any polymorphism. Among these three markers, *GJB3 G1182C* and *GJA4 I1297D* were almost completely concordant. Linkage disequilibrium (D'>0.9) was found between *GJA4 I1297D* and -1930C/T and between *GJA4 I1297D* and *C1019T*. The characteristics of the study subjects and genotype distributions of GJA4 polymorphisms are shown in Table 1.

Three previous studies have reported that the *C1019T* polymorphism in *GJA4* was associated with coronary artery disease

Correspondence to:
Naoharu Iwai MD, PhD
National Cardiovascular Center
5-7-1 Fujishirodai Suita
Osaka 565-8565
Japan
Tel.: +81-6-6833-5012, Fax: +81-6-6872-8091
E-mail: niwai@res.ncvc.go.jp

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Table 1: Characteristics of the study subjects and genotype distributions of GJA4 polymorphisms

	Controls (n=588)	Patients with MI (n=528)	p
Age, yrs	65(11)	58(10)	<.0001
Body mass index, kg/m2	23.3(2.9)	23.9(3.1)	<.0001
HTN, %	47.7	54.3	<.0001
DM, %	8.5	40.8	<.0001
HLP, %	13.4	56.3	<.0001
Smoker, %	35.1	65.5	<.0001
BMI (kg/m2)	22.8(3.1)	23.8(3.1)	<.0001
*GJA4 -1930C/T**	495/88/5	463/65/0	0.0162
(CC/CT/TT)	(84.2/15.0/0.9%)	(87.7/12.3/0%)	
GJA4 I1297D#	274/270/44	232/220/72	0.0028
(II/ID/DD)	(46.6/45.9/7.5%)	(44.3/42.0/13.7%)	

Values are expressed as the mean ± SD. *Number of subjects according to the GJA4 -1930C/T genotype (CC/CT/TT). #Number of subjects according to the GJA4 I1297D genotype (II/ID/DD).

in Asian populations as well as in Swedish men (4, 5). Kumari et al. investigated biophysical properties of the polymorphic variant and concluded that it may have little influence on several properties of GJA-mediated intercellular communication (6). The present findings indicate that previously reported associations between the *C1019T* polymorphism and ischemic heart disease might be due to linkage disequilibrium between the *C1019T* and *I1297D* polymorphisms. The *I1297D* polymorphism is located in the 3'-untranslated region of GJA4 mRNA and may be related to the stability of mRNA (7). Further studies are needed to elucidate the biological significance of this polymorphism.

In the present study, the MI and control groups were not age-matched. The mean age in the control group was 7 years greater than that in the MI group. Since subjects who had developed MI at a younger age were excluded from the controls, the controls in the present study may be a subset of subjects who are relatively unsusceptible to MI compared to the general population in Japan.

In conclusion, the present results suggest that the *I1297D* polymorphism is an important marker for a genetic risk of MI in a Japanese population and confirmed previous findings that the GJA4 gene contributes to MI.

Yoshitaka Iwanaga, Toshifumi Mannami, Yoichi Goto, Hiroshi Nonogi, Naoharu Iwai

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Association of Methylenetetrahydrofolate Reductase Gene Polymorphism With Carotid Atherosclerosis Depending on Smoking Status in a Japanese General Population

Nozomu Inamoto, MD; Tomohiro Katsuya, MD, PhD; Yoshihiro Kokubo, MD, PhD; Toshifumi Mannami, MD, PhD; Takashi Asai, MD; Shunroku Baba, MD, PhD; Jun Ogata, MD, PhD; Hitonobu Tomoike, MD, PhD; Toshio Ogihara, MD, PhD

Background and Purpose—The association of the *methylenetetrahydrofolate reductase* gene (*MTHFR*) with carotid atherosclerosis remains inconsistent. This may be due to small sample size and inappropriate analysis. We investigated the association of *C677T/MTHFR* with blood pressure and carotid atherosclerosis in a Japanese general population.

Methods—Subjects (30 to 89 years of age; 1693 women, 1554 men) who gave informed consent were randomly selected from a general population in Suita, Japan. *MTHFR* genotypes were determined by TaqMan polymerase chain reaction. Carotid atherosclerosis was evaluated by high-resolution ultrasonography with atherosclerotic indexes of intimal-medial thickness (IMT), maximum IMT in the common carotid artery (CCA), plaque score, and stenosis (>50%).

Results—Age-adjusted diastolic blood pressure was significantly higher in women with the *TT* genotype than in those with the *CC* genotype. In a recessive model (*CC+CT* versus *TT*), all adjusted odds ratios for hypertension and >50% stenosis in women were 1.42 and 3.42 (95% confidence intervals, 1.01 to 1.99 and 1.23 to 9.53), respectively. In women, maximum IMT in CCA for smokers with the *TT* genotype was significantly higher than for smokers with the *CC* genotype and nonsmokers with the *TT* genotype ($P<0.05$).

Conclusions—Our study suggests that the *MTHFR TT* genotype is a risk factor for hypertension and carotid stenosis in women. Significant interactions between *C677T/MTHFR* and smoking on maximum IMT in CCA were observed in women but not in men. Smoking cessation for subjects with the *TT* genotype is important in the prevention of cerebrovascular disease. **(*Stroke*. 2003;34:1628-1633.)**

Key Words: amine oxidoreductases ■ blood pressure ■ carotid arteries ■ Japan ■ risk factors

Hyperhomocysteinemia is associated with increased risk of atherosclerotic vascular disease.[1] The association of plasma total homocysteine concentration with atherosclerosis has been the subject of a number of clinical studies that have consistently linked moderate hyperhomocysteinemia with peripheral vascular disease, cerebrovascular disease, and coronary heart disease.[2-5]

Plasma total homocysteine levels are regulated mainly by 5,10-methylenetetrahydrofolate reductase, which is involved in the folate-dependent remethylation of homocysteine to methionine. Frosst et al[6] suggested that the *C677T* polymorphism in the *methylenetetrahydrofolate reductase* gene (*MTHFR*) is a candidate risk factor for vascular disease. The metabolic changes associated with *C677T/MTHFR* are postulated to modify the predisposition to diseases associated with folate deficiency.[7] Particular emphasis has been given to the role of *C677T/MTHFR* in cardiovascular[8] and cerebrovascular disease[9] and venous thrombosis.[10]

On the other hand, technical improvements in carotid ultrasonography have revealed new risk factors for stroke in its wide use. Some studies have demonstrated a close correlation between carotid ultrasound measurement, usually of carotid intimal-medial wall thickness (IMT), and the severity of extracranial carotid atherosclerosis.[11,12] Plasma total homocysteine levels have also been associated with more advanced carotid atherosclerosis in elderly subjects.[3,13] However, there have been controversies among their results. Most studies have failed to show an association between *C677T/MTHFR* and atherosclerotic disease.[14,15] These inconsistencies may be due to small sample size, combined-sex analysis, and lack of consideration of lifestyle. In this study, we examined the effect of *C677T/MTHFR* on carotid atherosclerosis and blood pressure (BP) in a large genetic epidemiological study, the Suita Study.

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From the Department of Preventive Cardiology, National Cardiovascular Center (N.I., Y.K., T.M., S.B., J.O., H.T.), and Department of Geriatric Medicine, Osaka University Graduate School of Medicine (N.I., T.K., T.A., T.O.),Suita, Osaka, Japan.

This article encompasses the doctoral dissertation of Nozomu Inamoto.

Correspondence to Tomohiro Katsuya, MD, PhD, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2 No. B6, Yamada-Oka, Suita, Osaka 565-0871, Japan. E-mail katsuya@geriat.med.osaka-u.ac.jp

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Materials and Methods

Subject Population

The Suita Study was based on a random sample of 14 200 Japanese residents of Suita, a city located in the second-largest urban area in Japan, Osaka.[16] These 14 200 residents between 30 and 89 years of age were arbitrarily selected from the municipality population registry, stratified by sex and 10-year age groups. We sent these residents letters to ask if they were willing to participate in this study from 1989 with a cohort base; by February 1007, 51.7% of the subjects (n=7347) had paid an initial visit to the National Cardiovascular Center (NCVC). The participants have visited NCVC every 2 years since then for regular health checkups. In addition to routine blood examinations that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose, glycosylated hemoglobin A_{1C} (HbA_{1C}), systolic BP (SBP), and diastolic BP (DBP), DNA was extracted from an extra 5 mL blood withdrawn from those who underwent general examinations at NCVC between May 1996 and February 1998. Ninety percent of the subjects who visited NCVC during this period gave informed consent for genetic analysis of 13 genes including *MTHFR* and storage of a DNA sample and were enrolled in the present study. The study protocol of genetic analysis was approved by the ethics committee of Osaka University. Three physicians performed the carotid ultrasonic examinations. Finally, the subjects in the present study included 1693 women and 1553 men 30 to 89 years of age who attended regular health checkups and subsequently underwent ultrasonic examinations and genetic analysis.

Measurements

The subjects' BPs were measured after at least 10 minutes of rest in the sitting position. The mean value of 2 measurements of SBP or DBP obtained by a physician using a mercury sphygmomanometer (recorded >3 minutes apart) was used for the analysis. Hypertension was defined as a mean SBP of ≥160 mm Hg, a mean DBP of ≥95 mm Hg, or current use of antihypertensive medication.

The subjects were classified as current smokers or drinkers if they smoked or drank. Hypercholesterolemia was defined as serum total cholesterol levels ≥220 mg/dL or current use of antihyperlipidemic medication. Diabetes was defined as fasting plasma glucose levels ≥7.0 mmol/L (126 mg/dL) or nonfasting glucose levels ≥11.1 mmol/L (200 mg/dL), HbA1C ≥6.5%, or current use of antidiabetic medication. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Blood samples drawn from the subjects after 12 hours of fasting were collected in EDTA-containing tubes. Total cholesterol and HDL cholesterol levels were measured with an autoanalyzer (Toshiba TBA-80) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.[17] Among 3247 subjects, 1541 (820 women, 721 men) underwent measurement of fasting total plasma homocysteine levels by high-performance liquid chromatography.[18]

Carotid Ultrasound Measurements

Details of the carotid ultrasonic examination methods have been previously published.[16] We used a high-resolution B-mode ultrasonic machine with a 7.5-MHz transducer yielding an axial resolution of 0.1 mm. The regions from 30 mm proximal to the beginning of the dilation of the bifurcation bulb to 15 mm distal to the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the electronic caliper and were recorded on photocopies. IMT was measured on a longitudinal scan of the CCAs at a point 10 mm proximal to the beginning of the dilation of each carotid artery bulb. IMT was defined as the mean of the IMT of the proximal and distal walls at the point of measurement. Maximum IMT in the CCA and maximum IMT were defined as the maximum IMT in the scanned CCA area and the maximum IMT in the entire scanned area, respectively. We defined a plaque, a focal IMT thickening, as an area where IMT ≥1.1 mm and calculated plaque score by totaling the maximum thickness of all the plaques in the scanned area. Finally, we defined stenosis as a condition in which a plaque occupied more than half of the lumen circumference of an artery on a cross-sectional scan. We performed color-flow Doppler examination to confirm the presence of stenosis.

MTHFR Genotype Determination With TaqMan Polymerase Chain Reaction Method

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures with a QIAamp DNA Blood Kit (Qiangen Inc). To deal with a large number of samples, we introduced the TaqMan polymerase chain reaction (PCR) method (Applied Biosystems). In the current investigation, we prepared 2 probes: C allele–specific probe, 5′ Tet-TCT GCG GGA GcC GAT TTC ATC ATC-Tamra-3′, and T allele–specific probe, 5′-Fam-TCT GCG GGA GtC GAT TTC ATC ATC-Tamra-3′. Primer design for PCR of the flanking region of *C677T/MTHFR* was as follows: forward, 5′-GGC TGA CCT GAA GCA CTT GAA-3′; reverse, 5′-GCG GAA GAA TGT GTC ATC CT-3′. PCR was carried out with a thermal cycler (GeneAmp, PCR System 9700, Applied Biosystems). PCR was performed according to the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The fluorescence level of PCR products was measured with an ABI PRISM 7200 and 7900 Sequence Detector (Applied Biosystems), resulting in clear identification of the 3 genotypes of *C677T/MTHFR*.

Statistical Analysis

The number of subjects was restricted to 3247 who had complete data, including *C677T/MTHFR* and carotid ultrasonographic measurements. 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.

Associations of *C677T/MTHFR* with BP were investigated by sex through logistic regression analysis considering potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. The genotype effect was examined according to a dominant (*TT*+*CT* versus *CC*) and a recessive (*TT* versus *CT*+*CC*) model. For multivariate risk predictors, the adjusted odds ratios (ORs) were given with the 95% confidence intervals (CIs). The relationships in men and women between *C677T/MTHFR* and hypertensive risk were expressed in terms of ORs adjusted for possible confounding effects. The association of *C677T/MTHFR* with carotid atherosclerotic index was also investigated by sex through logistic regression analysis considering potential confounding risk variables. Partial correlation coefficients between plasma total homocysteine and carotid atherosclerotic indexes by sex and *C677T/MTHFR* were determined. In addition, gene and environmental interactions were calculated with the following logistic regression model: logit $p=\beta_0+\beta_g x_g+\beta_c x_c+\beta_{gc} x_g x_c$, where x_g and x_c are genetic and environmental data, respectively; β_0 is an intercept term; β_g is the main effect due to genes; and β_c is the main effect of the environment. The coefficient β_{gc} of the product $x_g x_c$ estimates the gene and environmental interaction on the logit scale.[19] All analyses were performed with SAS statistical software (release 6.12, SAS Institute Inc).

Results

Basic Characteristics of Subjects in the Suita Study

As shown in Table 1, age, SBP, DBP, BMI, total cholesterol, HDL cholesterol, IMT, maximum IMT in CCA, plaque score, CCA stenosis (≥50%), percentage of current smokers, percentage of current drinkers, prevalence of hypertension, prevalence of diabetes mellitus, and total plasma homocysteine levels were significantly higher in men than in women.

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

	Women (n=1693)	Men (n=1554)
Age, y	58.2±12.2	60.4±12.8*
SBP, mm Hg	126.7±21.1	129.5±19.3*
DBP, mm Hg	78.0±11.0	80.7±11.0*
BMI, kg/m^2	22.3±3.2	23.0±2.8*
Total cholesterol, mmol/L	5.6±0.9	5.2±0.8*
HDL cholesterol, mmol/L	1.6±0.4	1.4±0.4*
IMT, mm	0.83±0.12	0.88±0.14*
Maximum IMT in CCA, mm	1.02±0.29	1.15±0.45*
Plaque score, mm	2.14±2.99	4.13±4.69*
Stenosis (≥50%), %	1.0	4.6†
Current smokers, %	8.0	39.6†
Current drinkers, %	28.2	70.6†
Present illness		
Hypertension	21.3	25.6†
Hyperlipidemia	48.0	30.2†
Diabetes mellitus	3.3	7.9†
Myocardial infarction	0.5	1.7†
Ischemic stroke	0.8	2.5†
Total plasma homocysteine, μmol/L	10.7±3.0 (n=820)	13.3±4.2* (n=721)

Values are mean±SD or percentage.

Hypertension indicates SBP ≥160 mm Hg and/or DBP ≥95 mm Hg 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), nonfasting plasma glucose ≥11.1 mmol/L (200 mg/dL), or antidiabetic medication.

*P<0.05 between female and male by Student's t test.

†P<0.05 between women and men by χ^2 test.

Only the frequency of hyperlipidemia was significantly higher in women than in men.

C677T/MTHFR, Hypertension, and Plasma Homocysteine Levels

The frequencies of *C677T/MTHFR* in women were 37.5% for *CC*, 47.2% for *CT*, and 15.3% for *TT* genotypes, whereas those in men were 36.2% for *CC*, 47.8% for *CT*, and 16.0% for *TT* genotypes. There was no significant difference in allele frequencies between age groups (χ^2=1.07, df=2, P=0.59). The genotype distribution of *C677T/MTHFR* was not significantly deviated from Hardy-Weinberg's expectation in men or women. In women, SBP and DBP increased according to the number of *T677* alleles of *MTHFR*, but the association was not statistically significant. Only DBP in *TT* women was significantly higher in those with the *C677* allele after age adjustment. In the recessive model (*CT*+*CC* versus *TT*), however, *C677T/MTHFR* was significantly associated with the prevalence of hypertension, and the all adjusted OR for hypertension was 1.42 (95% CI, 1.01 to 1.99) in women (Table 2).

Figure 1 shows plasma total homocysteine levels according to genotype of *C677T/MTHFR* in men and women. Mean plasma total homocysteine levels in subjects with the *TT* genotype was significantly higher than that in subjects with the *CC* or *CT* genotype.

TABLE 2. ORs of Presence of Hypertension in Men and Women by *C677T/MTHFR*

	Dominant Model		Recessive Model	
	CC	*CT*+*TT*	*CC*+*CT*	*TT*
Women (n=1693)				
Hypertensive, %	19.5	22.4	20.7	24.7
All adjusted OR*	1	1.15 (0.88–1.49)	1	1.42 (1.01–1.99)†
Men (n=1554)				
Hypertensive, %	25.9	25.3	25.5	25.7
All adjusted OR*	1	0.93 (0.73–1.20)	1	1.00 (0.72–1.40)

*Conditional logistic analysis, adjusted for age, BMI, SBP, smoking, drinking, antihypertensive drug use, hypercholesterolemia, and diabetes.

†P<0.05 vs *CC* or *CC*+*CT* subjects.

Carotid Atherosclerotic Index and *C677T/MTHFR*

Carotid atherosclerotic indexes (IMT, maximum IMT in CCA, maximum IMT, and plaque score) were evaluated in men and women separately, according to *C677T/MTHFR* genotype (Table 3). In women with the *CT* genotype, age-adjusted IMT, maximum IMT in CCA, and all adjusted maximum IMT in CCA were significantly thicker than in those with the *CC* genotype. However, there was no difference between subjects with the *TT* and *CC* genotypes in any atherosclerotic indexes.

In contrast, *C677T/MTHFR* gave a significantly increased risk for stenosis (>50%) of CCA in women. In a recessive model (*CC*+CT versus *TT*), the all adjusted OR for stenosis (>50%) was 3.42 (95% CI, 1.23 to 9.53) in women and 1.41 (95% CI, 0.76 to 2.63) in men.

Partial correlation coefficients between plasma total homocysteine levels and carotid atherosclerotic index by *C677T/MTHFR* genotype are shown in Table 4. Positive relationships were found between plasma total homocysteine levels and IMT in men with the *CC* genotype and maximum IMT in CCA for men. These associations were stronger in men than in women.

Interaction Between *C677T/MTHFR* and Lifestyle on Carotid Atherosclerotic Index According to Sex

Figure 2 shows the association of IMT and maximum IMT in CCA with *C677T/MTHFR* according to smoking and drink-

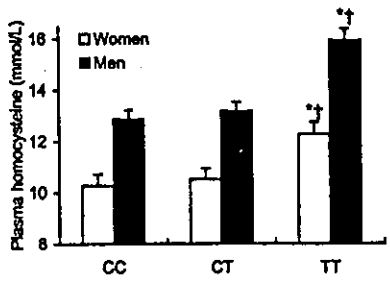

Figure 1. Plasma total homocysteine levels according to *C677T/MTHFR* by sex. Values are least-square mean±SE adjusted for age, BMI, smoking, drinking, antihypertensive drug use, hyperlipidemia, and diabetes. Bars indicate SE. *P<0.0001 vs CC subjects; †P<0.0001 vs CT subjects.

TABLE 3. Carotid Atherosclerotic Index in Men and Women by *C677T/MTHFR*

	MTHFR Genotype			
	CC	*CT*	*TT*	χ^2 *P*
Women (n=1693)				
IMT, mm				
Age adjusted	0.825±0.004	0.842±0.003‡	0.832±0.006	0.004
All adjusted*	0.861±0.009	0.874±0.009‡	0.866±0.010	0.030
Maximum IMT in CCA, mm				
Age adjusted	1.004±0.011	1.035±0.010†	1.023±0.018	0.122
All adjusted	1.075±0.026	1.100±0.025	1.094±0.029	0.231
Maximum IMT, mm				
Age adjusted	1.274±0.021	1.311±0.018	1.307±0.032	0.383
All adjusted	1.415±0.050	1.441±0.049	1.444±0.056	0.586
Plaque score, mm				
Age adjusted	1.990±0.108	2.259±0.096	2.141±0.169	0.178
All adjusted	2.915±0.262	3.114±0.254	3.026±0.293	0.369
Men (n=1554)				
IMT, mm				
Age adjusted	0.886±0.005	0.882±0.004	0.889±0.007	0.668
All adjusted	0.892±0.007	0.890±0.006	0.898±0.009	0.586
Maximum IMT in CCA, mm				
Age adjusted	1.162±0.020	1.140±0.017	1.144±0.030	0.713
All adjusted	1.173±0.027	1.163±0.026	1.165±0.036	0.916
Maximum IMT, mm				
Age adjusted	1.642±0.034	1.627±0.030	1.721±0.051	0.273
All adjusted	1.653±0.047	1.638±0.044	1.75±0.060	0.145
Plaque score, mm				
Age adjusted	4.201±0.178	4.010±0.155	4.308±0.268	0.550
All adjusted	4.413±0.246	4.215±0.232	4.625±0.316	0.363

*Values are least-square mean±SE adjusted for age, SBP, BMI, smoking, drinking, and medication (for hypertension, hyperlipidemia, or diabetes).
†$P<0.05$ vs *CC* subjects; ‡$P<0.005$ vs *CC* subjects.

ing status. In women with the *CC* or *CT* genotype, IMT in smokers was significantly higher than in nonsmokers. In women with the *TT* genotype, maximum IMT in CCA in smokers and drinkers was significantly higher than that in nonsmokers and nondrinkers, respectively (Figure 2–A2, $P<0.05$ for interaction; Figure 2–B2). In men with the *CC* or *TT* genotype, IMT and maximum IMT in CCA were significantly higher in smokers than in nonsmokers (Figure 2–A1).

TABLE 4. Partial Correlation Coefficient Between Plasma Total Homocysteine and Carotid Atherosclerotic Index by Sex and *C677T/MTHFR*

	CC	*CT*	*TT*
IMT			
Women	0.056 (0.334)	0.014 (0.784)	−0.027 (0.758)
Men	0.167 (0.001)	0.056 (0.300)	0.111 (0.253)
Maximum IMT in CCA			
Women	0.058 (0.398)	0.005 (0.935)	−0.098 (0.355)
Men	0.218 (0.003)	0.146 (0.016)	0.363 (0.002)

Figures in parentheses indicate *P* value adjusted for age, BMI, drinking, smoking, SBP, and medication for hypertension, hyperlidemia, and diabetes mellitus.

Discussion

The present study showed that the *TT* genotype of *C677T/MTHFR* was significantly associated with the prevalence of hypertension (OR, 1.15) and carotid stenosis (<50%) in women but not in men. In addition, the specific genotype of *C677T/MTHFR* affected maximum IMT in CCA in the interaction with smoking in women. These results show an association of *C677T/MTHFR* with BP and carotid atherosclerosis on the basis of gene and environmental interaction, which has not been previously reported.

Although previous studies showed that subjects with the *TT* genotype of *C677T/MTHFR* are associated with an increased risk of cardiovascular disease via an increase in plasma homocysteine levels,[2,6,20] the conclusion is still controversial.[7,14,15,21,22] The inconsistencies may be attributed to small sample size, combined-sex analysis, and no inclusion of lifestyle factors such as smoking and drinking. One should be aware that detecting gene and environmental interactions

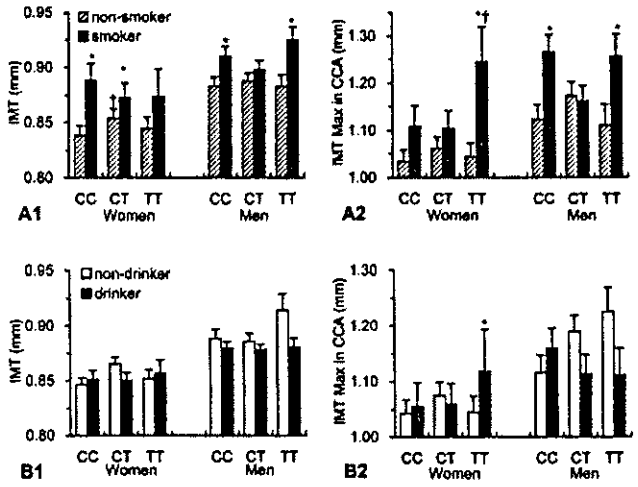

Figure 2. Association between *C677T/MTHFR* and carotid atherosclerotic indexes (IMT and maximum IMT in CCA) according to smoking (A) and drinking (B) status in men and women. Data are shown as the least-square mean±SE adjusted for age, BMI, SBP, smoking, drinking, and medication (for hypertension, hyperlipidemia, and diabetes). *$P<0.05$ vs nonsmokers (or nondrinkers) in subjects with the same genotype; †$P<0.05$ vs *CC* subjects with the same lifestyle (for smoking and drinking).

could require a substantially larger sample size than the sample size necessary for detecting genetic or environmental effects alone.[23] Thus, we examined the effect of *C677T/MTHFR* in a large general population with various phenotypes that included plasma homocysteine levels, atheromatous indexes, smoking and drinking status, and relevant basic characteristics.

It can be questioned why the *TT* genotype of *C677T/MTHFR* is not unequivocally associated with increased cardiovascular risk,[5] based on the argument that the gene is a strong predictor of hyperhomocysteinemia in general populations.[6,24,25] It could be attributed to the close relationship between plasma homocysteine levels and folate metabolism. Several reports revealed that plasma total homocysteine levels become elevated only in folate-deficient subjects with the *TT* genotype[7,25,26] and that the slope of regression lines relating total homocysteine to folate increases in the order of *CC*, *CT*, and *TT* genotypes.[15,24] In other words, if folate intake is sufficient, subjects with the *TT* genotype would not have increased risk of cardiovascular disease via hyperhomocysteinemia.

Under stratification by sex, we observed that the *TT* genotype was independently associated with DBP and carotid stenosis in women and showed a greater disadvantage in female smokers and drinkers. Even though homocysteine would injure the endothelium of small arteries at an early stage[27] and endothelial dysfunction plays a critical role in the early events of atherosclerosis,[28] we currently have no definitive answer to explain the results. However, it seems to be an important finding that most of the positive results in the present study were obtained only in women. As supporting data of our results, a female-specific significant association with the *TT* genotype was also reported in the predisposition to ischemic stroke[29] and asymptomatic carotid atherosclerosis.[30] Motti et al[31] reported that sex differentiation is independently associated with homocysteine. Plasma homocysteine levels are significantly higher in healthy men than in women, which is consistent with our results (Table 1). In addition, homocysteine levels are reported to be lower in premenopausal women than in men and postmenopausal women. Furthermore, a recent report suggested that total homocysteine levels were significantly correlated with fat-free mass and testosterone and inversely with estradiol. The sex difference with regard to total homocysteine levels was explained primarily by differences in fat-free mass but also by estradiol concentration. Those results might be a feasible explanation for the lack of association in men.[32] However, there was no association between *C677T/MTHFR* and carotid atherosclerosis in premenopausal and postmenopausal women (data not shown). This result suggests that estrogen might have a protective effect against homocysteinemia but not atherosclerosis via *C677T/MTHFR*. Indeed, previous reports did not find such a specific advantage in the relationship between *C677T/MTHFR* and coronary artery disease in young woman in a small Caucasian population.[33,34]

Disadvantages of our study design were that only half of the subjects had their total plasma homocysteine levels analyzed. This is not a serious limitation, however, because the association between *C677T/MTHFR* and plasma homocysteine levels has already been demonstrated in several large studies.[7,26] Another disadvantage was that we had no data on the physical activity and nutrition of the subjects, but these data were also supported by previous studies. The dietary intake of folate, vitamin B_6, and B_{12} is inversely (negatively) correlated with plasma homocysteine[35,36]; physical activity is also inversely associated with plasma homocysteine.[37] There is a need for additional prospective studies with data on relevant confounders that have sufficient power to examine the association between homocysteine concentration and stroke risk, whether linear or threshold, and to study interactions between homocysteine, other dietary markers, and established stroke risk factors such as smoking and hypertension. Similarly, the evidence linking hyperhomocysteinemia with hypertension is limited and inconsistent. Ultimately, the case for a causal role of elevated homocysteine levels in vascular disease, including hypertension and stroke, will depend on data from randomly controlled trials of homocysteine-lowering interventions.

In summary, the present study shows that the homozygous *T677* allele of *C677T/MTHFR* is a risk factor for hypertension and carotid stenosis in women. In addition, smoking increased IMT in CCA in women with the *TT* genotype. In the near future, physicians might use the genotypic data of *C677T/MTHFR* to modify their patients' lifestyles to prevent cardiovascular disease.

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Letter to the Editor

Association analysis between polymorphisms of the lymphotoxin-α gene and myocardial infarction in a Japanese population

Recently, a genome-wide association study revealed that variants in the lymphotoxin-α gene (*LTA*) are risk factors for myocardial infarction (MI), based on the multiplex PCR-Invader assay method at 92788 randomly selected gene-based SNPs [1]. It has also been shown that, in in vitro functional analyses, these variants might have some functional significance and that *LTA* may play a role in the pathogenesis of this disorder. However, association studies are plagued by the impression that they are not consistently reproducible [2,3]. Moreover, direct evidence of the contribution of *LTA* to atherogenesis is limited in both animals and humans [4]. Therefore, we performed an association analysis between polymorphisms of *LTA* and MI in a Japanese population.

Four hundred and seventy-seven male patients with MI (<70 years old) were recruited from the National Cardiovascular Center. The mean age was 56±8 years, with a range of 25–70 years. The control group consisted of 372 unrelated Japanese males <70 years old (mean age 59±9 years, range 30–70 years) recruited from the Suita study, which represents the general population in central Japan (Osaka) [5]. From the control group we excluded all subjects with a history of vascular diseases. Genomic DNA was isolated from leukocytes according to standard procedures. Polymorphisms were determined using the TaqMan system (PE Applied Biosystems). Three polymorphisms of *LTA*, *G10A* (exon1), *A252G* (intron1), and *C804A* (exon3), and one polymorphism of nuclear factor of κ light polypeptide gene enhancer in B cells, inhibitor-like 1 (*NFKBIL1*), and *T-63A* (promoter) were genotyped. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc., USA).

The pattern of the frequency distribution of the genotypes is summarized in Table 1. These polymorphisms were almost completely concordant (i.e., the same allele frequencies and almost complete positive linkage disequilibrium). No significant deviation from Hardy–Weinberg equilibrium was observed. The −63A allele in *NFKBIL1* and the 10A, 252G, and A804 alleles in *LTA* were more common in patients than in controls. For example, in *LTA G10A*, a multiple logistic regression analysis, while adjusting for age and the prevalence

Table 1
Distribution of LTA genotypes in MI patients and controls

SNPs			Controls (n = 372)	MI patients (n = 477)	P
NFKBIL1 (A-63T)	Genotype	TT	166 (44.6%)	160 (33.6%)	0.004
		TA	157 (42.2%)	236 (49.6%)	
		AA	49 (13.2%)	80 (16.8%)	
	Allele frequency	T	0.66	0.58	0.002
		A	0.34	0.42	
LTA (G10A)	Genotype	GG	166 (44.7%)	160 (33.5%)	0.004
		GA	156 (42.1%)	235 (49.3%)	
		AA	49 (13.2%)	82 (17.2%)	
	Allele frequency	G	0.66	0.58	0.001
		A	0.34	0.42	
LTA (A252G)	Genotype	AA	163 (44.9%)	159 (33.4%)	0.003
		AG	153 (42.2%)	236 (49.6%)	
		GG	47 (13.0%)	81 (17.0%)	
	Allele frequency	A	0.66	0.58	0.001
		G	0.34	0.42	
LTA (A804C)	Genotype	CC	164 (44.8%)	161 (33.7%)	0.004
		CA	153 (41.8%)	236 (49.4%)	
		AA	49 (13.4%)	81 (17.0%)	
	Allele frequency	C	0.66	0.58	0.002
		A	0.34	0.42	

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of smoking, diabetes mellitus and hypercholesterolemia, revealed that the frequency of the A allele was significantly higher in patients with MI than in controls. An analysis which assumed that the A allele had dominant effects showed a significant association ($AA + AG$ versus GG: $P = 0.0025$, odds ratio 1.7, 95% CI 1.2–2.3). Although Ozaki et al. reported a significant association between the risk of MI and these polymorphisms, the distribution of genotypes was different (for example, in *LTA G10A*, GG/GA/AA (%): 39.4/49.1/11.5 in Control versus 36.7/44.5/18.8 in MI) and as a result, a recessive association model was assumed [1]. It is well known that one of the weaknesses of a case–control study is the selection of the control subjects, and this might explain the difference between the two studies [6].

Although the precise in vivo mechanism by which *LTA* influences the susceptibility to MI is unknown, the present study supports the notion that this gene is one of the most important genetic determinants of susceptibility to MI that has been detected so far.

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Yoshitaka Iwanaga
Koh Ono
Shuichi Takagi
Masahiro Terashima
Yoshiaki Tsutsumi
Toshifumi Mannami
Naomi Yasui
Yoichi Goto
Hiroshi Nonogi
Naoharu Iwai*
National Cardiovascular Center, Suita, Japan
*Corresponding author. *National Cardiovascular Center*
5-7-1 Fujishirodai Suita, Osaka 565-8565, Japan
Tel.: +81-6-6833-5012; fax: +81-6-6872-8091
E-mail address: niwai@res.ncvc.go.jp (N. Iwai)

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An association analysis between genetic polymorphisms of matrix metalloproteinase-3 and methylenetetrahydrofolate reductase and myocardial infarction in Japanese

K. SHIOJI,*§ Y. KOKUBO,† Y. GOTO,‡ H. NONOGI‡ and N. IWAI*

**Department of Epidemiology, Research Institute, National Cardiovascular Center, †Department of Preventive Cardiology and ‡Division of Cardiology, National Cardiovascular Center; and §The Organization for Pharmaceutical Safety and Research of Japan, Osaka, Japan*

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Matrix metalloproteinases (MMPs), enzymes that degrade extracellular matrix, have been extensively found in human coronary atherosclerotic plaques, which suggests that MMPs play an important role in plaque instability [1]. Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in regulating the plasma homocysteine level, and hyperhomocysteinemia confers an increased risk of coronary artery disease [2]. Polymorphisms of stromelysin-1 (*MMP3*) and *MTHFR* have been reported to be related to an increased risk of myocardial infarction (MI), but the results have been controversial [3–7]. To assess whether these polymorphisms are associated with the incidence of MI, we conducted an association study.

The study population consisted of two groups: (i) 1857 (849 male and 1008 female) controls consecutively recruited from the Suita Study between April 2002 and February 2003 [8,9], and (ii) 548 (474 male and 74 female) patients with MI recruited from the National Cardiovascular Center between May 2001 and April 2003 [10]. The *MMP3* 5A/6A and *MTHFR* C677T polymorphisms were determined by the TaqMan system (the primer and probe sequences are available on request).

Univariate analysis showed that *MMP3* 5A/6A was not associated with the incidence of MI (Table 1). Logistic analysis indicated that the 5A/5A + 5A/6A genotype of *MMP3* only tended to be more susceptible to MI than the 6A/6A genotype [$P = 0.1004$, odds ratio (OR) = 1.23, 95% confidence interval (CI) 0.96, 1.59] in male subjects. *MTHFR* C677T was not associated with the incidence of MI (Table 1). Logistic analysis indicated that the CC genotype of *MTHFR* only tended to be more susceptible to MI than the CT + TT genotype ($P = 0.0911$, OR = 1.52, 95% CI 0.93, 2.48) in female subjects. None of the genotypes significantly influenced the secondary incidence of acute coronary syndrome (Kaplan–Meier method). Moreover, none of the genotypes significantly influenced the severity of coronary atherosclerosis as assessed by the number of stenotic lesions (>75%) by coronary arteriography. No significant deviation from Hardy–Weinberg equilibrium was observed for *MMP3* 5A/6A or *MTHFR* C677T.

It has been reported that individuals carrying the 6A/6A genotype of *MMP3* are predisposed to developing atherosclerotic plaques with significant stenosis, whereas those carrying the 5A allele are predisposed to developing unstable plaque [4]. In the Japanese population, *MMP-3* 5A/6A was initially

Table 1 Characteristics of subjects and the genotype frequencies in the present study

	Control	MI	*P*-value
Male			
N	849	474	
Age	66.2 ± 0.4	58.3 ± 0.5	<0.0001
Body mass index (kg m^{-2})	23.2 ± 0.1	23.7 ± 0.1	0.0044
MMP3 5A/6A			
5A5A/5A6A/6A6A	18/200/619	13/127/322	0.2474
	2.2%/23.9%/74.0%	2.8%/27.5%/69.7%	
MTHFR C677T			
CC/CT/TT	293/411/141	160/226/75	0.9799
	34.7%/48.6%/16.7%	34.7%/49.0%/16.3%	
Female			
N	1008	74	
Age	63.3 ± 0.3	62.8 ± 1.3	0.7238
Body mass index (kg m^{-2})	22.3 ± 0.1	23.5 ± 0.4	0.0011
MMP3 5A/6A			
5A5A/5A6A/6A6A	16/226/755	1/22/47	0.2677
	1.6%/22.7%/75.7%	1.4%/31.4%/67.1%	
MTHFR C677T			
CC/CT/TT	370/467/164	33/24/13	0.1202
	37.0%/46.7%/16.4%*	47.1%/34.3%/18.6%	

MMP3, Matrix metalloproteinase-3; MTHFR, methylenetetrahydrofolate reductase; control, control subjects; MI, myocardial infarction. Differences in numerical data among the groups were evaluated by an unpaired *t*-test. The genotype distributions in the groups were compared by the χ^2 test. *Due to rounding, the percentages may not total 100.

Correspondence: Naoharu Iwai, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai Suita, Osaka 565-8565, Japan.
Tel.: +81 6 6833 5012; fax: +81 6 6872 2088; e-mail: niwai@res.ncvc.go.jp

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reported to be associated with the incidence of MI [3]. However, a second report showed that this association was valid in females, but not in males [5]. We also did not observe any positive association in our male subjects. Thus, in Japanese male subjects, *MMP-3* 5A/6A does not seem to predict the incidence of MI. In females, it is possible that we did not observe any positive association, at least partially, due to the relatively small number of female patients with MI, and further investigations may be needed.

Several association studies and meta-analyses have investigated the association between the *MTHFR* gene and an increased risk of MI [6]. In the Japanese population, Yamada *et al.* did not find an association between *MTHFR* C677T and the incidence of MI [5]. Considering these and our present results, it is unlikely that *MTHFR* C677T is associated with an increased risk of MI in Japanese.

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Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese

T. SAKATA, A. OKAMOTO, T. MANNAMI,* H. MATSUO† and T. MIYATA‡

*Laboratory of Clinical Chemistry, *Department of Preventive Cardiology, †Department of Cardiology and ‡Research Institute, National Cardiovascular Center, Fujishirodai, Suita, Osaka, Japan*

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Correspondence: T. Sakata, Laboratory of Clinical Chemistry, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.
Tel.: +81 6 6833 5012 (ext 2296); fax: +81 6 6835 1176; e-mail: tsakata@hsp.ncvc.go.jp

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The frequency of factor (F)V Leiden mutation is relatively high among individuals of Caucasian descent, being from 2 to 15% in the general population and up to 50% in selected patients with thromboembolism [1]. The risk of the first episodes of thromboembolism as estimated in a large case–control study is 7-fold for heterozygous FV Leiden carriers [2]. Although the frequency of deficiencies of natural anticoagulants, protein C or antithrombin in the general population is low, prospective studies indicate that low levels of protein C and antithrombin

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SHORT COMMUNICATION

Keisuke Shioji · Toshifumi Mannami
Yoshihiro Kokubo · Nozomu Inamoto · Shuichi Takagi
Yoichi Goto · Hiroshi Nonogi · Naoharu Iwai

Genetic variants in *PCSK9* affect the cholesterol level in Japanese

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Abstract Mutations in the proprotein convertase subtilisin/kexin 9 (*PCSK9*) gene have been reported in affected members of two families with autosomal dominant hypercholesterolemia. To investigate the effects of common variants in *PCSK9* on the cholesterol level, we conducted an association study using a large cohort representing the general population in Japan ($n = 1,793$). Direct sequencing in all of the exonic regions identified 21 polymorphisms. After consideration of linkage disequilibrium among these polymorphisms, we selected and genotyped nine polymorphisms by the TaqMan method. The intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with levels of total cholesterol (TC) [C(-161)T, $P = 0.0285$; I474 V, $P = 0.0069$] and low-density lipoprotein cholesterol (LDL-C) [C(-161)T, $P = 0.0257$; I474 V, $P = 0.0007$]. The distributions of these polymorphisms in subjects with miocardial infarction (MI) ($n = 649$) were not different from those in the control population. These results provide the first evidence that common variants intron 1/C(-161)T and exon 9/I474 V in *PCSK9* significantly affect TC and LDL-C levels in the general population in Japan.

Keywords *PCSK9* · Cholesterol · Myocardial infarction · Polymorphisms · Association study

K. Shioji · N. Iwai (✉)
Department of Epidemiology,
Research Institute,
National Cardiovascular Center,
5-7-1 Fujishirodai, Suita,
Osaka 565-8565, Japan
E-mail: niwai@res.ncvc.go.jp
Tel.: +81-6-68335012
Fax: +81-6-68722088

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

T. Mannami · Y. Kokubo · N. Inamoto
Department of Preventive Cardiology,
National Cardiovascular Center,
Osaka, Japan

S. Takagi · Y. Goto · H. Nonogi
Division of Cardiology,
National Cardiovascular Center,
Osaka, Japan

Introduction

Proprotein convertase subtilisin/kexin 9 (*PCSK9*) in chromosome 1p34.1-p32 is a proprotein convertase that belongs to the subtilase subfamily (Seidah et al. 2003). A related protein is the subtilisin/kexin isoenzyme-1/site-1 protease, which plays a key role in cholesterol homeostasis by processing sterol regulatory element-binding protein (SREBP) (Brown and Goldstein 1999). The expression of PCSK9 mRNA has been reported to be down regulated by dietary cholesterol in C57BL/6 mice and to be up regulated in SREBP transgenic mice (Maxwell et al. 2003). Mutations in *PCSK9* have been reported in affected members of two families with autosomal dominant hypercholesterolemia (OMIM 603776) (Abifadel et al. 2003). These observations indicate that *PCSK9* plays an important role in cholesterol metabolism. Thus, it is possible that common genetic variations in *PCSK9* might affect the cholesterol level in the general population.

To investigate the effects of common variants in *PCSK9* on cholesterol level, we detected common variants in *PCSK9* by sequencing and conducted an association study using a large cohort representing the general population in Japan. We found that two polymorphisms, intron 1/C(-161)T and exon 9/I474V, were associated with levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). We next investigated the association between these polymorphisms and the incidence of myocardial infarction (MI).

Subjects and Methods

Subjects

1. The Suita population: Selection criteria and design of the Suita Study have been described previously (Shioji et al. 2004, in press; Mannami et al. 1997). The sample consisted of 14,200 men and women aged 30–79 years, stratified by gender and 10-year age groups, who were selected randomly from the municipal population registry. They were all invited by letter to attend regular cycles of follow-up examinations (every 2 years). The basic population sampling started in 1989 with a cohort study base, and 51.7% ($n = 7,347$) of the subjects responded to the invitation letter and had paid their initial visit to the National Cardiovascular Center by February 1997. The participants visited the center every 2 years for regular health checkups. DNA from leukocytes was initially collected from participants who visited the center between May 1996 and February 1998. In the present study, the genotypes were determined in 1,880 consecutive subjects who visited the center between April 2002 and February 2003 ($n = 1,880$, Table 1). Subjects with ischemic heart disease were excluded.
2. The MI group: Selection criteria and design of the MI group have been described previously (Takagi et al. 2002). This group consisted of 649 patients with MI (553 men and 96 women) who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003 (Table 2).

Written informed consent was obtained from each subject after a full explanation of the study, which was approved by the Ethics Committee and the Committee on Genetic Analysis and Genetic Therapy of the National Cardiovascular Center.

Table 1 Suita population characteristics. *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PR* pulse rate, *% CVA* percentage of subjects with cerebrovascular accident, *% OMI* percentage of subjects with old myocardial infarction, *% HT* percentage of subjects with hypertension, *% DM* percentage of subjects with diabetes mellitus, *% HLP* percentage of subjects with hyperlipidemia, *% drinking* percentage of subjects with a drinking habit, *% smoking* percentage of subjects with a smoking habit

Parameter	Men	Women	*P* value
Number	867	1013	
Age (years)	66.3 ± 0.4	63.3 ± 0.3	< 0.0001
BMI (kg/m^2)	23.2 ± 0.1	22.3 ± 0.1	< 0.0001
SBP (mmHg)	131.8 ± 0.7	128.1 ± 0.6	< 0.0001
DBP (mmHg)	79.7 ± 0.3	76.6 ± 0.3	< 0.0001
PR (beats/min)	66.0 ± 0.3	66.0 ± 0.3	0.9334
Total cholesterol (mmol/l)	5.13 ± 0.03	5.58 ± 0.02	< 0.0001
HDL cholesterol (mmol/l)	1.43 ± 0.01	1.68 ± 0.01	< 0.0001
Triglycerides (mmol/l)	1.38 ± 0.03	1.07 ± 0.03	< 0.0001
Blood glucose (mmol/l)	5.74 ± 0.04	5.30 ± 0.04	< 0.0001
% CVA	3.6	1.4	0.0018
% OMI	2.1	0.5	0.0015
% HT	45.9	37.2	< 0.0001
% DM	11.4	4.5	< 0.0001
% HLP	14.8	24.0	< 0.0001
% drinking	67.0	29.5	< 0.0001
% smoking	29.9	6.3	< 0.0001

P value was calculated by the unpaired *t*-test

Table 2 Miocardial infarction (MI) group characteristics. *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PR* pulse rate, *% CVA* percentage of subjects with cerebrovascular accident, *% OMI* percentage of subjects with old myocardial infarction, *% HT* percentage of subjects with hypertension, *% DM* percentage of subjects with diabetes mellitus, *% LP* percentage of subjects with hyperlipidemia

Parameter	Men	Women	*P* value
Number	553	96	
Age (years)	61.3 ± 0.5	64.8 ± 1.1	0.0028
BMI (kg/m^2)	23.7 ± 0.1	23.6 ± 0.3	0.7056
Total cholesterol (mmol/l)	5.17 ± 0.05	5.43 ± 0.11	0.0400
HDL cholesterol (mmol/l)	1.08 ± 0.02	1.23 ± 0.04	0.0006
Triglycerides (mmol/l)	1.55 ± 0.04	1.21 ± 0.09	0.0010
Blood glucose (mmol/l)	7.45 ± 0.67	6.75 ± 1.59	0.6832
% HT	53.5	61.5	0.1448
% DM	41.7	58.1	0.0034
% HLP	57.9	58.3	0.9402

P value was calculated by the unpaired *t*-test

DNA studies

All 12 exonic regions were sequenced for polymorphisms in 48 healthy subjects. Selected polymorphisms were determined by the TaqMan method. Detailed information will be provided upon request.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Since the distribution of triglyceride (TG) values was skewed, a logarithmic transformation was used for the statistical test; however, untransformed means are shown in Tables 1, 2, 5, 6. LDL-C was calculated by Friedewald's formula [(LDL-C) = (TC) - (HDL-cholesterol) - (TG/5). We excluded those whose HDL-cholesterol (HDL-C) or TG levels were ≥2.6 mM or 4.53 mM respectively]. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc.). Values of $P < 0.05$ were considered to indicate statistical significance. The residuals of lipid levels were calculated by adjusting for gender, age, body mass index (BMI), smoking (cigarettes/day), and consumption of alcohol (ethanol g/week). Data were analyzed using a contingency table analysis and Student's *t*-test. Hardy-Weinberg equilibrium was calculated by a chi-square test. *R*-square values between polymorphisms were analyzed using the SNPAlyze statistical package (Dynacom Inc.).

Results

Direct sequencing identified 21 polymorphisms (Table 3). We regarded $r^2 0.5$ as tight linkage (Table 4). Polymorphisms with frequencies of ≤ 0.03 in the intronic region and 3'-untranslated region were neglected in further analyses. Polymorphisms that were not accompanied by an amino acid change in the exonic regions were also neglected. Accordingly, we selected and genotyped nine polymorphisms for the following association study.

As shown in Table 5, intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with levels of

Table 3 Polymorphisms and nucleotide sequence in *PCSK9*

Region	Polymorphism	Allele frequency	Sequence
Exon 1	C(-64)A (5'-UTR)	0.13	CCCACCGCAAGGCTCAAGGCGCCGC[C/A]GGCGTGGACCGCGCACGGCCTCTAG
	V4I	0.01	CTCTCCCCTGGCCCTCATGGGCACC[G/A]TCAGCTCCAGGCGGTCCTGGTGGCC
	15–16 ins (+L)	0.13	GCGGTCCTGGTGGCCGCTGCCACTG[CTG/-]CTGCTGCTGCTGCTGCTGCTCCTGG
	A53V	0.13	TTGCGTTCCGAGGAGGACGGCCTGG[C/T]CGAAGCACCCGAGCACGGAACCACA
Intron 1	**C(-161)T**	0.04	TAATAATAGTTGGCCTATATGAGTT[C/T]TTTAATTTGCTTTTTGGTCCGCATT
Exon 2	L112L	0.05	GCCGGGGATACCTCACCAAGATCCT[G/A]CATGTCTTCCATGGCCTTCTTCCTG
Intron 2	**T357C**	0.13	GCACAGTAACTACTGGCTTTCTGTA[T/C]AGAATTCCCTTTAAGCCTGGCCATG
Intron 3	G(-10)A	0.04	CATTCCCTCCTCTCCCACAAATGTC[G/A]CCTTGGAAAGACGGAGGCAGCCTGG
Intron 4	**G-36A**	0.05	CTGATTTGTTATAGGGTGGAGGGGG[G/A]GTCTTTCTCATGTGGTCCTTGTGTT
Exon 6	Q275Q	0.01	GCCTGGAGTTTATTCGGAAAAGCCA[G/A]CTGGTCCAGCCTGTGGGGCCACTGG
	P331P	0.01	GCCTCTACTCCCCAGCCTCAGCTCC[C/T]GAGGTAGGTGCTGGGGCTGCTGCCC
Exon 8	**I424V**	0.01	GATCCACTTCTCTGCCAAAGATGTC[A/G]TCAATGAGGCCTGGTTCCCTGAGGA
Intron 8	T276C	0.03	TCCCTTGTCTGTGTAAGGAGGATGA[T/C]GCCACCTTAAATAGGATTAAATGAG
	T(-57)C	0.03	CTCTCCTACCATGAACTAAAGATTT[T/C]TGTGGAGGTCCCCTCACTCCCAGCA
Exon 9	V460V	0.03	GTTGGCAGCTGTTTTGCAGGACTGT[G/A]TGGTCAGCACACTCGGGGCCTACAC
	I474V	0.03	GGGGCCTACACGGATGGCCACAGCC[A/G]TCGCCCGCTGCGCCCCAGATGAGGA
Intron 10	**A241G**	0.11	CTTTCTCCTTATGCACCCACTGCCC[G/A]CGAGGCTTGGTCCTCACAAGTGTGA
Exon 12	G67A (3'-UTR)	0.02	CAGTGCCCTCCCTGGGACCTCCCAC[G/A]TCCTGGGGGCCTACGCCGTAGACAA
	C291T (3'-UTR)	0.03	AGCTTTAAAATGGTTCCGACTTGTC[C/T]CTCTCTCAGCCCTCCATGGCCTGGC
	C448T (3'-UTR)	0.03	GTGGAGGTGCCAGGAAGCTCCCTCC[C/T]TCACTGTGGGGCATTTCACCATTCA
	T787C (3'-UTR)	0.07	TCTAGCCAGAGGCTGGAGACAGGTG[T/C]GCCCCTGGTGGTCACAGGCTGTGCC

Bolded polymorphisms were genotyped by the TaqMan method
Allele frequencies described are based on TaqMan data (*bolded* polymorphisms, the Suita population, 1,793 subjects) or sequence data (48 subjects)

Table 4 Linkage disequilibrium among polymorphisms in *PCSK9*

Polymorphism		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
C(-64)A	1		*0.80*	*1.00*	*1.00*	0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.07
V4I	2			*0.80*	*0.80*	0.00	0.40	*0.80*	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.02	0.00	0.20
15–16 ins (+L)	3				*1.00*	0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
A53V	4					0.00	0.38	*1.00*	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
C(-161)T	5						0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.09	0.09	0.09	0.09	0.15	0.09	0.08	0.03
L112L	6							0.38	0.02	0.01	0.19	0.00	0.00	0.06	0.06	0.06	0.06	0.05	0.00	0.04	0.00	0.00
T357C	7								0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.01	0.08
G(-10)A	8									*0.79*	0.00	0.00	0.00	0.06	0.06	0.06	0.06	0.05	0.00	0.06	0.00	0.03
G-36A	9										0.00	0.00	0.00	0.04	0.04	0.04	0.04	0.03	0.00	0.04	0.00	0.01
Q275Q	10											0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P331P	11												0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
I424V	12													0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.33	0.00
T276C	13														*1.00*	*1.00*	*1.00*	0.10	0.00	*1.00*	0.00	0.00
T(-57)C	14															*1.00*	*1.00*	0.10	0.00	*1.00*	0.00	0.00
V460V	15																*1.00*	0.10	0.00	*1.00*	0.00	0.00
I474V	16																	0.10	0.00	*1.00*	0.00	0.00
A241G	17																		0.00	0.09	0.00	0.36
G67A	18																			0.00	*0.66*	0.00
C291T	19																				0.00	0.00
C448T	20																					0.00
T787C	21																					

R^2 values are shown (*italics* indicates $r^2 > 0.5$)
Values are based on the genotypes of 48 subjects used for sequence analyses
Bold polymorphisms were selected for genotyping
All values refer to the variant allele indicated in the table

Table 5 Lipid levels among the *PCSK9* polymorphisms (Suita population). *BMI* body mass index, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol. *TG* triglycerides, *LDL-C* low-density lipoprotein cholesterol, *% drinking* percentage of subjects with a drinking habit, *% smoking* percentage of subjects with a smoking habit

	Intron 1/C(-161)T		*P* value	Exon 9/I474V		*P* value
	CC	CT+TT		II	IV+VV	
Number (%)	1,665 (92.9)	128 (7.1)		1,704 (95.0)	89 (5.0)	
Men/women	754/911	54/74		772/932	38/51	
Age[a]	64.4±0.3	62.8±1.0	0.1054	64.3±0.3	64.1±1.2	0.8125
BMI (kg/m^2)[a]	22.7±0.1	22.9±0.3	0.5178	22.8±0.1	22.5±0.3	0.4568
TC (mM)[b]	5.36±0.02	5.24±0.08	0.0285	5.38±0.02	5.14±0.09	0.0069
HDL-C (mM)[b]	1.57±0.01	1.56±0.04	0.4431	1.56±0.01	1.63±0.04	0.1324
TG (mM)[b]	1.20±0.02	1.21±0.08	0.8826	1.20±0.02	1.15±0.10	0.7617
LDL-C (mM)[b]	3.29±0.02	3.14±0.07	0.0257	3.29±0.02	3.01±0.08	0.0007
% drinking[c]	46.8	45.3	0.1238	46.8	44.9	0.7277
Ethanol (g/week)[a]	75.7±3.2	86.0±11.6	0.3953	77.4±3.2	60.6±14.0	0.2404
% smoking[c]	17.1	22.7	0.7472	17.4	19.1	0.6891
Cigarettes (day)[a]	8.3±0.3	7.5±1.1	0.5378	8.2±0.3	7.9±1.4	0.8145

Values are expressed as the mean±SEM.
The formula for calculating LDL-C is described in "Subjects and methods"
Student's *t*-test was performed on residual values adjusted for age, gender BMI, smoking (cigarettes/day), and alcohol consumption (ethanol, g/week)
For triglyceride values, although a logarithmic transformation was applied for the statistical test, untransformed values are shown

[a] Student's *t*-test was performed
[b] Subjects receiving hypolipidemic medication were excluded (intron 1/C-161T: CC $n=1512$, CT+TT$n=122$; exon 9/I474 V: II$n=1,550$, IV+VV $n=83$)
[c] Chi-square test was performed

TC and LDL-C in the Suita population. Since we only found one subject each who was homozygous for minor alleles, these subjects were categorized as heterozygotes. A gender-based subanalysis indicated that the exon 9/I474 V polymorphism significantly influenced the LDL-C level in both male and female subjects (Table 6). TC level in the IV(+VV) genotype of exon 9/I474 V was also lower than that in the II genotype in both male ($P=0.1656$) and female subjects ($P=0.0133$). Although *P*-values were not statistically significant, partially due to low statistical power, TC and LDL-C levels in the CT(+TT) genotype of intron 1/C(-161)T were lower than those in the CC genotype in both male and female subjects. No significant deviation from Hardy-Weinberg equilibrium was observed in these polymorphisms [C(-161)T: $P=0.8290$, I474 V: $P=0.9971$].

We next evaluated whether intron 1/C(-161)T and exon 9/I474 V polymorphisms were associated with the incidence of MI. Distribution of these polymorphisms in subjects with MI were no different from those in the Suita population (Table 7). A gender-based subanalysis indicated that these polymorphisms did not influence the incidence of MI in either male or female subjects (data not shown), nor were they associated with lipid levels in

Table 6 Lipid levels among the PCSK9 polymorphisms (gender-based subanalysis). *TC* total cholesterol,*HDL-C* high-density lipoprotein cholesterol,*TG* triglycerides, *LDL-C* low-density lipoprotein cholesterol

	Intron 1/C(-161)T			Exon 9/I474V		
Men	CC	CT+TT	*P* value	II	IV+VV	*P* value
Number (%)	742 (93.1)	55 (6.9)		757 (95.0)	40 (5.0)	
TC (mM)	5.10±0.03	4.98±0.10	0.1769	5.10±0.03	4.95±0.12	0.1656
HDL-C (mM)	1.43±0.01	1.43±0.05	0.9723	1.42±0.01	1.45±0.06	0.2599
TG (mM)	1.36±0.04	1.43±0.15	0.9598	1.37±0.04	1.41±0.17	0.7717
LDL-C (mM)	3.09±0.03	2.89±0.09	0.0554	3.08±0.03	2.88±0.11	0.0317
Women	CC	CT	*P* value	II	IV	*P* value
Number (%)	770 (92.0)	67 (8.0)		793 (94.9)	43 (5.1)	
TC (mM)	5.58±0.03	5.40±0.10	0.1042	5.59±0.03	5.26±0.12	0.0133
HDL-C (mM)	1.68±0.01	1.65±0.05	0.2716	1.67±0.01	1.77±0.06	0.3345
TG (mM)	1.04±0.02	1.03±0.07	0.7957	1.05±0.02	0.91±0.09	0.1487
LDL-C (mM)	3.44±0.03	3.30±0.10	0.1964	3.45±0.03	3.09±0.12	0.0081

Values are expressed as the mean±SEM
The formula for calculating LDL-C is described in "Subjects and methods"
Subjects receiving hypolipidemic medication were excluded
Student's *t*-test was performed on residual values adjusted for age, BMI, smoking (cigarettes/day), and alcohol consumption (ethanol, g/week)
For triglyceride values, although a logarithmic transformation was applied for the statistical test, untransformed values are shown in the table

Table 7 Association between *PCSK9* polymorphisms and the incidence of myocardial infarction (MI)

	Intron 1/C(-161)T		*P* value	Exon 9/I474V		*P* value
	CC	CT+TT		II	IV+VV	
Suita population, number (%)	1665 (92.9)	128 (7.1)		1704 (95.0)	89 (5.0)	
Patients with MI, number (%)	593 (92.2)	50 (7.8)	0.5943[a]	609 (95.9)	26 (4.1)	0.3684[a]

[a]Genotype distributions in the Suita population and patients with MI were compared using the chi-square test

patients with MI. One possible reason for this lack of association may be that a substantial proportion of the MI group had dyslipidemia and had been treated with hypolipidemic drugs.

Discussion

While C(-161)T and I474 V polymorphisms have been reported previously (Abifadel et al. 2003), association studies have not been reported. The present study clarified that the C(-161)T and I474V polymorphisms were significantly associated with TC and LDL-C levels in the total population. Even in a gender-based subanalysis, the I474V polymorphism significantly influenced the LDL-C level in both male and female subjects. It is unclear whether these polymorphisms are functional variations or just in linkage disequilibrium with other important variants, and this question requires further investigation. Since Ile at amino acid number 474 was not conserved in either rats or mice, another polymorphism in tight linkage with I474 V may be influential. In fact, a polymorphism in the polypyrimidine-rich tract in intron 8/T(-57)C was almost completely concordant with I474V ($r^2 = 1.00$, Tables 3 and 4).

The minor allele frequencies of intron 1/C(-161)T and exon 9/I474 V polymorphisms were low. However, variances between residuals of TC in genotypes [C(-161)T: CC versus CT+TT, I474 V: II versus IV+VV] were similar [C(-161)T: F-ratio = 0.2368, $P = 0.6266$; I474 V: F-ratio = 2.418, $P = 0.1201$ (Levene's test)]. Variances between residuals of LDL-C in the genotypes were also similar [C(-161)T: F ratio = 0.1060, $P = 0.7448$; I474 V: F ratio = 0.4436, $P = 0.5055$]. The sample power was 0.9234 (α-value: 0.05, sigma: 27.70, delta: 2.35, adjusted power: 0.8990, confidence limit: 0.2978–0.9996). Thus, these associations were thought to have adequate statistical power. It has been recommended that a single, nominally significant association should be viewed as tentative until it has been independently replicated at least once and preferably twice (Ioannidis et al. 2001). Accordingly, it will be necessary to verify the association between these *PCSK9* polymorphisms and the levels of TC and LDL-C using a larger number of subjects from the Suita cohort or another population.

We found two polymorphisms that were associated with TC and LDL-C levels among nine polymorphisms of *PCSK9* in the Suita population. However, if we apply Bonferroni's correction for multiple tests, only exon 9/I474 V polymorphism can be considered significantly associated with the HDL level [intron 1/C(-161)T, TC: $P = 0.2565$, LDL-C: $P = 0.2313$; exon 9/I474 V, TC: $P = 0.0621$, LDL-C: $P = 0.0063$, *P*-values are corrected by multiplying by 9 (nine polymorphisms)]. Again, it will be necessary to verify the association between these *PCSK9* polymorphisms and the levels of TC and LDL-C using a larger number of subjects from the Suita cohort or another population.

A high LDL-C level is a well-known coronary risk factor (Kannel et al. 1979). Although *PCSK9* polymorphisms affected the LDL cholesterol level, they did not affect the incidence of MI. The intron 1/C(-161)T polymorphism was inversely associated with LDL-C level and incidence of MI, although these associations were not significant. This was thought to be due, at least in part, to the low statistical power. A much larger group of MI subjects might be necessary to detect the influence of these variants on the incidence of MI.

In conclusion, the present study provides the first evidence that common variants intron 1/C(-161)T and exon 9/I474 V in *PCSK9* significantly affect TC and LDL-C levels in the general Japanese population.

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