Sex Hormone and Gender Difference—Role of Testosterone on Male Predominance in Brugada Syndrome

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Testosterone in Brugada Syndrome. ***Introduction:*** **The clinical phenotype is 8 to 10 times more prevalent in males than in females in patients with Brugada syndrome. Brugada syndrome has been reported to be thinner than asymptomatic normal controls. We tested the hypothesis that higher testosterone level associated with lower visceral fat may relate to Brugada phenotype and male predominance.**

Methods and Results: **We measured body-mass index (BMI), body fat percentage (BF%), and several hormonal levels, including testosterone, in 48 Brugada males and compared with those in 96 age-matched control males. Brugada males had significantly higher testosterone (631 ± 176 vs 537 ± 158 ng/dL; P = 0.002), serum sodium, potassium, and chloride levels than those in control males by univariate analysis, and even after adjusting for age, exercise, stress, smoking, and medication of hypertension, diabetes, and hyperlipidemia, whereas there were no significant differences in other sex and thyroid hormonal levels. Brugada males had significantly lower BMI (22.1 ± 2.9 vs 24.6 ± 2.6 kg/m^2; P < 0.001) and BF% (19.6 ± 4.9 vs 23.1 ± 4.7%; P < 0.001) than control males. Testosterone level was inversely correlated with BMI and BF% in both groups, even after adjusting for the confounding variables. Conditional logistic regression models analysis showed significant positive and inverse association between Brugada syndrome and hypertestosteronemia (OR:3.11, 95%CI:1.22–7.93, P = 0.017) and BMI (OR:0.72, 95%CI:0.61–0.85, P < 0.001), respectively.**

Conclusions: **Higher testosterone level associated with lower visceral fat may have a significant role in the Brugada phenotype and male predominance in Brugada syndrome.** *(J Cardiovasc Electrophysiol, Vol. 18, pp. 415-421, April 2007)*

Brugada syndrome, gender, sex hormones, testosterone, body mass index

Introduction

Brugada syndrome is characterized by coved-type ST-segment elevation in the right precordial electrocardiographic (ECG) leads (V1–V3) and an episode of ventricular fibrillation (VF) in the absence of structural heart disease.[1-5] The prevalence of the disease is estimated to be up to 5 per 10,000 inhabitants and is one of the important causes of sudden cardiac death of middle-aged males, particularly in Asian countries including Japan.[4]

More than eight dozen distinct mutations in *SCN5A*, the gene encoding the α subunit of the sodium channel, have been so far identified in patients with Brugada syndrome and all mutations display an autosomal-dominant mode of transmission.[6,7] Therefore, males and females are expected to inherit the defective gene equally. However, more than 80% of patients in Western countries and more than 90% of patients in Asian countries affected with Brugada syndrome are males.[8] Recent experimental studies have unveiled the cellular mechanism of Brugada phenotype. The male predominance in the Brugada syndrome is suggested to be due, at least in part, to intrinsic differences in ventricular action potential (AP) between males and females.[9]

A male hormone, testosterone is reported to increase net outward currents[10-12] and is expected to accentuate Brugada phenotype, such as ST-segment elevation and subsequent episodes of VF in patients with Brugada syndrome. Testosterone is also known to decrease visceral fat.[13-15] Since patients with Brugada syndrome have been reported to be

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thinner than asymptomatic normal controls by Matsuo et al.,[16] we speculated that higher testosterone level associated with lower visceral fat may modulate Brugada phenotype and may relate to male predominance in patients with Brugada syndrome.

Methods

Patient Population and Data Collection

The study population consisted of 48 males with Brugada syndrome who agreed to participate in this study and showed Type 1 "coved" ST-segment elevation in V1–V3 leads[17] ranging in age from 30 to 69 years with a mean age of 50 ± 11 years (mean ± SD). Brugada males who were less than 30 years old and more than 70 years old were excluded from this study to minimize the influence of age on the basal sex hormonal levels including testosterone. Forty of the forty-eight Brugada males have been included in our previous clinical studies.[18-20] In all patients, physical examination, chest roentgenogram, laboratory values, echocardiography with wall motion analysis, and Doppler screening excluded structural heart diseases. The clinical, electrocardiographic, and electrophysiologic characteristics of the 48 Brugada males are shown in Table 1. Average age of the 48 Brugada males at diagnosis was 47 ± 12 years old. Aborted cardiac arrest or VF was documented in 21 males (44%), syncope alone in 11 males (23%), and 16 males (33%) were asymptomatic. Family history of sudden cardiac death (SCD) was observed in eight males (17%). An *SCN5A* coding region mutation was identified in seven (17%) of 42 males in whom genetic screening was conducted. Implantable cardioverter defibrillator (ICD) was implanted in all 32 symptomatic males with documented VF and/or syncope. ICD was also implanted in nine of 16 asymptomatic males due to induction of VF during the electrophysiologic study. Type 1 ST-segment elevation was recorded spontaneously in 43 males (90%) and was induced by sodium channel blockers in five males (10%). Complete right bundle branch block was observed in three males (6%). Late potential was recorded by a signal-average ECG system in 27 (59%) of 46 males. During the electrophysiologic study, VF requiring direct cardioversion for termination was induced in 32 (73%) of 44 males. Average HV interval was 46 ± 11 msec.

We first obtained data, such as the hormonal levels, visceral fat parameters, and ECG parameters in the 48 Brugada males prospectively between January and July in 2003, mainly at regular outpatient clinics for checking ICD. Only a Brugada male refused to participate during the recruitment of the case.

Thereafter, age-matched control males were randomly selected from the municipal population registry in Suita City. The hormonal and visceral fat data were collected sequentially between August and December in 2003. The municipal population registry in Suita City included 5,846 control subjects, among whom 1,052 males were age-matched to the 48 Brugada males. The 96 control males with a mean age of 50 ± 11 years were sequentially recruited from the age-matched 1,052 males. None of the recruited 96 control males refused to participate in this study. There were no significant differences in the clinical characteristics between the 96 control males and the remaining 956 age-matched males. Therefore, we had no way of knowing the body weight of the individuals who were selected to serve as controls from a very large database. Although K. Matsuo is a co-author of this study, none of the Brugada males and control males who appeared in the article by Matsuo[16] are included in the present study population.

All protocols were approved by the Ethical Review Committee in the National Cardiovascular Center. Written informed consent was obtained from all subjects.

TABLE 1

Clinical, Electrocardiographic, and Electrophysiologic Characteristics in the 48 Brugada Males

Clinical characteristics	
Age at diagnosis (years)	47 ± 12
Aborted cardiac arrest or VF (%)	21/48 (44%)
Syncope alone (%)	11/48 (23%)
Asymptomatic (%)	16/48 (33%)
Family history of SCD	8/48 (17%)
SCN5A mutation	7/42 (17%)
ICD implantation	41/48 (85%)
Follow-up period (month)	41 ± 2
Arrhythmic event (%)	9/48 (19%)
Electrocardiographic characteristics	
Spontaneous coved-type ST elevation	43/48 (90%)
CRBBB (%)	3/48 (6%)
RR (msec)	939 ± 113
PQ interval (II) (msec)	186 ± 34
QRS duration (V2) (msec)	104 ± 18
Corrected QT interval (V5) (msec)	394 ± 27
ST amplitude at J point (V2) (mV)	0.32 ± 0.16
Late potential (%)	27/46 (59%)
Electrophysiologic characteristics	
Induction of VF	32/44 (73%)
Mode (Triple/Double/Single)	16/15/1
HV interval (msec)	46 ± 11

CRBBB = complete right bundle branch block; ICD = implantable cardioverter defibrillator; SCD = sudden cardiac death; VF = ventricular fibrillation.

Sex and Thyroid Hormonal Levels and Serum Electrolytes

Blood samples for analysis of basal hormone levels and serum electrolytes were obtained between 8:00 and 9:00 AM after an overnight fast. Plasma sex hormonal levels including testosterone, estradiol, DHEA-S, LH, and FSH were measured using commercially prepared immunoassay kits (testosterone, LH, and FSH: Chemiluminescent immunoassay [Bayer HealthCare, New York, NY, USA]; estradiol: Electrochemiluminescent immunoassay [Roche Diagnostics GmbH, Mannheim, Germany]; DHEA-S: Radioimmunoassay [Diagnostic Products Corporation, Los Angeles, CA, USA]). Thyroid hormonal levels including free T3, T4, and TSH, and serum electrolyte levels including sodium, potassium, and chloride were also measured.

Body Mass Index and Body Fat Percentage

Body weight (BW) was measured to the nearest 0.1 kg and height to the nearest cm. Body-mass index (BMI) was calculated as weight/height^2 (kg/m^2) as a parameter of visceral fat. We also measured body-fat percentage (BF%) by using body composition analyzer (Biospace Co., Ltd. Tokyo, Japan). These visceral fat parameters were measured just after blood sampling. In the 32 symptomatic Brugada males who had had documented VF and/or syncope, the BW and BMI were also measured within 48 hours after their clinical events during admission in our hospital or other emergent hospitals.

ECG Parameters

In the 48 males with Brugada syndrome, 12-lead ECG was recorded just before blood sampling, and ECG parameters were assessed by an investigator (WS) blinded to clinical information. The ECG parameters included RR interval, PQ interval measured in lead II, QRS interval measured in lead V2, QT interval, corrected QT (QTc) interval measured in leads V5, and ST amplitude at J point measured in lead V2.

Statistical Analysis

We first conducted univariate analysis by using unpaired *t*-test to compare each data between the Brugada males and the control males. Since several confounding variables, such as age, exercise (none, sometimes, regularly), stress (none, sometimes, regularly), current smoking (no, yes), and medication (no, yes) of hypertension, diabetes, and hyperlipidemia may affect the hormonal levels including testosterone level and the visceral fat parameters, analysis of covariance (ANCOVA) was used to compare least square mean values between the Brugada males and the control males adjusting for these confounding variables. Pearson's correlation coefficients were calculated between the testosterone level and the visceral fat parameters. Partial correlation coefficients were calculated between the testosterone level and the visceral fat parameters after adjusting for age, exercise, stress, current smoking, and medication. Moreover, conditional logistic regression models were used to calculate odds ratios and 95% confidence intervals adjusting for age, BMI, exercise, stress, current smoking, hypertension, diabetes, and hyperlipidemia. Hypertestosteronemia was defined as serum testosterone levels $\geq$700 ng/dL, which is 75 percentiles of testosterone levels among case and control combined groups. In the 32 Brugada males with documented VF and/or syncope, a paired *t*-test was used to compare the visceral fat parameters at the clinical cardiac events and at the measurement of hormonal and visceral fat data. A two-sided P value below 0.05 was considered to indicate significance. All statistical analyses were performed by using SAS software, Ver 8.2.

TABLE 2

Sex and Thyroid Hormonal Levels, Serum Electrolytes, and Visceral Fat Parameters in the 48 Brugada Males and the 96 Age-Matched Control Males

	Brugada Males (n = 48)	Control Males (n = 96)	P Value
Sex hormones			
Testosterone (ng/dL)	631 ± 176	537 ± 158	0.002
Estradiol (pg/mL)	28.9 ± 7.6	31.1 ± 12.6	0.263
DHEA-S (ng/mL)	1,901 ± 850	1,966 ± 861	0.668
LH (mIU/mL)	4.6 ± 2.6	3.9 ± 2.0	0.073
FSH (mIU/mL)	6.2 ± 4.9	5.0 ± 2.9	0.066
Thyroid hormones			
Free T3 (pg/mL)	3.3 ± 0.4	3.4 ± 0.3	0.360
Free T4 (ng/dL)	1.3 ± 0.1	1.3 ± 0.2	0.089
TSH (μIU/mL)	1.9 ± 1.4	1.7 ± 1.4	0.619
Serum electrolytes			
Sodium (mEq/L)	143.7 ± 2.0	142.6 ± 2.0	0.003
Potassium (mEq/L)	4.6 ± 0.3	4.3 ± 0.3	<0.001
Chloride (mEq/L)	105.1 ± 2.1	103.6 ± 2.1	<0.001
Viceral fat			
BMI (kg/m^2)	22.1 ± 2.9	24.6 ± 2.6	<0.001
BF% (%)	19.6 ± 4.9	23.1 ± 4.7	<0.001
BW (kg)	62.9 ± 9.7	70.0 ± 8.6	<0.001

Values are mean ± SD where indicated.

BMI = body-mass index; BF% = body-fat percentage; BW = body weight.

Results

Hormonal Levels, Serum Electrolytes, and Visceral Fat

Table 2 illustrates univariate analysis for comparing sex and thyroid hormonal levels, serum electrolytes, and visceral fat parameters between the two groups. Testosterone level was significantly higher in the Brugada males than in the control males, whereas there were no significant differences in other sex hormonal levels; estradiol, DHEA-S, LH, FSH, and thyroid hormonal levels; T3, T4, and TSH. Serum sodium, potassium, and chloride levels were all significantly higher in the Brugada males than in the control males. BMI, BF%, and BW were all significantly lower in the Brugada males than in the control males. All variables followed normal distribution, both in the 48 Brugada and 96 control males.

The comparison of the confounding variables that may affect the hormonal levels and the visceral fat parameters between the 48 Brugada males and the 96 control males was shown in Table 3. Even after adjusting for age, exercise, stress, current smoking, and medication (hypertension, diabetes, and hyperlipidemia), the testosterone level, serum sodium, potassium, and chloride levels were all significantly higher, and the visceral fat parameters were significantly lower in the 48 Brugada males than in the 96 control males (Table 4). There were also significant differences in these parameters between the 24 definite Brugada males with documented VF and/or *SCN5A* mutations and the 96 control males after adjusting for the confounding variables (Table 4).

Correlation between Testosterone, Visceral Fat, and Serum Electrolytes

Testosterone level was inversely correlated with all visceral fat parameters, BMI, BF%, or BW in both the Brugada males and the control males, even after adjusting for age,

TABLE 3

Comparison of the Confounding Variables Between the 48 Brugada Males and the 96 Age-Matched Control Males

	Brugada Males (n = 48)	Control Males (n = 96)	P Value
Exercise			
None (%)	39.6	44.8	
Sometimes (%)	41.6	43.8	
Regularly (%)	18.8	11.5	0.482
Stress			
None (%)	27.1	21.9	
Sometimes (%)	54.2	54.2	
Regularly (%)	18.8	24.0	0.684
Current smoking (%)	25.0	27.1	0.789
Medication			
Hypertension (%)	20.8	19.8	0.883
Diabetes (%)	2.1	13.5	0.028
Hyperlipidemia (%)	10.4	5.2	0.246

TABLE 4

Testosterone, Serum Electrolytes, and Visceral Fat Parameters in the Brugada Males and the 96 Age-Matched Control Males after Adjusting for Confounding Variables

	Brugada Males	Control Males (n = 96)	P Value
ALL Case (n = 48)			
Testosterone (ng/dL)	631 ± 44	538 ± 40	0.003
Sodium (mEq/L)	144.2 ± 0.5	143.2 ± 0.5	0.007
Potassium (mEq/L)	4.6 ± 0.1	4.3 ± 0.1	<0.001
Chloride (mEq/L)	105.5 ± 0.5	103.9 ± 0.5	<0.001
BMI (kg/m^2)	22.3 ± 0.7	24.9 ± 0.7	<0.001
BF% (%)	20.0 ± 1.3	23.9 ± 1.1	<0.001
BW (kg)	63.4 ± 2.4	70.1 ± 2.1	0.001
Definite Brugada case with VF and/or *SCN5A* (n = 24)			
Testosterone (ng/dL)	656 ± 59	550 ± 48	0.009
Sodium (mEq/L)	143.9 ± 0.7	142.9 ± 0.6	0.042
Potassium (mEq/L)	4.7 ± 0.1	4.4 ± 0.1	<0.001
Chloride (mEq/L)	105.2 ± 0.7	103.9 ± 0.6	0.006
BMI (kg/m^2)	21.5 ± 1.0	24.5 ± 0.8	<0.001
BF% (%)	19.9 ± 1.7	24.1 ± 1.4	<0.001
BW (kg)	60.5 ± 3.1	69.2 ± 2.5	0.001

Values are mean ± SE adjusted for age, exercise, stress, current smoking, and medication of hypertension, diabetes and hyperlipidemia. BMI = body-mass index; BF% = body-fat percentage; BW = body weight; VF = ventricular fibrillation.

exercise, stress, current smoking, and medication (Brugada: BMI, r = –0.394, P = 0.011; BF%, r = –0.390, P = 0.012; BW, r = –0.335, P = 0.032; Control: BMI, r = –0.333, p = 0.002; BF%, r = –0.333, P = 0.001; BW, r = –0.305, P = 0.004), suggesting that Brugada males had higher testosterone level associated with lower visceral fat compared with control males (Fig. 1). No significant correlations were observed between other serum electrolytes and testosterone level or visceral fat parameters. Testosterone level was not correlated with age, even after adjusting for exercise, stress, current smoking, and medication (r = 0.007, P = 0.947).

Conditional Logistic Regression Models Analysis

Conditional logistic regression models analysis showed significant positive and inverse association between Brugada syndrome, hypertestosteronemia (Odd Ratio (OR): 3.11, 95%CI: 1.22–7.93, P = 0.017), and BMI (OR: 0.72, 95%CI: 0.61–0.85, P < 0.001), respectively (Table 5). Other variables did not significantly increase or decrease risks of Brugada syndrome (Table 5).

Visceral Fat at Clinical Cardiac Events in Brugada Males

In the 32 symptomatic Brugada males with documented VF and/or syncope, the time-span between the clinical cardiac events and the measurement of hormonal and the visceral fat data was 42 ± 32 months (mean ± SD, 1-99 months). The BMI and BW at the clinical cardiac events (VF or syncope) were significantly lower than those at the measurement of hormonal and visceral fat data (BMI, 21.0 ± 2.6 vs 22.1 ± 2.9 kg/m^2; BW, 60.0 ± 8.9 vs 62.9 ± 9.7 kg: P < 0.001, respectively).

Testosterone versus ECG Parameters, Symptoms or SCN5A Mutation in Brugada Males

Baseline electrocardiographic data of the 48 Brugada males are shown in Table 1. No significant correlations were observed between testosterone level and ECG parameters, including ST amplitude (r = –0.123, P = 0.406) and QTc interval (r = –0206, P = 0.160), in the 48 Brugada males. There was no significant difference in testosterone level between 32 symptomatic and 16 asymptomatic Brugada males (649 ± 185 vs 593 ± 157 ng/dL: P = 0.298). No significant difference was observed in testosterone level between 43 Brugada males with spontaneous Type 1 ST-segment elevation and five Brugada males with sodium channel blocker-induced Type 1 ST-segment elevation (624 ± 171 vs 688 ± 230 ng/dL: P = 0.448). Testosterone level was also no different between seven Brugada males with *SCN5A* mutation and 41 Brugada males without *SCN5A* mutation (700 ± 198 vs 619 ± 172 ng/dL: P = 0.261).

Follow-Up

Arrhythmic events occurred in nine (19%) of 48 Brugada males during average follow-up periods of 41 ± 2 months after blood sampling for the present study (Table 1). In more detail, arrhythmic events appeared in eight (38%) of 21 Brugada males with a history of aborted cardiac arrest or VF, in one (9%) of 11 Brugada males with syncope alone, but did not appear in any (0%) of 16 asymptomatic Brugada males.

Discussion

The major findings of the present study were: (1) Brugada males had significantly higher testosterone level, serum sodium, potassium, and chloride level, and significantly lower BMI, BF%, and BW than those in control males by univariate analysis, even after adjusting for age, exercise, stress, current smoking, and medications related to hypertension, diabetes and hyperlipidemia. (2) Testosterone level was inversely correlated with the BMI, BF%, and BW in both Brugada males and control males, even after adjusting for the confounding variables. (3) Conditional logistic regression models analysis showed strong positive association between Brugada syndrome and higher testosterone level (hypertestosteronemia) and strong inverse association between Brugada syndrome and BMI.

Testosterone in Brugada Phenotype and Male Predominance

For the past decade, numerous clinical, experimental, and molecular genetic studies have elucidated Brugada syndrome as a distinct clinical entity.[1-5,17] However, several problems remain unresolved, such as genetic heterogeneity, ethnic difference, and gender difference.[7] Di Diego and Antzelevitch recently suggested the cellular basis for male predominance in Brugada syndrome by using arterially perfused canine right ventricular wedge preparations.[9] Transient outward current (I_{to})-mediated phase 1 AP notch was larger in male dogs than in female dogs in the right ventricular epicardium, but not in the left ventricular epicardium, responsible for the male predominance in the Brugada phenotype. Recent clinical studies suggested that male hormone testosterone might be attributable to gender difference of the prevalence in this

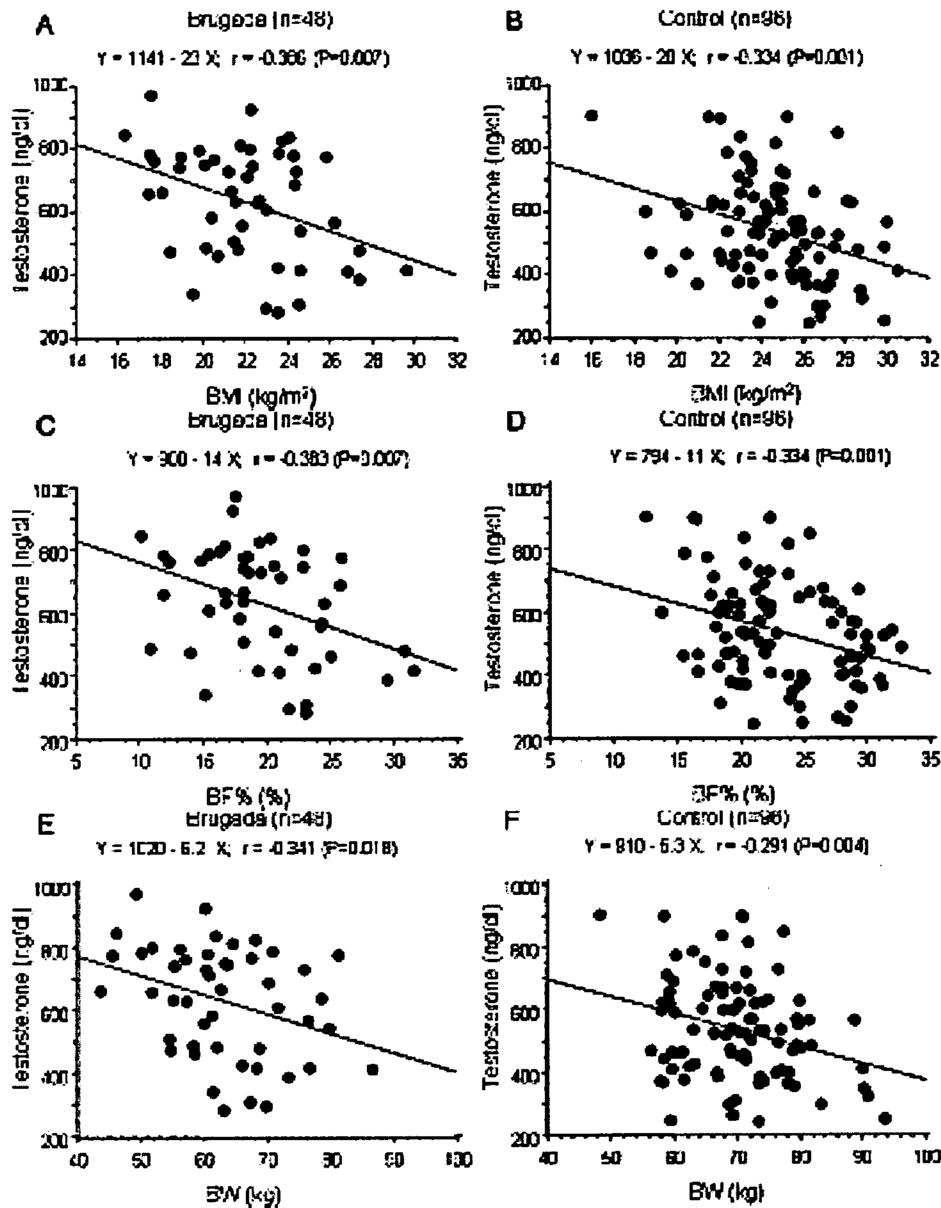

Figure 1. *Correlation between testosterone level and visceral fat parameters; body mass index (BMI) (A and B), body fat percentage (BF%) (C and D), and body weight (BW) (E and F) in the 48 Brugada males and the 96 age-matched control males. Testosterone level was inversely correlated with the BMI, BF%, or BW in both Brugada males and control males.*

syndrome. Matsuo et al. reported two cases of asymptomatic Brugada syndrome in whom typical coved ST-segment elevation disappeared following orchiectomy as therapy for prostate cancer,[21] indicating that testosterone may contribute to the Brugada phenotype in these two cases. Several experimental studies reported that testosterone increased outward potassium currents, such as the rapidly activating component (I_{Kr})[10,11] and the slowly activating component (I_{Ks})[12] of the delayed rectifier potassium current, and the inward rectifier potassium current (I_{K1}),[11] or decreased inward L-type calcium current (I_{Ca-L}).[12] Since the maintenance of the AP dome is determined by the fine balance of currents active at the end of phase 1 of the AP (principally I_{to} and I_{Ca-L}),[22,23] any agents that increase outward currents or decrease inward currents can increase the magnitude of the AP notch, leading to loss of the AP dome (all-or-none repolarization) in the epicardium, but not in the endocardium, contributing to a significant voltage gradient across the ventricular wall during ventricular activation, thus augmenting ST-segment elevation, the Brugada phenotype.[24] Therefore, testosterone would be expected to accentuate the Brugada phenotype. In the present study, males with Brugada syndrome had significantly higher testosterone level than age-matched control males, even after adjusting for age, exercise, stress, current smoking, and medication (hypertension, diabetes, and hyperlipidemia), which may affect the testosterone level. Moreover, conditional logistic regression models analysis showed strong positive association between Brugada syndrome and higher testosterone level (OR: 3.11). Our data suggest a significant role of testosterone, male hormone, in the Brugada phenotype. The

TABLE 5

Odds Ratios of Presence of Hypertestosteronemia and Confounding Risk Factors for Brugada Syndrome in Males

Variable	Odd Ratio	95% Confidence Interval	P Value
Hypertestosteronemia	3.11	1.22–7.93	0.017
Age	0.99	0.95–1.03	0.637
BMI	0.72	0.61–0.85	<0.001
Exercise	1.57	0.87–2.83	0.135
Stress	0.69	0.35–1.35	0.277
Current smoking	0.71	0.26–1.90	0.493
Hypertension	3.12	0.85–11.45	0.087
Diabetes	0.13	0.01–1.27	0.079
Hyperlipidemia	2.14	0.44–10.49	0.348

Hypertestosteronemia was defined as serum testosterone levels ≥ 700 ng/dL.

data also indicate that the male predominance in the Brugada phenotype is at least in part due to testosterone, which is present only in males.

Lower Visceral Fat May Be a Predictor for Brugada Phenotype

Matsuo et al. recently reported in their epidemiologic study that cases with the Brugada-type ECG had significantly lower BMI than that in control subjects.[16] Similarly, in the present study, males with Brugada syndrome had significantly lower visceral fat parameters, BMI, BF%, and BW than those in age-matched control males, even after adjusting for several confounding variables. Moreover, conditional logistic regression models analysis showed strong inverse association between Brugada syndrome and BMI (OR: 0.72). All of the visceral fat parameters were inversely correlated with testosterone level in both Brugada and control males, even after adjusting for the confounding variables. It has been well demonstrated that testosterone level in obese males is decreased compared to normal males of similar age.[13] Tsai et al. reported that lower baseline total testosterone level independently predicted an increase in visceral fat in the Japanese-American male cohort for 7.5 years.[15] Reversely, Marin et al. reported that testosterone treatment of middle-aged abdominally obese males was followed by a decrease of visceral fat mass measured by computerized tomography.[14] These data suggest that primarily higher level of testosterone in Brugada males compared to that in control males may result in lower visceral fat in Brugada males, which would be an "innocent bystander" sign of Brugada phenotype. In reverse, if primary lower visceral fat (body weight loss) would result in higher testosterone level, the weight loss could be a trigger for Brugada phenotype, just like fever is.[25] It is noteworthy that the visceral fat parameters at the clinical cardiac events (VF or syncope) in the 32 symptomatic Brugada males were significantly lower than those at the time of blood sampling for this study. This indicates that testosterone level is expected to be additively higher at the clinical cardiac events, which may contribute to spontaneous episodes of VF or syncope.

Other Hormonal Levels and Serum Electrolytes

Estradiol, female hormone, is reported to reduce the expression of Kv4.3 channels, which are important molecular components of I_{to} currents.[26] However, in contrast to testosterone, other sex hormonal levels including estradiol were not different between the Brugada males and the control males in the present study. Although thyroid hormones are also demonstrated to alter membrane currents, such as I_{to} and I_{Ca-L},[27,28] no significant differences were observed in the thyroid hormonal levels between the two groups in the present study.

On the other hand, serum sodium, potassium, and chloride levels were all significantly higher in the Brugada males than in the control males, even after adjusting for several confounding variables. Recently, many agents and conditions that cause an outward shift in current activity at the end of phase 1 AP have been known to unmask ST-segment elevation, as found in the Brugada syndrome, leading to the acquired form of this disorder.[4,29] Electrolyte abnormalities, such as hyperkalemia, are reported to amplify ST-segment elevation like that in Brugada syndrome.[30] The lower visceral fat found in the Brugada males is expected to decrease serum level of insulin, leptine, a novel adipocyte-derived hormone, or ghrelin, a novel growth hormone-releasing peptide, suppressing β-adrenergic receptor or plasma norepinephrine level, resulting in an increase of serum potassium level.[31,32] Further studies including measurement of levels of insulin, leptine, and ghrelin will be required to elucidate the precise mechanism.

Study Limitations

Although the testosterone level was significantly higher in the Brugada males than in the control males, no statistically significant correlations were observed between the testosterone level and the ST amplitude in the Brugada males. The degree of the ST-segment elevation is variable between Brugada patients because it is influenced by several factors other than sex hormonal levels or electrolytes levels, such as basal autonomic tone, presence of *SCN5A* mutation, or probably intrinsic current density of I_{to}, etc., in the right ventricular epicardial cells. The threshold of ST-segment elevation for spontaneous induction of VF also varies between Brugada patients. Therefore, the Brugada phenotype, such as ST-segment elevation or spontaneous induction of VF, may correlate with the testosterone level day to day individually (intra-personally) in each Brugada male, but may not correlate among the pooled data obtained from many Brugada males, probably due to inter-person difference of the ST-segment elevation.

There were no significant differences in testosterone level between symptomatic and asymptomatic Brugada males, between Brugada males with spontaneous ST elevation and those with sodium channel blocker-induced ST elevation, or between Brugada males with and without *SCN5A* mutation, all of which are probably due to a relatively small number of Brugada males in the present study. Further evaluation with increasing number of Brugada males will be required.

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Age- and gender-related differences of plasma prothrombin activity levels

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Dear Sir,

Advancing age is an important risk factor for venous or arterial thrombosis in both sexes (1–3). Moreover, gender is associated with differences in the prothrombotic state and in the progression of atherosclerosis that occurs with aging (4, 5). Prothrombin is one of the dominant factors influencing thrombin generation (6), and the prothrombin G20210A mutation accompanied by an increased level of prothrombin poses a risk factor for venous or arterial thrombosis (7, 8). However, gender differences in age-related changes in plasma prothrombin activity have not been investigated until now. In the present study, we measured prothrombin activity in 742 individuals derived from a general Japanese population which was supposed to be free of prothrombin G20210A mutation (9).

The study population was composed of samples randomly selected from the residents of Suita, a city located in the second largest urban area in Japan (the Suita Study) (4). All subjects had been visiting the National Cardiovascular Center every two years since 1989 for regular health checkups. Only subjects who provided written informed consent to have a blood examination were enrolled in this study. We excluded subjects treated with oral anticoagulant therapy. Finally, 742 subjects, aged 36 to 85 years (mean age: 64 years), were included in this study. Spearman correlation analysis was used to assess the association between aging and the level of prothrombin activity within a given gender. For comparison between the two gender groups, the Mann-Whitney U test was used. Differences with a value of $p<0.01$ for the Spearman correlation analysis and $p<0.05$ for the Mann-Whitney U test were considered to be significant. Statistical calculations were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL, USA). Prothrombin activity was measured according to a published method (10) with a modification. Briefly, 200 µl of 20 mM Tris-HCl, 0.14 M NaCl, pH 7.5 buffer containing 1 mg/ml of bovine serum albumin (TBSA) was added to 50 µl of plasma anticoagulated with 0.13% sodium citrate. Then, diluted plasma was incubated for 150 seconds at 37°C, and we detected ΔA/min at 405 nm after adding 50 µl of the reagent containing 6 mM $CaCl_2$, 0.5 mM Boc-Val-Pro-Arg-pNA as a thrombin substrate, 500 pM carinactivase-1 as a thrombin activator, and TBSA. Calibration was performed with a standard-human-plasma (Dade Behring GmbH, Marburg, Germany). The coefficient of intra-assay variation for prothrombin activity assay was 2.0%.

The mean ± SD of prothrombin activity level in men and women was 110.2 ± 17.0 (range: 54.5–158.5%) and 120.4 ± 17.4 (range: 57.5–194.4%), respectively. Figure 1 shows the age-related distribution (36–85 years) of prothrombin activity in 348 men (Fig. 1A) and 394 women (Fig. 1B). As a whole, a linear decrease of prothrombin activity level with age was observed in

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men (r=-0.34, p<0.0001), but not in women (r=-0.04, p=0.47). When prothrombin activity level was analyzed in 10-year age groups, significant decreases were observed in the men aged 46–55 years and 56–65 years (p<0.0001), aged 56–65 years and 76–85 years (p<0.05), and in the women aged 66–75 years and 76–85 years (p<0.0001). Levels of prothrombin activity were decreased in both sexes in the oldest age group (aged 76–85 years). With regards to gender-related change, the prothrombin activity level in the age group of 56–65 years, 66–75 years, and 76–85 years was significantly lower in men than in women.

In the present study, we showed the age-related decrease in the plasma prothrombin activity of men and gender-related change in the plasma prothrombin activity. These results contribute to the understanding of age-related hypercoagulability and to the practical institution of anticoagulant therapy in older patients. It has been established that thrombin generation increases with age in both sexes, evidenced by plasma prothrombin fragment F1+2 levels produced by the cleavage of prothrombin by factor Xa (11, 12). Age-related hypercoagulability does not likely stem from the prothrombin activity, because the prothrombin activity of men showed the age-related decrease, but it may result from some other mechanisms including decreased levels of anticoagulant proteins such as protein C and S (11, 13). We presented here the gender-related change of significantly lower prothrombin activity levels in men in the age of 56–85 years than in women. Men tend to develop thrombotic events including recurrent venous thrombosis (14), but this tendency was not related to the plasma level of prothrombin activity. Our work sheds further light on the point that, when considering relative hypercoagulability, gender-adjustment is necessary for the comparison of prothrombin activity levels.

With regards to anticoagulant therapy, the plasma levels of vitamin K-dependent coagulation factors decrease with increasing intensity of anticoagulation therapy (15). At the same time, the risks of major haemorrhage increase according to the intensity of anticoagulation therapy, especially in patients older than 80 years (16). Given our current study results, the markedly decreased prothrombin level in the age group of 76–85 years, especially in men, provides a potential mechanistic explanation for the increased rate of major haemorrhage observed in elderly patients receiving anticoagulant therapy.

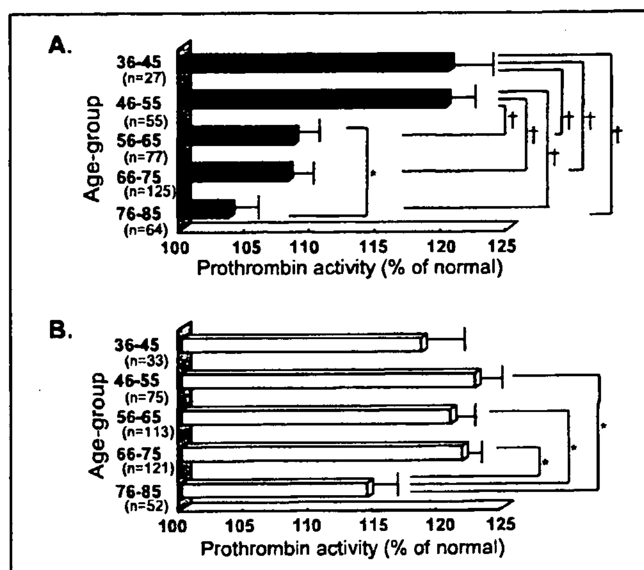

Figure 1: Age-related changes of plasma prothrombin activity levels according to gender (A: men, B: women). Populations aged from 36 to 85 years old were divided into five age groups by gender. Data are expressed as the mean ± SEM. *: P<0.05, †: P<0.0001, compared between two age groups of the same gender.

In conclusion, there are significant age- and gender-related differences in plasma prothrombin activity levels. In particular, the prothrombin activity level in men in the age group of 76–85 years was lower than that of any other age group in either gender.

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Genetic variants in *PCSK9* in the Japanese population: Rare genetic variants in *PCSK9* might collectively contribute to plasma LDL cholesterol levels in the general population

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Abstract

The aim of this study was to investigate whether plasma low-density lipoprotein cholesterol (LDL-C) levels in the general population are influenced by rare sequence variations in the *PCSK9* gene. We sequenced the promoter and coding regions of the *PCSK9* gene in individuals from the general population ($n = 3655$) with the lowest ($n = 78$) and highest ($n = 96$) LDL-C levels and in individuals taking antihypercholesterolemia medication ($n = 96$). We identified 33 sequence variants in the *PCSK9* gene among which 24 were specific for Japanese. Statistical analysis showed that one missense mutation, R93C, was associated with low LDL-C levels. The other variants had no association with LDL-C levels or the numbers of individuals with the variants were too small for statistical analysis. A comparison of the numbers of individuals with nonsynonymous mutations between the low LDL-C and high LDL-C/treatment groups found that four missense mutations and one nonsense mutation were identified only in the low LDL-C group and six missense mutations were identified only in the high LDL-C/treatment group. As we have analyzed groups at opposite ends of the LDL-C spectrum, it is likely that some of these nonsynonymous mutations may be associated with either low or high LDL-C in the Japanese population. Based on the extremely high frequencies of the nonsynonymous mutations in *PCSK9* compared with those of *LDLR* or *apoB-100*, *PCSK9* mutations could be important factors that cumulatively influence plasma LDL-C levels in the general population.
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Keywords: *PCSK9*; Plasma LDL cholesterol; Rare genetic variants; General population; Missense mutation

1. Introduction

Elevated plasma concentration of low-density lipoprotein cholesterol (LDL-C) is a major risk factor for the development and progression of atherosclerosis. Plasma concentrations of LDL-C are determined primarily by the activity of the LDL receptor (LDLR) in the liver. Recently, the *proprotein convertase subtilisin/kexin type 9* (*PCSK9*) gene has been found to be involved in the post-transcriptional regulation of the LDLR. *PCSK9* encodes a protein of 692 amino acids which is a member of the subtilisin-like protein convertase family [1,2] and is expressed most abundantly in the liver, kidneys and small intestine [2]. PCSK9 consists of several domains: a signal peptide, a prosegment, a subtilase-like catalytic domain and a C-terminal domain [3]. It is synthesized as a soluble zymogen which undergoes autocatalytic intramolecular cleavage in the endoplasmic reticulum (ER) between the prosegment and the catalytic domain [1,2].

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After cleavage, the mature PCSK9 exits the ER and is efficiently secreted [2]. The only known substrate of PCSK9 is itself; no other substrate(s) for PCSK9 have yet been identified. Although even the physiological substrate remains unknown, PCSK9 has been shown to play a role in cholesterol metabolism by regulating the number of cell-surface LDLRs [3–5].

Overexpression of the wild-type *Pcsk9* gene in mice results in hypercholesterolemia because of a reduced number of LDLRs [3–5]. The reduced number of LDLRs due to PCSK9 is not accompanied by changes in *LDLR* mRNA levels; therefore, it is likely that PCSK9 is involved in the post-transcriptional regulation of the LDLR [4,5]. Degradation of the LDLR by PCSK9 is dependent on the catalytic activity of PCSK9 [5,6]. In contrast, mice expressing no PCSK9 have markedly increased hepatic LDLR levels, resulting in accelerated LDL clearance [7]. These findings indicate that PCSK9 normally acts to limit the number of LDLRs at the cell surface. Thus, *PCSK9* mutations which disrupt normal function, i.e., loss-of-function mutations, are presumed to increase the number of LDLRs, resulting in hypocholesterolemia. In fact, the nonsense mutations identified by Cohen et al. are associated with a 40% reduction in mean plasma levels of LDL-C [8]. On the other hand, some mutations in the *PCSK9* gene cause hypercholesterolemia [9–11], which are probably due to gain-of-function mechanisms. These mutations in *PCSK9* might promote the degradation of LDLRs in hepatocytes [3–5]. Recently, Cameron et al. demonstrated that loss-of-function mutations in *PCSK9* increase the number of cell-surface LDLRs, while gain-of-function mutations decrease the number of LDLRs, based on studies on HepG2 cells transfected with mutant *PCSK9* constructs [12].

Since mutations in *PCSK9* can cause severe hypercholesterolemia [9–11] as well as hypocholesterolemia [8,13], sequence variants of *PCSK9* might contribute to variations in the plasma levels of LDL-C. Shioji et al. [14] have identified the two common single nucleotide polymorphisms (SNPs), and Chen et al. [15] identified a haplotype associated with differences in plasma LDL-C levels. Kotowski et al. performed a systematic examination of the relationship between sequence variations in *PCSK9* and plasma levels of LDL-C in the general population [16]. They analyzed sequence variations in *PCSK9* in individuals of the examined population who had lower and higher LDL-C levels and found that three missense mutations and two noncoding sequence variants were significantly associated with lower levels of LDL-C, while a single noncoding variant was associated with a modest increase in LDL-C levels. They concluded that sequence variants in *PCSK9* contribute significantly to interindividual variations in plasma LDL-C levels, and report that the spectrum of *PCSK9* alleles associated with LDL-C levels spanned a wide range of allele frequencies and magnitude of phenotypic effects.

In order to verify whether sequence variants in *PCSK9* could be a determinant of LDL-C plasma levels in the Japanese general population, we performed sequence analyses in the proximal promoter and all exons of *PCSK9* in individuals from the general population with the lowest and highest LDL-C levels and also in individuals taking antihypercholesterolemia medication since these individuals are presumed to have originally high levels of plasma LDL-C. Finally, we performed statistical analyses and compared the numbers of individuals with certain genetic variants between groups.

2. Methods

2.1. General population and the three investigated groups of individuals

DNA analysis was performed in individuals selected from the participants of the Suita cohort study, whose total sample included 3655 subjects. The study design of the Suita study has been described previously [17–19]. Briefly, the individuals were randomly selected from the municipal population registry, taking into consideration group stratification by gender and 10-year age divisions. The subjects visited the National Cardiovascular Center every 2 years for general health checkups. In addition to performing routine blood examinations, including lipid profiles, glucose levels, blood pressure and anthropometric measurements, a physician or nurse administered questionnaires regarding the individual's personal history of cardiovascular disease, including angina pectoris, myocardial infarction and stroke. Leukocyte DNA was collected from individuals who visited the National Cardiovascular Center between April 2002 and February 2004, and written informed consent was obtained from each individual before proceeding with genetic analysis. All clinical data, sequencing results and genotyping results were anonymous, and the study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

From the 3655 participants of the Suita study, we selected the 96 individuals who showed the lowest levels of LDL-C to form the low LDL-C group. After analysis, it became evident that 18 of these individuals were under antihypercholesterolemia treatment; these subjects were excluded, leaving a total of 78 individuals in this group. Additionally, there were 498 individuals who were under antihypercholesterolemia medication in the total population. From this treated population we selected the 96 individuals with the highest LDL-C levels to form the treatment group. Sixteen individuals in the treatment group had ischemic heart diseases. From the untreated 3139 individuals, we selected the 96 showing the highest LDL-C levels to form the high LDL-C group.

2.2. Lipid measurements

Total serum cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-C) levels were measured with an autoanalyzer (Toshiba TBA-80; Toshiba, Tokyo, Japan) using a fasting blood sample. LDL-C levels were calculated

using the Friedewald formula [20]. Individuals with triglyceride levels higher than 400 mg/dl were omitted.

2.3. DNA analysis

Genomic DNA was isolated from blood leukocytes using an NA-3000 nucleic acid isolation system (Kurabo, Osaka, Japan). The proximal promoter (−380 bp upstream) and all coding regions (including 10 bases of each exon–intron boundary sequence) were directly sequenced. Descriptions of the primers used for amplifying and sequencing fragments are given in the electronic appendix. Polymerase chain reaction (PCR) was performed with 10 ng of genomic DNA as the template in a 10-μl reaction mixture using a HotStar Taq Master Mix Kit (Qiagen, Hilden, Germany) as follows: *Taq* polymerase was activated at 95 °C for 15 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s. The PCR products were then treated with shrimp alkaline phosphatase and exonuclease I (PCR product pre-sequencing kit; USB Corporation, Cleveland, OH, USA), and used as templates for direct single-pass sequencing with a BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The reaction products were purified with Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden) and analyzed on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). The acquired sequences were examined for the presence of variants using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA) followed by visual inspection. Each detected variant was confirmed by repeat sequencing from the opposite direction. The A of the initiating ATG codon is denoted as nucleotide +1, and the nucleotide sequence (GenBank accession no: NT032977) was used as the reference sequence.

2.4. Statistical analysis

Lipid levels and ages were compared by Student's *t*-test. Deviations in the distributions of the genetic variants in the low LDL-C and high LDL-C/treatment groups were assessed by Fisher's two-sided exact test using the version 4 SNPAlyze statistical software (Dynacom Co. Ltd., Mobara, Japan). Pairwise linkage disequilibrium (LD) between two polymorphisms was evaluated by r^2 using SNPAlyze version 4.

3. Results

3.1. Participant characteristics

The characteristics of the individuals in the low LDL-C, high LDL-C and treatment groups, and those of the treated and untreated individuals in the total population, are shown in Table 1. The LDL-C levels of the individuals in the low LDL-C group ranged from 29.2 to 88.0 mg/dl (mean ± S.D., 70.3 ± 13.2 mg/dl), and those in the high LDL-C group ranged from 169.8 to 300.8 mg/dl (mean ± S.D., 196.7 ± 19.2 mg/dl). The LDL-C levels of

Table 1
Characteristics of individuals in the low LDL-C, high LDL-C and treatment groups, and of the total population of the Suita study

	Low LDL-C group	High LDL-C group	Treatment* group	Total population	
				Treated*	Untreated
No. (men/women)	78 (35/43)	96 (36/60)	96 (23/73)	516 (171/345)	3139 (1538/1601)
Age					
Range	39–88	40–85	44–85	44–90	35–93
Mean ± S.D.	64.2 ± 12.1	64.3 ± 10.2	69.2 ± 7.9+,++	69.9 ± 8.5	63.9 ± 11.5
LDL-C (mg/dl)					
Range	29.2–88.0	169.8–300.8	148.0–204.4	52.4–204.4	29.2–300.8
Mean ± S.D.	70.3 ± 13.2	196.7 ± 19.2**	165.1 ± 13.4+,++	126.9 ± 27.0	126.8 ± 29.8
Total cholesterol (mg/dl)					
Range	88–223	229–380	208–318	124–350	87–380
Mean ± S.D.	156.6 ± 22.9	277.4 ± 23.5**	246.3 ± 20.1+,++	210.4 ± 30.1	207.6 ± 32.9
Triglycerides (mg/dl)					
Range	21–396	40–230	49–289	33–435	18–1868
Mean ± S.D.	100.7 ± 71.4	113.3 ± 43.6	118.4 ± 48.1	119.5 ± 61.9	106.2 ± 76.2
HDL-C (mg/dl)					
Range	27–125	32–106	31–91	26–108	22–140
Mean ± S.D.	66.1 ± 20.6	58.0 ± 12.4**	57.5 ± 12.6+	59.4 ± 14.6	60.3 ± 15.8

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

* Treatment refers to antihypercholesterolemia medication. The lipid values of the individuals in the treatment group were taken under treatment. *P* values were obtained by Student's *t*-test.

** $P < 0.005$, low LDL-C group vs. high LDL-C group.

+ $P < 0.005$, low LDL-C group vs. treatment group.

++ $P < 0.005$, high LDL-C group vs. treatment group.

the subjects in the treatment group ranged from 148.0 to 204.4 mg/dl (mean ± S.D., 165.1 ± 13.4 mg/dl), which were lower than those of the high LDL-C group but significantly higher than those of the untreated population (mean ± S.D., 126.8 ± 29.8 mg/dl, P value by t-test <0.005).

Triglyceride levels did not differ significantly between the three groups and these values were also similar to those of treated and untreated individuals in the total population. HDL-C levels in the low LDL-C group were significantly higher than those of all other groups. Lower LDL-C levels in general tended to be accompanied by higher HDL-C levels, however, the reason for this remains unclear. There was no difference in age between the low and high LDL-C groups, however, ages in the treatment group were higher than those of the other groups. In the total population, the treated individuals also had higher ages than the untreated individuals. This may be because exercise or diet therapy is preferred for the treatment of hypercholesterolemia in younger patients, while lipid-lowering drug therapy is often adopted in older patients.

3.2. PCSK9 polymorphisms found

We sequenced 156 alleles from 78 individuals with low LDL-C levels, 192 alleles from 96 individuals with high LDL-C levels, and 192 alleles from the individuals taking antihypercholesterolemia medication, identifying a total of 33 genetic variants. A list of the genetic variants and their genotype distribution for each group are shown in 2Table 2. 'Allele 1' refers to the allele shown in the GenBank reference (accession no. NT032977), and 'Allele 2' is the variant allele. An LD defined by an r^2 value greater than 0.5 is indicated in the LD column; there are four LD groups (a–d). The minor allele frequencies of the variants in groups a (−64C > T, L21-22ins, and A53V) and b (c.658 − 7C > T and c.799 + 3A > G) were greater than 0.1, indicating that these variants are relatively common. With respect to group d, the two missense mutations, A514T and V624M, were found in the same individual. The r^2 values in groups a and c were both 1.00, indicating tight LD, and the value in group b was 0.83.

Fourteen of the 33 detected variants have previously been reported (see the columns for dbSNP ID and the references in Table 2), leaving 19 novel variants. Among 14 of the variants that have been already reported, five were found to date only in the Japanese population [14], thus, a total of 24 variants are specific to the Japanese population. The genetic variants that are specific to the Japanese population are shown in boldface type in Tables 2 and 3 and Fig. 1. Twenty of the 33 variants were nonsynonymous and resulted in an amino acid change, including one nonsense mutation (W428X) in exon 8 and one trinucleotide insertion resulting in the addition of an extra leucine in a leucine repeat (L21-22ins) in exon 1. The others were either synonymous variants in coding regions or variants in untranslated regions. The sites of the nonsynonymous mutations found in the present study are shown in Fig. 1; they were distributed along the entire structure of the *PCSK9* gene.

3.3. Contribution of the PCSK9 gene variants to plasma LDL-C levels

Statistical analysis was carried out in order to determine whether sequence variants in the *PCSK9* gene affect plasma LDL-C levels. Deviations in the distribution of each genetic variant in either the low LDL-C group or the high LDL-C/treatment group were assessed by Fisher's two-sided exact test. Of the tested 33 genetic variants, only a single missense mutation, R93C, was found to be significantly associated with the low LDL-C group ($P = 0.003$).

Next, we compared the numbers of individuals with nonsynonymous mutations in the low LDL-C and high LDL-C/treatment groups (Table 3), since nonsynonymous mutations are presumed to have stronger and more direct effects than synonymous or noncoding sequence variants. The allele frequencies of R93C in the low LDL-C group and in the high LDL-C/treatment group were 0.051 and 0.008, respectively (Table 3). Although the other nonsynonymous mutations did not show significant statistical values in distribution, several nonsynonymous mutations were found only in the low LDL-C group (Q219E, A239D, W428X, G452D and S668R) or only in the high LDL-C/treatment group (V4I, E32K, E54A, R104C, A514T and V624M). The sequence analysis on the *LDLR* gene was performed in the individuals who had the *PCSK9* mutations found only in the high/treatment group, confirming that there was no *LDLR* mutation in these individuals. L21-22ins, A53V, A68T, G263S, I424V, V474I, V644I and G670E were found in both the low LDL-C and high LDL-C/treatment groups.

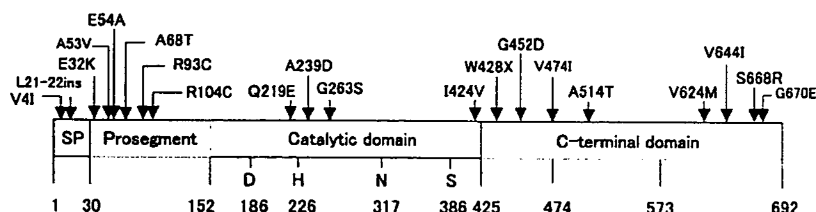

Fig. 1. Sites of the nonsynonymous mutations found in the present study. SP indicates signal peptide. The positions of the canonical aspartic acid (D), histidine (H), serine (S) catalytic triad, and oxyanion-hole asparagine (N) [3], are shown. The amino acid numbers are indicated at the bottom, and the sites of mutations found in the present study are indicated by arrows. Mutations shown in boldface type are those found only in the Japanese population.

Table 2
Genetic variants found and their genotypic distribution in each group

SNPs (allele 1 > allele 2)	LD ($r^2 > 0.5$)	Region	Amino acid change	No. of subjects: Low LDL-C group			High LDL-C group			Treatment group			Allele frequency		Flanking sequences	dbSNP ID	Reference
				Allele 1 Homo	Hetero	Allele 2 Homo	Allele 1 Homo	Hetero	Allele 2 Homo	Allele 1 Homo	Hetero	Allele 2 Homo	Allele 1	Allele 2			
−253G > A		Exon1		77	1	0	95	1	0	96	0	0	0.996	0.004	GCGGGCGCC[G/A]CCGTTCAGT		
−64C > T	a	Exon 1		63	15	0	76	18	2	72	22	2	0.883	0.117	AGGCGCCGC[C/T]GGCGTGGAC		[9,11,14,15]
c.10G > A		Exon 1	V4I	78	0	0	95	1	0	90	6	0	0.987	0.013	ATGGGCACC[G/A]TCAGCTCCA		[14]
c.63–64insCTG	a	Exon 1	L21-22ins	63	15	0	76	18	2	72	22	2	0.883	0.117	CTGCTGCTG[–/CTG]CTCCTGGGT		[9,11,14,15]
c.94G > A		Exon1	E32K	78	0	0	91	5	0	93	3	0	0.985	0.015	CGTGCGCAG[G/A]AGGACGAGG		
c.158C > T	a	Exon 1	A53V	63	15	0	76	18	2	72	22	2	0.883	0.117	ACGGCCTGG[C/T]CGAAGCACC	rs11583680	[9,11,14]
c.161A > C		Exon 1	E54A	78	0	0	96	0	0	95	1	0	0.998	0.002	GCCTGGCCG[A/C]AGCACCCGA		
c.202G > A		Exon 1	A68T	77	1	0	95	1	0	96	0	0	0.996	0.004	CACCGCTGC[G/A]CCAAGGTGC		
c.277C > T		Exon 2	R93C	70	8	0	95	1	0	94	2	0	0.980	0.020	CAGTCAGAG[C/T]GCACTGCCC		
c.310C > T		Exon 2	R104C	78	0	0	95	1	0	96	0	0	0.998	0.002	CAGGCTGCC[C/T]GCCGGGGAT		
c.336G > A		Exon 2	L112L	74	4	0	85	10	1	90	5	1	0.957	0.043	CAAGATCCT[G/A]CATGTCTTC		[14]
c.420C > T		Exon 3	V140V	78	0	0	96	0	0	95	1	0	0.998	0.002	GCCCCATGT[C/T]GACTACATC		
c.655C > G		Exon 4	Q219E	77	1	0	96	0	0	96	0	0	0.998	0.002	TTCCACAGA[C/G]AGGTAAGCA		
c.657 + 9G > A		Intron 4		73	5	0	91	5	0	93	3	0	0.976	0.024	GGTAAGCAC[G/A]GCCGTCTGA	rs11800243	[9,11,15]
c.658 − 7C > T	b	Intron 4		47	28	3	54	34	8	48	44	4	0.748	0.252	TGTGTTCGT[C/T]GAGCAGGCC	rs2483205	[9,11,15]
c.716C > A		Exon 5	A239D	77	1	0	96	0	0	96	0	0	0.998	0.002	GCCGGGATG[C/A]CGGCGTGGC		
c.787G > A		Exon 5	G263S	77	1	0	94	2	0	94	2	0	0.991	0.009	ACGGTTAGC[G/A]GCACCCTCA		
c.799 + 3A > G	b	Intron 5		42	32	4	48	39	9	46	44	6	0.715	0.285	CTCATAGGT[A/G]AGTGATGGC	rs2495477	[9,11,15]
c.993C > T		Exon 6	P331P	76	1	1	95	0	1	94	2	0	0.987	0.013	CTCAGCTCC[C/T]GAGGTAGGT		[14]
c.1227C > T		Exon 8	A409A	78	0	0	96	0	0	95	1	0	0.998	0.002	CACCCTGGC[C/T]GAGTTGAGG		
c.1270A > G		Exon 8	I424V	75	3	0	95	1	0	96	0	0	0.993	0.007	AAAGATGTC[A/G]TCAATGAGG		[14]
c.1284G > A		Exon 8	W428X	77	1	0	96	0	0	96	0	0	0.998	0.002	TGAGGCCTG[G/A]TTCCCTGAG		
c.1355G > A		Exon 9	G452D	77	1	0	96	0	0	96	0	0	0.998	0.002	TTTTTGCAG[G/A]TTGGCAGCT		
c.1380A > G	c	Exon 9	V460V	0	6	72	0	5	91	0	6	90	0.031	0.969	CAGGACTGT[A/G]TGGTCAGCA	rs540796	[9,11,14,15]
c.1420G > A	c	Exon 9	V474I	0	6	72	0	5	91	0	6	90	0.031	0.969	GCCACAGCC[G/A]TCGCCCGCT	rs562556	[9,11,14,15]
c.1540G > A	d	Exon 10	A514T	78	0	0	96	0	0	95	1	0	0.998	0.002	GCCCACAAC[G/A]CTTTTGGGG		
c.1863 + 6G > A		Intron 11		78	0	0	96	0	0	95	1	0	0.998	0.002	GCAGGTGAA[G/A]AGGCCCGTG		
c.1870G > A	d	Exon 12	V624M	78	0	0	96	0	0	95	1	0	0.998	0.002	CAGGTGACC[G/A]TGGCCTGCG		
c.1878C > T		Exon 12	C626C	78	0	0	95	1	0	96	0	0	0.998	0.002	CGTGGCCTG[C/T]GAGGAGGGC		
c.1930G > A		Exon 12	V644I	77	1	0	94	2	0	96	0	0	0.994	0.006	ACCTCCCAC[G/A]TCCTGGGGG		[14]
c.1947C > T		Exon 12	A649A	78	0	0	96	0	0	95	1	0	0.998	0.002	GGCCTACGC[C/T]GTAGACAAC		
c.2004C > A		Exon 12	S668R	77	1	0	96	0	0	96	0	0	0.998	0.002	CAGCACCAG[C/A]GAAGGGGCC		
c.2009G > A		Exon 12	G670E	0	6	72	0	13	83	0	6	90	0.046	0.954	CCAGCGAAG[G/A]GGCCGTGAC	rs505151	[9,11,15]

The mutations found only in Japanese are shown in boldface type. SNP, single nucleotide polymorphism; LD, linkage disequilibrium; homo, homozygote; hetero, heterozygote.

Table 3
No. of individuals with nonsynonymous mutations in the low LDL-C and high LDL-C/treatment groups

Amino acid change	Domain	No. of subjects			Allele frequency		LDL-C (mean ± S.D.) (mg/dl)	Identity with rodents
		Low LDL-C group ($n=78$)	High LDL-C group ($n=96$)	Treatment group ($n=96$)	Low LDL-C group ($n=78$)	High LDL-C/treatment group ($n=192$)		
Mutation significantly associated with the low LDL-C group								
R93C	**Prosegment**	**8**	**1**	**2**	**0.051***	**0.008***	**96.2 ± 43.0**	**N**
Mutations found only in the low LDL-C group								
Q219E	**Catalytic**	**1**	**0**	**0**	**0.006**	**0.000**	**83.6**	**Y**
A239D	**Catalytic**	**1**	**0**	**0**	**0.006**	**0.000**	**49.8**	**Y**
W428X	**C-terminal**	**1**	**0**	**0**	**0.006**	**0.000**	**70.4**	**Y**
G452D	**C-terminal**	**1**	**0**	**0**	**0.006**	**0.000**	**73.6**	**Y**
S668R	**C-terminal**	**1**	**0**	**0**	**0.006**	**0.000**	**87.6**	**Y**
Mutations found only in the high LDL-C/treatment group								
V4I	**Signal peptide**	**0**	**1**	**6**	**0.000**	**0.018**	**168.5 ± 14.4**	**N**
E32K	**Prosegment**	**0**	**5**	**3**	**0.000**	**0.021**	**167.5 ± 10.4**	**N**
E54A	**Prosegment**	**0**	**0**	**1**	**0.000**	**0.003**	**153.0**	**N**
R104C	**Prosegment**	**0**	**1**	**0**	**0.000**	**0.003**	**192.2**	**Y**
A514T	**C-terminal**	**0**	**0**	**1**	**0.000**	**0.003**	**192.2**	**Y**
V624M	**C-terminal**	**0**	**0**	**1**	**0.000**	**0.003**	**192.2**	**Y**
Mutations found in both groups								
L21-22ins	Signal peptide	15	18,2**	22,2+	0.096	0.125	146.3 ± 50.3++	N
A53V	Prosegment	15	18,2**	22,2+	0.096	0.125	146.3 ± 50.3++	N
A68T	**Prosegment**	**1**	**1**	**0**	**0.006**	**0.003**	**129.3 ± 89.2**	**N**
G263S	**Catalytic**	**1**	**2**	**2**	**0.006**	**0.010**	**172.4 ± 52.8**	**Y**
I424V	**Catalytic**	**3**	**1**	**0**	**0.019**	**0.003**	**104.0 ± 52.6**	**Y**
V474I	C-terminal	6	5	6	0.038	0.029	141.2 ± 53.6	N
V644I	**C-terminal**	**1**	**2**	**0**	**0.006**	**0.005**	**145.9 ± 50.4**	**N**
G670E	C-terminal	6	13	6	0.038	0.049	158.8 ± 55.2	Y

Catalytic, catalytic domain; C-terminal, C-terminal domain; * P-value by Fisher's exact test was 0.003; N, amino acid residue in human wild-type has no identity with those of mouse and rat; Y, amino acid in human wild-type has identity with those of mouse and rat; ** 18 heterozygotes and 2 homozygotes; + 22 heterozygotes and 2 homozygotes; ++ mean ± S.D. was calculated from the values of the heterozygotes. The mutations specific to Japanese are shown in boldface type.

4. Discussion

In order to identify the genetic variants in *PCSK9* affecting plasma LDL-C levels in the Japanese population, we screened the proximal promoter and the entire coding region sequences in 78 individuals with low LDL-C levels, 96 individuals with high LDL-C levels, and 96 individuals currently taking anti-hypercholesterolemia medication. All subjects were selected from a large sample of the general population ($n = 3655$).

Among the 33 detected sequence variants, only one missense mutation, R93C, was found to be significantly associated with the low LDL-C group ($P = 0.003$). This mutation has not been detected to date in the other populations; thus, it may be a specific genetic factor causing low LDL-C levels in the Japanese population. The other variants showed no statistical significance in their association to either low or high LDL-C levels. However, when the numbers of individuals with nonsynonymous mutations were compared between the low LDL-C group and the high LDL-C/treatment group (Table 3), four missense mutations, Q219E, A239D, G452D and S668R, and one nonsense mutation, W428X, were found only in the low LDL-C group. All of these have not been detected to date in the other populations. Five (or six) missense mutations, V4I, E32K, E54A, R104C, A514T (and/or V624M) were found only in the high LDL-C/treatment group. Since A514T and V624M were found in the same individual, the contribution of each mutation to the individual's high LDL-C level is less clear. All of these mutations are also specific to Japanese. The numbers of individuals bearing each mutation were very small and nine of these mutations were identified in single individuals and therefore they might have arisen by chance, however, because we have analyzed groups at opposite ends of the LDL-C spectrum, it is likely that some of these rare variants in the *PCSK9* gene are associated with either low or high LDL-C levels in the Japanese population. Family studies or similar investigations in other Japanese populations are necessary to fully understand the effect of each mutation.

Although at present it is difficult to determine the effect of each mutation, the frequencies of nonsynonymous mutations in the *PCSK9* gene were extremely high compared to those of *LDLR* and *apo B-100*. Heterozygotes with the *LDLR* mutations are found in approximately 0.2% of most general populations, and those with *apoB-100* are found in about 0.1% [21]. *ApoB-100* mutations have not yet been detected in the Japanese population [22]. Among the 20 nonsynonymous *PCSK9* mutations detected in the present study, L21-22ins, A53V, G670E and V474I were found to be distributed almost equally between the two examined groups (Table 3), and their allele frequencies were 0.117, 0.117, 0.046 and 0.031, respectively (Table 2). As these mutations have been found with high frequencies in several populations, they are known as common polymorphisms [11]. The allele frequencies of the remaining 16 nonsynonymous mutations ranged from 0.002 to 0.02. Collectively, these rare nonsynonymous mutations appeared in 47 of the 270 examined individuals (17%) in the present study, indicating extraordinary high frequencies of the mutations in this gene. Because of these high frequencies, the *PCSK9* gene might be an important determinant of plasma LDL-C levels in the general population.

When we examined the sites of the nonsynonymous mutations, the mutations detected only in the low LDL-C group were found to be concentrated in the catalytic and C-terminal domains, while the mutations found only in the high LDL-C/treatment group were distributed in the signal peptide, the prosegment and the C-terminal domain (Table 3). The amino acid residues of the mutations found only in the low LDL-C group are all conserved between rodents and humans (Table 3). Although the numbers of each mutation were too small to reach any definitive conclusions, we speculate that the mutations occur in the catalytic or C-terminal domain, which are expected to be important for the function of the protein, are prone to be hypocholesterolemic. These mutations, together with nonsense mutations, are prone to be hypocholesterolemic due to a loss-of-function mechanism. This speculation is consistent with the finding that the two nonsense mutations found in African Americans, Y142X and C679X, are hypocholesterolemic [8]. The present W428X mutation was also found in low the LDL-C group and the individual heterozygous for this mutation had 70.4 mg/dl of plasma LDL-C (Table 3), corresponding to a 44% decrease from the average LDL-C level in the untreated population (126.8 mg/dl, Table 1). This observation is consistent with the finding that the two African-American nonsense mutations were associated with a 40% reduction in plasma LDL-C levels [8].

Although selected mutations in the *PCSK9* gene have been found to cause severe phenotypes of hypercholesterolemia [9–11], more frequent genetic variants in *PCSK9* are associated with modest differences in plasma LDL-C [8,16]. Even if the effect of each mutation is small, *PCSK9* mutations might cumulatively contribute to differences in LDL-C levels in the general population.

We previously performed large-scale sequence analyses of five hypertension candidate genes (*WNK4*, *SCNN1B*, *SCNN1G*, *NR3C2*, and *RGS2*) to evaluate whether rare genetic mutations contribute collectively to quantitative trait variations, such as those found in blood pressure, finding that a low but significant subset of hypertensive subjects had missense/frameshift mutations [23–26]. Therefore, rare mutations collectively and partially contribute to quantitative trait variations, such as plasma levels of HDL-C [27,28] and LDL-C, and hypertension.

Based on the present analyses, nonsynonymous mutations in the *PCSK9* gene might contribute to either high or low levels of LDL-C in the Japanese general population.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2006.12.035.

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REGULAR ARTICLE

Genotypes of vitamin K epoxide reductase, γ-glutamyl carboxylase, and cytochrome P450 2C9 as determinants of daily warfarin dose in Japanese patients

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Abstract The dose required for the anticoagulant effect of warfarin exhibits large inter-individual variations. This study sought to determine the contribution of four genes, vitamin K epoxide reductase (*VKORC1*), γ-glutamyl carboxylase (*GGCX*), calumenin (*CALU*), and cytochrome P450 2C9 (*CYP2C9*) to the warfarin maintenance dose required in Japanese patients following ischemic stroke. We recruited 93 patients on stable anticoagulation with a target International Normalized Ratio (INR) of 1.6–2.6. We genotyped eleven representative single nucleotide polymorphisms (SNPs) in the three genes involved in vitamin K cycle and the 42613A>C SNP in *CYP2C9*, known as *CYP2C9*3*, and then examined an association of these genotypes with warfarin maintenance doses (mean±SD=2.96±1.06 mg/day). We found an association of effective warfarin dose with the −1639G>A (p=0.004) and 3730G>A genotypes (p=0.006) in *VKORC1*, the 8016G>A genotype in *GGCX* (p=0.022), and the 42613A>C genotype in *CYP2C9* (p=0.015). The model using the multiple regression analysis including age, sex, weight, and three genetic polymorphisms accounted for 33.3% of total variations in warfarin dose. The contribution to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9*

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42613A>C, and 4.6% for *GGCX* 8016G>A. In addition to polymorphisms in *VKORC1* and *CYP2C9*, we identified *GGCX* 8016G>A, resulting in the missense mutation R325Q, as a genetic determinant of warfarin maintenance dose in Japanese patients.
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Warfarin is the most widely prescribed anticoagulant for long-term prevention of thromboembolic events. The dose of warfarin required to achieve target levels of anticoagulation varies dependent on dietary intake and individual variations in pharmacokinetics. Management of warfarin therapy is difficult because of significant inter-individual and intra-individual variability and the narrow therapeutic range. The effectiveness and safety of warfarin must be monitored by serial determinations of prothrombin time using the standardized international normalized ratio (INR).

Warfarin exerts an anticoagulant effect by interfering with the regeneration of reduced vitamin K from the epoxide form, which is required for the enzymatic activity of vitamin K epoxide reductase subunit 1 (VKORC1) [1,2]. γ-Carboxylation of a wide variety of proteins, including numbers of factors in the clotting cascade, is catalyzed by γ-glutamyl carboxylase (GGCX), a vitamin K-dependent enzyme. This reaction incorporates a carbon dioxide molecule into specific glutamic acid residues with the help of the reduced form of vitamin K and oxygen, generating γ-carboxylglutamic acid and vitamin K 2,3-epoxide. When reduced vitamin K cannot be regenerated, the biosynthesis of vitamin K-dependent coagulation/anticoagulation factors, including prothrombin, factors VII, IX, and X, and proteins C and S, is suppressed. The endoplasmic reticulum resident protein calumenin (CALU) associates with γ-glutamyl carboxylase, inhibiting its activity [3]. Recent studies on the genetic aspects of the inter-individual variability of warfarin have demonstrated that single nucleotide polymorphisms (SNPs) in the *VKORC1* gene influence warfarin responses [4–15]. Haplotype analysis demonstrated that individuals who can be controlled by the low dose of warfarin showed the low hepatic expression of *VKORC1* mRNA [6].

The inter-individual variability of warfarin can also be explained by the genetic variability of the warfarin metabolizing enzyme, *CYP2C9*. The missense mutations R144C and I359L in the *CYP2C9* gene known as *CYP2C9*2* and *CYP2C9*3* are known to associate with warfarin dose [16]. These two genetic variations exhibited ethnic specificity. Asian population does not have the *CYP2C9*2* allele but carries the *CYP2C9*3* allele [17].

In this study, we investigated the influence of SNPs in four genes controlling γ-carboxylation (*VKORC1*, *GGCX*, *CALU*, and *CYP2C9*) on the inter-individual variability of warfarin dose requirements in Japanese patients. We identified SNPs in *VKORC1*, *GGCX*, and *CYP2C9* associated with the inter-individual differences in warfarin dosage.

Materials and methods

Subjects

The study population consisted of 93 unrelated Japanese patients admitted to the Cerebrovascular Division of the National Cardiovascular Center between November 2003 and March 2004. The patients had all experienced an ischemic stroke within the 7 days prior to admission. Stroke subtype consisted of cardioembolic infarction ($n=48$) and the embolic infarction of unknown origin with non-valvular atrial fibrillation ($n=45$). Anticoagulation of all patients was stably controlled with a target INR of 1.6–2.6 for the prevention of stroke recurrence [18,19]. Inclusion criteria were a confirmed date of initial exposure to warfarin, and current anticoagulation therapy. Data collection consisted of inpatient and outpatient medical records. The anticoagulant database was used to obtain information on daily warfarin doses. This study was approved by the Ethical Review Committee of the National Cardiovascular Center. All patients who participated in the study provided written informed consent for genetic analysis.

DNA analyses

We previously performed DNA sequence analyses of 3 genes (*VKORC1*, *GGCX*, and *CALU*) involved in vitamin K cycling in 96 Japanese stroke patients; that study identified genetic polymorphisms and pair-wise linkage disequilibrium (LD) [20]. Using the minor allele frequency (over 4%), LD (r^2 more than 0.5), and possible functional change (missense mutation) as guidance, we selected nine representative SNPs for genotyping: 523G>A, 1338A>G (H68R), and 3730G>A in *VKORC1*, 412G>A, 8016G>A (R325Q), and 8445C>T in *GGCX*, and 11G>A (R4Q), 344G>A, and 20943T>A in *CALU*. In *CYP2C9*, only the 42613A>C (I359L) SNP,

known as the *CYP2C9*3* genotype, was analyzed. In addition, recent studies have demonstrated the significant association of the *VKORC1* polymorphisms −1639G>A and 1173C>T with warf polymorphisms. We adopted the numbering standards of the Nomenclature Working Group, wherein the A of the initiator Met codon (ATG) is denoted nucleotide +1 [21].

The genotypes of the 12 SNPs in our subjects were identified by the TaqMan-PCR system. TaqMan genotyping methodology has been described previously [22]. The PCR primers and probes used for the TaqMan system are available on request.

Statistical analysis

The significance level for all statistical tests was set at $P<0.05$. Pair-wise LD between two polymorphisms was evaluated by r^2 using SNPAlyze v4.0 software (DYNACOM, Kanagawa, Japan). Statistical analyses were performed using JMP v 5.1 software and the SAS release 8.2 (SAS Institute Inc., Cary, NC). Associations between genotypes and warfarin daily doses were examined by one-way analysis of variance or univariate regression analysis. In addition, the relative contributions of age, sex, weight, and selected genetic variations to inter-individual variations in warfarin dose were estimated by using the multiple regression analysis. An index P_i, for estimating the relative contribution of a specific independent variable, x_i, was employed and given by

$$P_i = R^2 - R^2_{-i},$$

where R was the multiple correlation coefficient from the model with all of the selected independent variables ($x_1, x_2, \ldots, x_p$) and R^2_{-i} was that of the model excluding x_i from the independent variables.

Results

We analyzed the frequency of 11 SNPs in three genes involved in the vitamin K cycle and one polymorphism in *CYP2C9* 42613A>C (*CYP2C9*3*) in 93 stroke patients under stable anticoagulation with warfarin. Characteristics of the patients are summarized in Table 1. The mean±SD daily warfarin dose was 2.96±1.06 mg/day (1.00–5.50 mg/day).

Table 1 Characteristics of patients

Number	93
Number of men (%)	66 (71.0)
Age (years)	68.1±10.6
Weight (kg)	59.8±9.7
Warfarin dose (mg/day)	2.96±1.06
Warfarin dose range (mg/day)	1.00–5.50

Age, weight, and warfarin dose are shown as mean±SD.

Table 2 Differences in daily warfarin dose for each genotype of the *VKORC1*, *GGCX*, and *CYP2C9* genes

Gene	SNP	Genotype	*n*	Mean±SD (mg/day)	*P*
VKORC1	−1639 G>A*	AA	79	2.83±1.00	
		GA	14	3.70±1.11	0.004
		GG	0	–	
VKORC1	1173 C>T*	TT	79	2.83±1.00	
		CT	14	3.70±1.11	0.004
		CC	0	–	
VKORC1	3730 G>A*	GG	79	2.84±1.00	
		GA	14	3.68±1.12	0.006
		AA	0	–	
GGCX	8016 G>A (R325Q)	GG	48	3.25±1.19	
		GA	39	2.63±0.77	0.022
		AA	6	2.79±1.07	
CYP2C9	42613 A>C (CYP2C9*3) (I359L)	AA	83	3.06±1.05	
		AC	9	2.17±0.84	0.015
		CC	0	–	

P values were calculated by one-way ANOVA. *These SNPs were in linkage disequilibrium. Rieder et al. reported that the hepatic expression levels of *VKORC1* mRNA were significantly decreased in the carriers with the *VKORC1* −1639A allele [6]. As for the *GGCX* R325Q mutation, there were no available data on its function. CYP2C9 mutant carrying the missense mutation, I359L (*CYP2C9*3*), showed a markedly high *Km* for the 7-hydroxylation of S-warfarin [28].

We examined the association of the genotype data with maintenance warfarin doses by one-way analysis of variance (ANOVA). Of the 12 SNPs examined, five SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, 8016G>A (R375Q) in *GGCX*, and *CYP2C9*3* exhibited a significant association with daily warfarin dose (Table 2). The *VKORC1* 1338G>A allele could not be evaluated due to the low minor allele frequency. None of the other SNPs demonstrated a significant association with warfarin dosage.

The mean warfarin dose was higher ($p=0.004$) in patients with the *VKORC1* −1639GA or 1173CT genotypes (3.70 mg/day) than in those with the −1639AA or 1173TT genotypes (2.83 mg/day). The mean warfarin dose was higher ($p=0.006$) in patients with the *VKORC1* 3730GA genotype (3.68 mg/day) than in those with the 3730GG genotype (2.84 mg/day). For *CYP2C9*, the mean warfarin dose was higher ($p=0.015$) in patients with the *CYP2C9*1*1* (*CYP2C9* 42613AA) genotype (3.06 mg/day) than in those with the **1*3* (42613AC) genotype (2.17 mg/day).

A significant association was observed between warfarin dosage and the 8016G>A SNP of *GGCX*. The mean warfarin dose was higher ($p=0.022$) among patients with the *GGCX* 8016GG genotype (3.25 mg/day) than in those with the GA (2.84 mg/day) or AA (2.79 mg/day) genotypes. The *GGCX* 8016G>A SNP,