ated. To further select genes that are expressed almost exclusively in heart, expression values for the candidate genes were retrieved in 24 major tissues for analysis from GeneExpress database (Gene Logic Inc.) containing GeneChip expression profiles of human samples.

RNA extraction, RT-PCR, and quantification. Rat tissues (20–50 mg) and zebrafish embryos at 72 hpf were homogenized in 1 ml RNA-Bee reagent (Tel-Test Inc.), and total RNA was isolated and converted to cDNA using Omniscript RT kit (QIAGEN) according to the manufacturer's instructions. Specific primers to amplify rat ANP, β myosin heavy chain, cardiac-MLCK, and GAPDH mRNA were purchased from Applied Biosystems. Quantitative RT-PCR reactions were run in duplicate using the ABI Prism 7700 Sequence Detector System (Applied Biosystems). The level of each transcript was quantified by the threshold cycle (Ct) method using GAPDH as an endogenous control. For RT-PCR, specific primers that cover the region of targeted exons were designed to amplify the transcripts of z-cardiac-MLCK and z-MLC2v. See Supplemental Methods for primer sequences.

Northern blot analysis. Commercially available human multiple tissue Northern blot and polyA$^+$ RNA of human heart and skeletal muscle were purchased from Clontech. Each polyA$^+$ RNA was reverse transcribed and amplified using an Omniscript RT kit (QIAGEN) according to the manufacturer's protocol. Hybridization probes of human cardiac-MLCK and smMLCK were amplified by PCR from cDNA of human heart, and a hybridization probe of human skMLCK was amplified by PCR from cDNA of human skeletal muscle. Membrane was hybridized to ^{32}P-labeled probe in Rapid-Hyb buffer (Amersham Bioscience) at 65°C for 1 hour. Final wash conditions were 0.1× SSC with 0.1% SDS at 65°C for 5 minutes. Hybridized membrane was visualized by autoradiography using the BAS system (Fuji).

Preparation and transfection of adenovirus constructs. Adenovirus constructs were generated using ViraPower Adenoviral Expression System (Invitrogen) essentially as instructed by the manufacturer. Adenovirus vectors encoding murine cardiac-MLCK and LacZ were infected to cultured cardiomyocytes for 12 hours in various MOIs. Protein collection and immunostaining were performed 48 hours after adenovirus infection.

Identification of the substrate of cardiac-MLCK. Recombinant cardiac-MLCK was expressed in HEK293T cells as FLAG-tagged protein. HEK293T cells expressing FLAG-tagged cardiac-MLCK were lysed with cell lysis buffer (20 mM MOPS, pH 7.0, 0.15 M NaCl, 10% glycerol, and 1% CHAPS) and recombinant cardiac-MLCK was purified by immunoprecipitation using anti-FLAG-M2 affinity gel (Sigma-Aldrich). Hearts dissected from male C57BL/6 mice (10–12 weeks of age) were mechanically homogenized using a Polytron homogenizer in 10 ml of tissue lysis buffer (30 mM MOPS, pH 6.8, 5% glycerol, 0.1% 2-mercaptoethanol, and 1 mM EGTA). Lysate was centrifuged for 40 minutes at 100,000 *g*, and 9 ml of supernatant was collected. Murine heart extracts were then applied to SP650 cation exchange column. The column was equilibrated with elution buffer A (30 mM MOPS, 5% glycerol, 0.1% 2-mercaptoethanol) at pH 6.8, and the extracts were eluted with a linear gradient of NaCl (0–0.5 M) at a flow rate of 1 ml/min. Each 1-ml fraction collected was incubated for 30 minutes with activated recombinant cardiac-MLCK, commercially available recombinant calmodulin (Upstate), 2 mM $CaCl_2$, and [γ-P^{32}]ATP and then subjected to SDS-PAGE. After drying, the gel was autoradiographed and visualized with BAS (Fuji). The fractions containing 20-kDa substrate (fractions 10 and 11) labeled with [γ-P^{32}]ATP were pooled and applied to a phenyl-RPLC column (5Ph-AR-300; nacalai tesque) equilibrated with 0.3% trifluoroacetic acid and 5% acetoritrile. Fractions were eluted with a linear gradient of 100% acetonitrile at flow rate of 1 ml/min. After separation with SDS-PAGE, the gel was simultaneously silver stained and autoradiographed. After identifying the 20-kDa substrate with silver-stained gel, the bands were excised from the gel, and proteins were identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry and peptide mass fingerprinting.

Preparation of cultured neonatal rat cardiomyocytes and gene silencing via RNA interference. Primary cultures of neonatal cardiomyocytes were prepared from Wistar rats as described previously (29). Cardiomyocytes were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Equitech-Bio). At 6 hours after isolation of cardiomyocytes, cells were transfected with siRNAs (100 nmol/l) using Optifect reagent (Invitrogen) according to the manufacturer's instructions. Both si-cMK (see Supplemental Methods) and si-smMK (see Supplemental Methods) were purchased from B-bridge. As a negative control, cells were transfected with siControl Non-Targeting siRNA#1 (B-bridge). Isolation of mRNA was performed at 24 hours after transfection and protein experiments were performed at 72 hours after transfection. For immunostaining, the same procedures of siRNA transfection were performed in one-fifth scale on Lab-Tek Chamber Slides (nunc).

Cloning of z-cardiac-MLCK. We generated an adult zebrafish cDNA library in Lambda Zap II (Stratagene) using polyA$^+$ RNA from adult zebrafish. The cDNA library was screened with the probe designed to the 5′ side in the ORF of the putative zebrafish ortholog of cardiac-MLCK sequence. Positive phage clone was determined by using phage plaque screen method and single clone excision protocol according to the manufacturer's instructions (Stratagene).

Gene accession numbers. DDBJ accession numbers for the zebrafish MLCK family were as follows: cardiac-MLCK, AB267907; smMLCK, AB267908; skMLCK, AB267909.

Whole-mount in situ hybridization. The digoxigenin-labeled antisense and sense RNA probes (see Supplemental Methods) were transcribed using SP6 and T7 RNA polymerase. Zebrafish embryos at 24 and 48 hpf were fixed with 4% paraformaldehyde, digested with proteinase K, and hybridized with each probe at 68°C. Alkaline-conjugated anti-digoxigenin antibody was used to detect the signals. After staining, embryos were refixed with 4% paraformaldehyde and stored in PBS.

Injection of MO. All MOs were synthesized by Gene-Tools. At cell stages 1–4, 4–10 ng of these MOs were injected into zebrafish embryos. Several data were collected before the 96-hpf stage. Sequences of MOs are available in the Supplemental Methods.

Analysis of zebrafish cardiac histology and cardiac function. We studied hearts of control mock-injected zebrafish embryos and z-cMKaugMO-injected zebrafish embryos at 72 hpf by routine histopathology including transmission electron micrography. To visualize the motion of zebrafish cardiac ventricle, the SAG4A strain of zebrafish, which specifically expresses GFP in its cardiac ventricular wall (14), was applied to MO-mediated gene knockdown experiments. GFP-expressed control mock-injected and z-cMKaugMO-injected zebrafish hearts at 72 hpf were imaged with Leica digital camera DFC 350 FX on a Leica MZ 16 FA fluorescence stereomicroscope. Acquired images were compiled as digital movie files using Leica FW4000 software. Each recorded movie was converted to M-mode image using our original software, and Dd, Ds, FS, and heart rate were measured from the M-mode images.

Experimental protocols of rats. Male Wistar rats (0 days, 1 week, 2 weeks, and 10 weeks for mRNA and protein expression analysis; 8 weeks for production of MI rats; Japan Animals) were used in these experiments. MI was induced by permanent ligation of the left anterior descending coronary artery as previously described (29). The same surgical procedure was performed in a sham-operated group of rats except that the suture around the coronary artery was not tied. Isolation of total RNA was performed at 4 weeks after the onset of MI from noninfarcted myocardiums of resected LVs.

Statistics. Statistical analysis was performed using Mann-Whitney *U* test and single regression analysis. Data are presented as mean ± SEM. A *P* value less than 0.05 was considered significant.

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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.

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.

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.

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.

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 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.

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

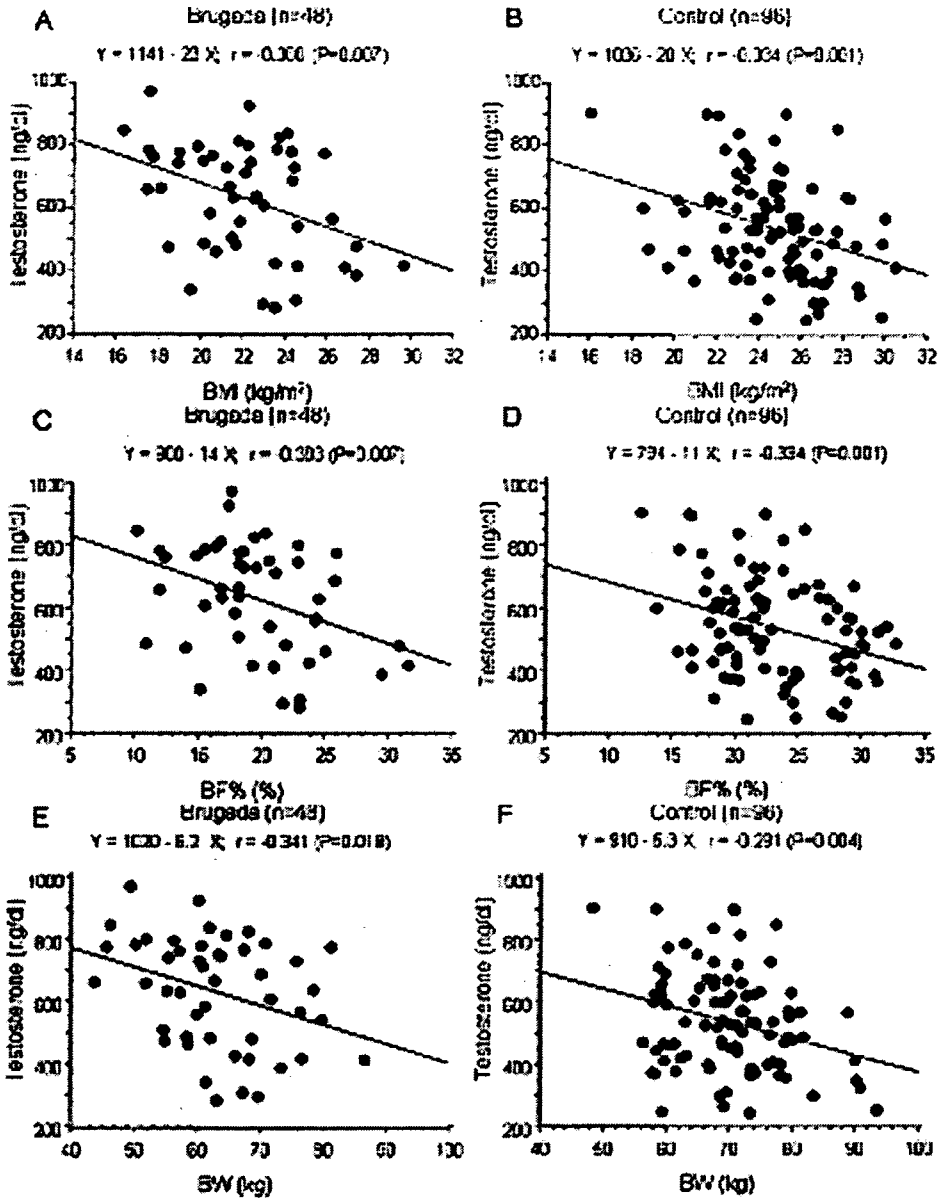

Figure 1. *Correlation between testosterone level and visceral fat parameters; body mass index (BMI) (A and B), body fat percentage (BF%) (C and D), and body weight (BW) (E and F) in the 48 Brugada males and the 96 age-matched control males. Testosterone level was inversely correlated with the BMI, BF%, 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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Polymorphism of CYP11B2 Determines Salt Sensitivity in Japanese

Naoharu Iwai, Kazuaki Kajimoto, Hitonobu Tomoike and Naoyuki Takashima

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Polymorphism of *CYP11B2* Determines Salt Sensitivity in Japanese

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Abstract—Aldosterone plays essential roles in body fluid and electrolyte homeostasis and blood pressure. However, the association between polymorphisms in the *CYP11B2* gene and hypertension is controversial. We resequenced *CYP11B1* and *CYP11B2* and identified 35 polymorphisms in this region. We performed association studies between the plasma aldosterone concentration and 13 polymorphisms in this region in 1443 subjects. The subjects were all obtained from the Suita Cohort Study. Multiple regression analysis indicated that aldosterone levels were determined by renin activity, age, total cholesterol, and hematocrit. Residuals of the aldosterone levels after adjusting for these confounding factors were nominally associated with the T(−344)C (P=0.0026), C(595)T (P=0.0180), −(4837)C (P=0.0310), and G(4936)A (P=0.0498) polymorphisms. Only the T(−344)C polymorphism was significantly associated with the aldosterone level after a correction for multiple testing (Bonferroni). A significant interaction was observed between the T(−344)C polymorphism and renin activity in determining aldosterone levels. Moreover, a significant interaction was observed in 2063 subjects between urinary sodium excretion, which reflects sodium intake, and the T(−344)C polymorphism in determining systolic blood pressure. Only subjects with the TT genotype showed a positive correlation between urinary sodium excretion and systolic blood pressure. In vitro experiments confirmed the functional significance of this T(−344)C polymorphism in terms of angiotensin II reactivity. Thus, the T(−344)C polymorphism in *CYP11B2* appears to affect salt sensitivity in Japanese and to have clinical significance. (***Hypertension.* 2007; 49:825-831.)**

Key Words: aldosterone ■ polymorphism ■ hypertension ■ salt ■ genetics

Aldosterone synthase (*CYP11B2*), a cytochrome P450 enzyme that is mainly expressed in the zona glomerulosa of the adrenal cortex, is a key enzyme in aldosterone synthesis.[1,2] Aldosterone controls the sodium balance and, thus, influences blood pressure regulation. Rare mutations of this gene are associated with either markedly elevated aldosterone levels and hypertension or insufficient aldosterone synthesis and sodium wasting.[3–6] Thus, *CYP11B2* is a candidate gene that may contribute to salt-sensitive hypertension.

The T(−344)C polymorphism, which is located at the putative binding site for the steroidogenic transcriptional factor, has been reported to be associated with hypertension[7–12] and/or hypertension-related phenotypes, such as the plasma aldosterone level,[12,13] urinary aldosterone excretion,[8,14] aldosterone:renin ratio,[7,11,12,15–17] and left ventricular hypertrophy.[18]

We reported previously that the T(−344)C polymorphism was unlikely to influence the blood pressure status in the Japanese population based on an analysis of 4049 subjects recruited from the Suita Study.[19] However, in the previous study, we had not determined sodium and potassium intakes, aldosterone levels, and plasma renin activity levels, which all seem to be important factors in determining the significance of *CYP11B2* polymorphisms. Moreover, other polymorphisms than the T(−344)C in the *CYP11B2* region may be associated with aldosterone production and/or hypertension. Thus, in the present study, we resequenced the *CYP11B2* gene regions and performed extensive association studies between the polymorphisms in this region and phenotypes related to possible functions of *CYP11B2*.

Methods

Study Population

The selection criteria and study design of the Suita Study have been described previously.[20–22] The sample consisted of 14 200 men and women (30 to 79 years of age at enrollment), stratified by gender and 10-year age groups (10 groups and 1420 subjects in each group), who had been randomly selected from the municipal population registry. They were all invited, by letter, to attend regular cycles of follow-up examination (every 2 years). We routinely check 10 to 15 participants per day. DNA from leukocytes was collected from participants who visited the National Cardiovascular Center between April 2002 and March 2005. The present study included 2 study populations. Study population I consisted of 2779 subjects whose DNA was collected between April 2002 and October 2003. In this population, we collected spot urine specimens for the assessment of urinary sodium and potassium excretion. Study population II consisted of 1995 subjects whose DNA was collected between November 2003 and March 2005. In this study population, we measured

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TABLE 1. Characteristics of Study Population I

Phenotype	Genotype of the T(−344)C			P
	TT	TC	CC	
No.	931	920	212	
Male, %	46	44.5	41.5	n.s.
Age, y	62.6±11.2	63.3±11.5	62.1±12.0	n.s.
BMI, kg/m^2	22.43±2.85	22.44±3.20	22.40±3.07	n.s.
SBP, mm Hg	125.4±18.8	125.5±17.9	124.9±18.7	n.s.
DBP, mm Hg	76.9±10.0	76.4±10.1	76.7±11.1	n.s.
Total cholesterol, mg/dL	206.6±32.3	208.2±32.1	209.9±33.7	n.s.
Hct, %	40.99±3.72	41.05±3.87	40.72±3.92	n.s.
Residual SBP, mm Hg	0.15±16.77	−0.13±16.8	−0.04±16.84	n.s.
Residual DBP, mm Hg	0.16±9.53	−0.22±9.53	0.27±10.51	n.s.
24-h Na, mEq/day	136.01±32.2	135.37±33.0	135.68±31.6	n.s.
24-h K, mEq/day	39.47±7.52	39.24±7.60	39.33±7.82	n.s.

Characteristics of the study population I are shown according to the genotypes of the T(−344)C polymorphism of *CYP11B2*. BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Hct, hematocrit; 24-h Na, urinary sodium excretion; 24-h K, urinary potassium excretion; n.s., not significant. Residuals of SBP and DBP were calculated by adjusting for age and BMI.

plasma renin activity (PRA) and plasma aldosterone concentrations (PACs). All of the participants were Japanese, and only those who gave their written informed consent for genetic analyses of cardiovascular diseases were included. The study protocol was approved by the institutional ethics committee.

Blood pressure was measured after 10 minutes of rest in a sitting position. Systolic (SBP) and diastolic blood pressure (DBP) values were the means of 2 physician-obtained measurements. Physicians obtained detailed personal medical information directly from the participants. The diagnosis of hypertension was based on blood pressure measurement (SBP ≥140 mm Hg, or DBP ≥90 mm Hg) or the current use of antihypertensive medication.

Twenty-four–hour urinary sodium and potassium excretion was estimated from spot urine specimens as reported previously.[23] Spot urine specimens were collected between 9:00 AM and 10:00 AM. The relationship between blood pressure levels and the urinary excretion of sodium and potassium was assessed in study population I. We excluded subjects who were receiving antihypertensive medication. Thus, the total number of subjects in this study was 2063 (Table 1).

PACs (nanograms per milliliter) and PRA (nanograms of angiotensin I per milliliter per hour) were measured in study population II. PAC was measured by radioimmunoassay (intra-assay variance <6%; interassay variance <5%). PRA was measured by radioimmunoassay for angiotensin II (nanograms per milliliter per hour; PRA, intra-assay variance <8%, interassay variance <9%). Blood was collected between 9:30 AM and 10:30 AM after an overnight fast and after a 10-minute rest in the sitting position. To investigate the relationship between genotypes and PAC, we excluded subjects whose PAC appeared to be modulated by secondary causes. First, subjects who were receiving antihypertensive treatment were excluded, because antihypertensive medication can obscure genetic effects on aldosterone levels. Of the remaining 1474 subjects, 246 had hypertension, and of these, 5 (2.0%) had possible renovascular hypertension (hypertensive with PRA ≥3.0 and PAC ≥120) and 26 (10.6%) had possible primary aldosteronism (hypertensive with PRA ≤1.0 and PAC ≥120). There were no subjects with possible hyperreninemic hypoaldosteronism in our study population. The criteria for secondary hypertension, including renovascular hypertension or aldosteronism, were based on the proposal by Omura et al.[24] Thus, the final number of subjects was 1443 (Table 2). Of these 1443 subjects, 696 subjects had visited us between April 2002 and October 2003, and their clinical data at that time were included in study population I.

TABLE 2. Characteristics of Study Population II

Phenotype	Genotypes of the T(−344)C			P
	TT	TC	CC	
No.	699	568	176	
Male, %	42.2	42.3	42.5	n.s.
Age, y	63.5±11.3	63.4±10.9	64.5±11.1	n.s.
BMI	22.50±2.95	22.46±3.06	22.39±3.06	n.s.
Log(PAC)	4.48±0.37	4.45±0.38	4.40±0.40	0.0471
Log(PRA)	−0.25±0.83	−0.16±0.78	−0.12±0.81	0.0525
SBP, mm Hg	121.5±18.0	119.4±17.2	120.0±18.5	0.0994
DBP, mm Hg	74.7±10.0	73.8±9.8	74.5±9.7	n.s.
Total cholesterol, mg/dL	211.7±32.3	210.3±32.3	210.9±36.0	n.s.

Characteristics of the study population II are shown according to the genotypes of the T(−344)C polymorphism of *CYP11B2*. Residual of log(PAC) was calculated by adjusting for age, total cholesterol, hematocrit, and log(PRA). BMI indicates body mass index.

Sequence Analysis of *CYP11B2* and *CYP11B1*

The promoter and exon regions of *CYP11B2* and *CYP11B1* were resequenced in 96 subjects with a high or low aldosterone/renin ratio (top 48+bottom 48 in 1995 subjects in study population II). Because the homology between these 2 genes is very high, sequence amplification primers were designed to correspond with gene-specific regions (Table S1, available online at http://hyper.ahajournals.org). The linkage disequilibrium structure among the polymorphisms in this region was calculated (R^2 and D′) using the SNPAlyze statistical package (Dynacom). Based on this linkage disequilibrium structure (Figure 1), 13 polymorphisms were selected for genotyping by the TaqMan method (Table S2).

Genotype Determination and Statistical Analysis

All of the statistical analyses were performed using the JMP statistical package (SAS Institute Inc). PRA and PAC levels were logarithmically transformed to obtain a normal distribution. Multiple logistic and regression analyses were performed with other covariates. Residuals were the observed values minus predicted values based on confounding factors. Residuals of SBP and DBP were calculated by adjusting for age and body mass index. Because the relationship between age and DBP was not linear, a polynomial equation (n=2) was applied to accommodate a decline in DBP in elderly subjects. Haplotype-based association analyses were performed using the SNPAlyze statistical package (Dynacom).

Functional Analysis of the T(−344)C Polymorphism

To examine whether the T(−344)C polymorphism modulates the expression level of the *CYP11B2* gene, the region from −435 (promoter) to +812 (exon2) of *CYP11B2* (nucleotide number indicates the relative position from the translation initiation codon) was amplified and subcloned into the *Hind*III and *Xba*I sites of pGL3-Basic vector (Promega). This promoter region has been reported to be enough to maintain promoter activity and angiotensin II responsiveness.[25] We included intron 1 in the construct, because our preliminary experiments indicated that this construct enhanced the expression level of the reporter transcript. Primers used for amplification were as follows: *Hind*III-forward, 5′-aagcttggtacgtggacattttctgca, and *Xba*I-reverse, 5′-tctagaccagctctcagctcccaac. We constructed 4 types of expression vectors corresponding with the 4 haplotypes defined by the T(−344)C and C(595)T polymorphisms. We used 2 cell lines, namely, Y1 and

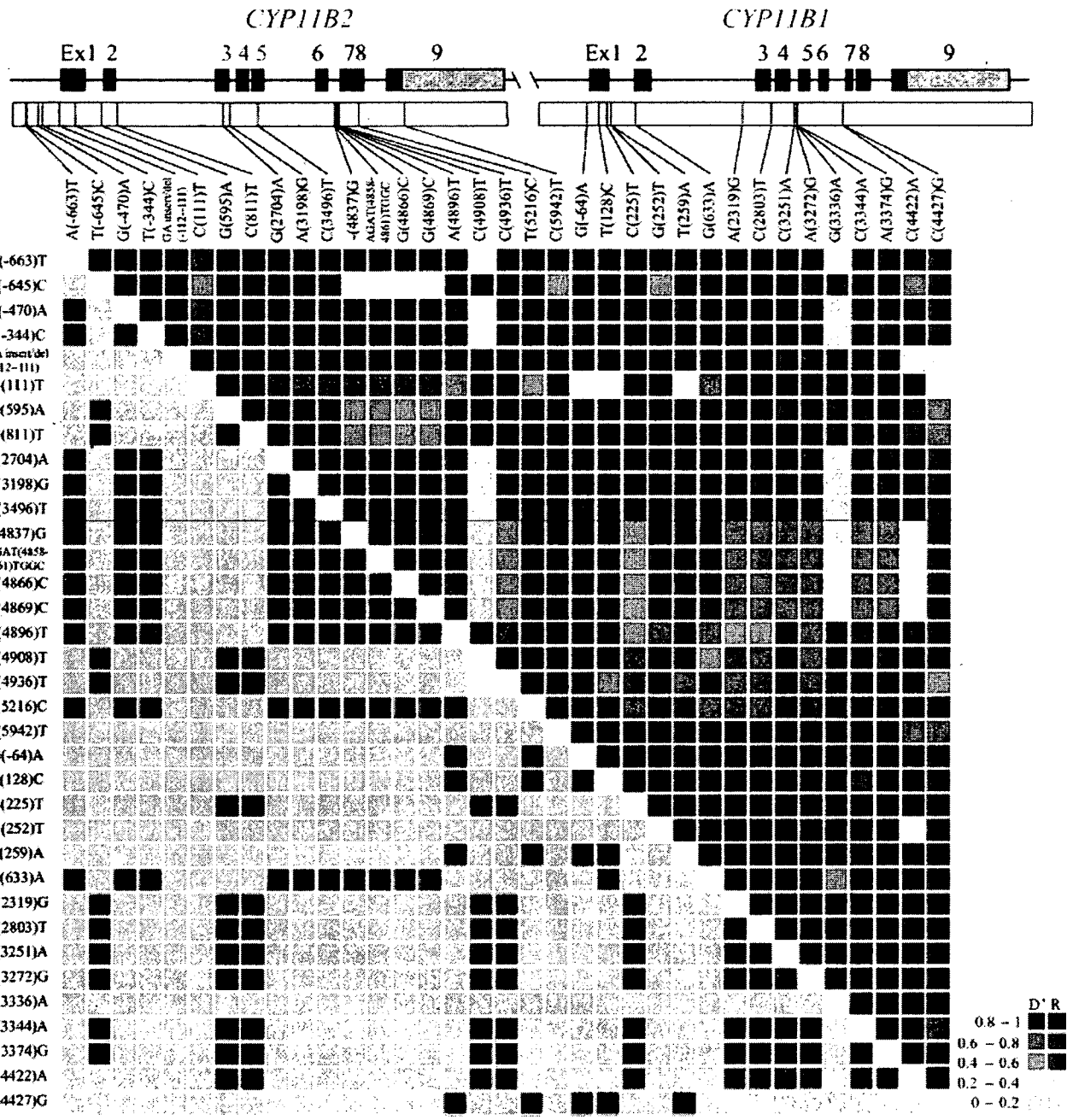

Figure 1. Linkage disequilibrium among the single nucleotide polymorphisms in the *CYP11B1* and *CYP11B2* genes. In this schematic of the *CYP11B2* and *CYP11B1* genes, ▨ indicates 5′- and 3′-untranslated regions, and ■ indicate coding regions. Two measures of linkage disequilibrium are shown: D′-values in the upper right triangle and R^2 values in the lower left triangle. Color-coded scales for D′-values or R^2 values (measures of linkage disequilibrium (LD strength) are on the right.

H295R adrenocortical cells, for promoter reporter analyses. No significant modulation of promoter activity by angiotensin II was observed in Y1 cells (data not shown). H295R adrenocortical cells were cultured in a medium containing a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 15 mmol/L of HEPES, 0.5 mmol/L of sodium pyruvate, 1.2g/L of sodium bicarbonate, 6.25 μg/mL of insulin, 6.25 μg/mL of transferrin, 6.25 ng/mL of selenium, 1.25 mg/mL of bovine serum albumin, 5.35 μg/mL of linoleic acid, and 2.5% of ν-Serum (BD Bioscience). Transient transfection was performed using Lipofectamine2000 reagent (Invitrogen). Cells were seeded onto 6-well plates to 70% to 80% confluence and, 24-hour later, transiently cotransfected with 4 μg of a reporter plasmid and 1 μg of pAcGFP1-C1 vector, which expresses green fluorescence protein (GFP; Clontech), per well. After incubation for 6 hours, cells were washed with 1× PBS and further incubated in fresh medium for 24 hour in the presence or absence of angiotensin II (0.5 μmol/L). This incubation period of 6 hours was based on the previous study.[25] Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol and was briefly treated with RNase-free DNaseI. RT-PCR was carried out with the following amplimers: Ex1-S (5′-gcaaaggcagaggtgtgcgt) and Ex2-AS (5′-cgccacatttgtgcccacga) for CYP11B2 and AcGFP-S (5′-cctgatcgagctgaatggcg) and AcGFP-A (5′-tgctggtagtggtcggccag) for GFP. PCR products were subjected to 1.5% agarose gel

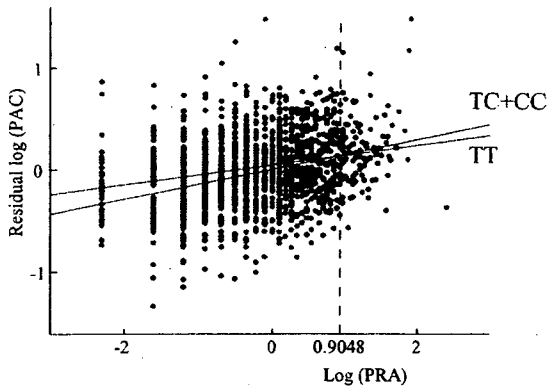

Figure 2. Interaction between the T(−344)C polymorphism and PRA in determining PAC. Residual [log(PAC)] in this figure was calculated by adjusting for age, total cholesterol, and hematocrit. Significant correlations were observed between residual [log(PAC)] and log(PRA) in subjects with the TT genotype and in subjects with the TC or CC genotype. However, there were significant differences in the correlation coefficients between the 2 groups. Subjects with the TT genotype are indicated by red dots, and those with TC or CC genotype are indicated by blue dots.

electrophoresis and visualized by ethidium bromide. The intensity of the PCR product from the CYP11B2 reporter plasmid (374 bp) relative to that from pAcGFP1-C1 (510 bp) was assessed by a densitometer.

Results

Sequence Analysis of *CYP11B2* and *CYP11B1*

Sequence analysis in this region identified 35 polymorphisms (Table S3). Based on the linkage disequilibrium structure in this region, 13 polymorphisms were selected for genotyping (Table S2). These 13 polymorphisms were sufficient to cover all of the major haplotypes in this region (Figure 1).

Association Analysis Between Polymorphisms and Renin/Aldosterone Profiles

Multiple regression analysis indicated that log(PAC) was determined (R^2=0.219; P<0.0001) by log(PRA) (t=11.40; P<0.0001), age (t=−8.82; P<0.0001), total cholesterol level (t=2.70; P=0.0070), and hematocrit (t=7.92; P<0.0001). Cholesterol is a substrate for aldosterone synthesis. Higher hematocrit may reflect dehydration and, thus, may be positively associated with higher aldosterone levels. Residuals of log(PAC) were calculated by adjusting for the above confounding factors. Of the 13 polymorphisms genotyped, T(−344)C (P=0.0026), C(595)T (P=0.0180), −(4837)C (P=0.0310), and G(4936)A (P=0.0498) showed a P<0.05 association with aldosterone levels. Only the T(−344)C polymorphism was significantly associated with the residuals of log(PAC) levels after the Bonferroni correction (P= 0.0026×13=0.0338). Haplotype analysis did not increase the power of the association with aldosterone levels (data not shown).

Intriguingly, a significant interaction (P=0.0277) was observed between the T(−344)C (TT/TC+CC) polymorphism and log(PRA) in determining log(PAC) levels (Figure 2). Aldosterone levels were linearly correlated with renin activity. However, the slope was different between the TT and TC+CC genotypes. Subjects with the TT genotype seem to have inappropriately higher aldosterone levels under high salt intake (low renin activity).

Association Analysis Between Blood Pressure and the T(−344)C Polymorphism

Next, we investigated whether the T(−344)C polymorphism might influence blood pressure levels in Japanese. Although the TT genotype tended to be associated with higher SBP in study population II, we may not be able to assess the significance of this polymorphism without knowing the sodium balance (see Discussion section). Thus, this assessment was performed in study population I, which included 2063 subjects whose sodium and potassium excretion was estimated from a spot urine specimen.

Multiple regression analysis indicated that SBP levels were determined (R^2=0.175; P<0.0001) by age (t ratio=18.26; P<0.0001), body mass index (t ratio=9.76; P<0.0001), and urinary sodium (t ratio=3.78; P=0.0002) and potassium (t ratio=−2.81; P=0.0050) excretion. Intriguingly, whereas the T(−344)C genotype had no significant influence on SBP (P=0.7179), a significant interaction was observed between urinary sodium excretion and the T(−344)C polymorphism (TT/TC+CC; P=0.0137). This means that a significant positive correlation between SBP and sodium excretion was observed in subjects with the TT genotype but not in subjects with the TC or CC genotype (Figure 3). However, a similar interaction was not observed for DBP.

Functional Analysis of the T(−344)C Polymorphism

To investigate whether the T(−344)C polymorphism might modulate the expression of *CYP11B2*, we constructed reporter plasmids that expressed a fusion transcript under the *CYP11B2* promoter, which contained the T(−344)C poly-

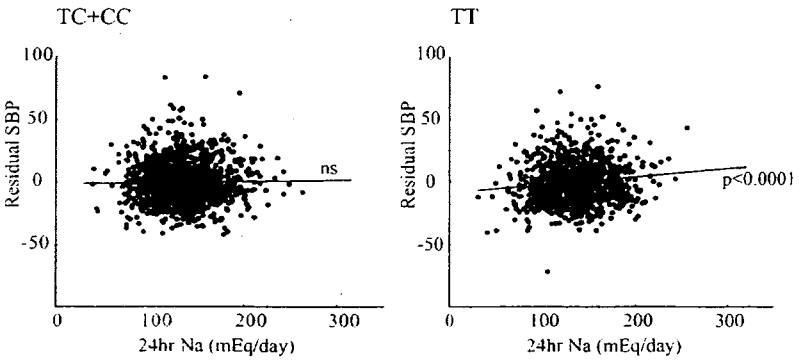

Figure 3. Interaction between the T(−344)C polymorphism and 24-hour Na in determining SBP. Residual SBP in this figure was calculated by adjusting for age, body mass index, and 24-hour K. Significant correlations were observed between residual SBP and 24-hour Na in subjects with the TT genotype but not in subjects with the TC or CC genotype.

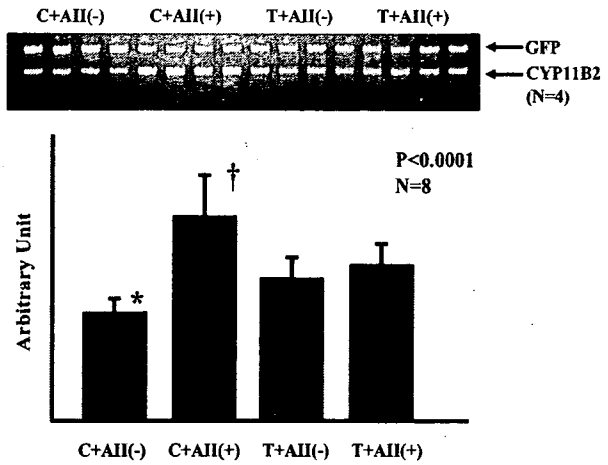

Figure 4. Promoter reporter assay on the T(−344)C polymorphism. H295R cells were transiently cotransfected with a reporter construct (see Methods section) and AcGFP1 expression vector. The promoter activities were assessed under the presence or absence of angiotensin II (0.5 μmol/L). The relative intensity of the PCR product from the *CYP11B2* reporter plasmid to the PCR product from AcGFP1 vector was assessed by a densitometer. Data are mean±SD (N=8 for each group). Representative photograph is shown above (N=4 for each group). C+AII(−): C(−344) type promoter under the absence of angiotensin II; C+AII(+): C(−344) type promoter under the presence of angiotensin II; T+AII(−): T(−344) type promoter under the absence of angiotensin II; T+AII(+): T(−344) type promoter under the presence of angiotensin II. *Significantly different from T+AII(−) [$P<0.05$], T+AII(+) [$P<0.05$], and C+AII(+) [$P<0.01$]. † indicates significantly different from T+AII(−) [$P<0.01$], T+AII(+) [$P<0.01$], and C+AII(−) [$P<0.01$].

morphic site. The expression levels of the fusion transcript were assessed by RT-PCR with the GFP transcript as an internal standard. The promoter activities of the T(−344) and C(−344) types were assessed in the presence or absence of angiotensin II (Figure 4). One-way ANOVA indicated that there was a significant difference among the 4 groups ($P<0.0001$). Subsequent analysis indicated that the promoter activity of the C(−344) type in the absence of angiotensin II stimulation was significantly less than that in the presence of angiotensin II stimulation ($P<0.01$). However, such modulation by angiotensin II was not observed in the promoter activity of the T(−344) type. Intron 1 had another C(595)T polymorphic site. The constructs in Figure 4 corresponded with the C(595) type. Almost identical results were obtained with the constructs that corresponded with the T(595) type (data not shown).

Discussion

Aldosterone plays essential roles in body fluid and electrolyte homeostasis and blood pressure. However, the association between polymorphisms in the *CYP11B2* gene and hypertension is controversial.[7-19]

In the present study, we performed extensive association studies between polymorphisms in *CYP11B2* regions and renin–aldosterone profiles and blood pressure values. Our findings in study population II indicate that subjects with the TT genotype of the T(−344)C polymorphism appear to have higher aldosterone levels in response to PRA levels than those with the TC or CC genotype when renin activity is <2.45 ($e^{0.9}$=2.45; Figure 2). This raises the possibility that subjects with the TT genotype might have inappropriately high aldosterone levels under high salt intake (low renin activity state) and might have higher blood pressure under high salt intake.

There are at least three possibilities with regard to the aldosterone/sodium/volume/pressure relationship. First, relatively higher aldosterone levels in subjects with the TT genotype are offset by suppressing renin activity, and appropriate aldosterone levels are maintained. Thus, the genotype is not associated with a tendency for volume retention and, thus, not associated with higher blood pressure. Second, relatively higher aldosterone levels in subjects with the TT genotype are offset by suppressing renin activity. However, the degree of suppression of renin activity may not be enough to prevent sodium retention, and the TT genotype is associated with higher blood pressure. Third, the tendency for sodium retention in subjects with the TT genotype may be offset by the reduced intake of sodium. A similar situation has been reported in subjects with Gitelman's syndrome mutations, who develop higher sodium intake to prevent volume loss and low blood pressure levels.[26] According to the observation in study population II, the first possibility seems to be unlikely, because subjects with the TT genotype had higher aldosterone levels under low renin activity (reflecting high sodium intake) as shown in Figure 2. To evaluate the second and third possibilities, we should know the sodium intake status of the study population. Therefore, the relationship between the polymorphism and blood pressure was assessed in study population I, in which sodium intake status was assessed by spot urine. The observation in study population I indicated that only subjects with the TT genotype showed a positive correlation between SBP and urinary sodium excretion, which supports the second hypothesis inferred from the observation in study population II. However, urinary sodium excretion was not different among subjects with different genotypes, which excludes the third hypothesis (Table 1).

The present study confirmed the hypothesis that the T(−344)C polymorphism determines salt sensitivity through the use of 2 separate study populations with different phenotypes: renin–aldosterone profile (study population II) and sodium–blood pressure relationship (study population I). In this sense, it may be appropriate to say that the observation in study population II is validated by the observation in study population I. However, genetic studies have been fraught with inconsistent results, probably in part from trying to attribute small and likely chance differences in blood pressure to specific polymorphisms. Therefore, validation in other study populations is warranted to establish the present hypothesis.

The in vitro reporter analysis indicated that only the C(−344) type promoter was responsive to angiotensin II stimulation (Figure 4). Moreover, promoter activity of the C type was lower than that of the T type in the absence of angiotensin II. The absence of angiotensin II may be considered to be equivalent to high salt intake. Thus, the results of the in vitro experiment were in good agreement with the epidemiological data presented in Figures 2 and 3.

Previously, we reported that the T(−344)C polymorphism in *CYP11B2* was unlikely to influence blood pressure levels in Japanese.[19] However, that previous conclusion was based on observations that did not consider sodium and potassium intake in the analysis. Our present conclusion is that the relationship between the T(−344)C polymorphism and blood pressure depends on the sodium intake status. This is not inconsistent with the previous study. Only subjects with the TT genotype appear to be salt sensitive and have higher SBP under high sodium excretion, which reflects high sodium chloride intake, than those with the TC or CC genotype (Figure 3).

Study Strengths and Limitations

The strengths of the present study are as follows: (1) a large community-based sample, (2) assessment of various possible confounding factors, (3) resequencing of *CYP11B1* and *CYP11B2* genes in 96 subjects to catalog polymorphisms in Japanese, and (4) functional confirmation of the significance of the T(−344)C polymorphism in vitro. However, the present study also has some limitations. The mean ages of male and female subjects were 62±11 and 63±11 years, respectively. Because the phenotypes of elderly subjects are suspected to be influenced more by environmental factors than those of younger subjects, younger subjects might be more appropriate for a genetic association analysis.

Although the aldosterone levels were corrected by renin activity, several environmental factors are known to affect aldosterone levels, and these are difficult to control in an epidemiological research setting. In fact, we did not measure urine sodium excretion over a 24-hour period or serum potassium levels in this population (study population II). These 2 factors are the most important environmental factors that affect aldosterone levels.

We excluded subjects with possible renovascular hypertension or primary aldosteronism from the analysis based on the screening criteria proposed by Omura et al.[24] However, precise diagnostic procedures were not performed on these suspected cases because of the constraints of the epidemiological research setting. Because we did not measure PAC or PRA in study population I, hypertensive subjects with possible renovascular hypertension or primary aldosteronism were not excluded from study population I. This may have obscured the influence of the T(−344)C polymorphism on blood pressure.

The estimation of sodium and potassium excretion in study population I was based on a spot urine specimen. The correlation between the estimated and measured values has been reported to be high when urine specimens are collected in the morning,[23] and spot urine specimens were collected between 9:00 AM and 10:00 AM in the present study. We need to assess the sodium intake status more precisely in future studies.

Perspectives

The T(−344)C polymorphism may be useful for identifying hypertensive subjects to whom a low NaCl diet or diuretics should be recommended.

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We express our gratitude to the participants of the present study. We thank Dr Chun-chen Shen for technical assistance.

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Disclosures

None.

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吹田市基本健診での生活習慣とメタボリックシンドロームに関する研究

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伊達 ちぐさ*6　岡山 明*3　友池 仁暢*7

目的　都市住民のメタボリックシンドローム（Mets）有病率とMets定義病態に関連する生活習慣の特徴を性・年齢ごとに評価した。

方法　平成16年度吹田市基本健康診査受診者のうち問診票で有効回答が得られた30～89歳の26,522人の男女を対象とした。MetsはUS National Cholesterol Education Program: Adult Treatment Panel Ⅲの基準を改変して診断した。Mets有病率，Mets有病者での構成因子の有病率を求め，さらにMetsと関連する生活習慣の検討を行った。

結果　30～89歳でのMetsの有病率は，男性19.4%，女性10.7%であった。Mets有病者のうち，若年群では肥満の有病率が高く（30歳代：男性82%，女性90%），高齢群では血圧高値の有病率が高い傾向にあった（80歳代：男性99%，女性98%）。生活習慣では，「他の人より食べる量が多い」「早食いである」「睡眠が不規則である」「立位・歩行時間が1時間未満である」は，男女ともすべての年代でMetsと関連していた。4項目のいずれにも該当しない対象者と1項目該当の対象者のMetsの多変量調整オッズ比は1.29～2.17の値をとり，2個では1.66～4.60，3個では3.13～5.09で，4個すべてに該当する対象者では5.36であった。

結論　Metsの構成因子は年齢により異なっていたが，過食・早食い・不規則な睡眠・運動不足はすべての年代でMetsとの関連がみられ，これらを多く満たす人ほどMetsのリスクが高かったことから，これら4つの項目はMetsの予防・改善の保健指導の項目となりうる生活習慣と考えられた。

キーワード　メタボリックシンドローム，有病率，生活習慣

Ⅰ はじめに

メタボリックシンドローム（Metabolic Syndrome: 以下，Mets）は，肥満，高血糖，脂質代謝異常，血圧高値などの循環器疾患危険因子が集積しやすく，循環器疾患やⅡ型糖尿病を予防する上で目標を定めやすい病態として公衆衛生・予防医学の分野でも注目されている[1]。前向きコホート研究では，Metsの循環器疾患・Ⅱ型糖尿病に対するリスクがこれまでに確認されてきた[2)-8)]。Metsの原因については，遺伝要因と近年の生活習慣における近代化・欧米化といった環境要因の両面の関与が指摘されており，特にアジア人は欧米化した生活習慣によってMetsになりやすい遺伝要因を有していることが知られている[9)10)]。わが国でも，戦後より脂肪摂取量の増加や労働の機械化・交通網の発達による運動量の減少など生活習慣が著しく変化しており，肥満や代謝性疾患の増加も顕著で，Mets有病率の上昇が指摘されている[11)]。しかし，Mets有病率と関連する生活習慣をわ

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＊4 吹田市医師会副会長　＊5 同会長
＊6 奈良女子大学生活環境学部教授　＊7 国立循環器病センター病院長

が国の都市住民で検討した報告はみられない。

Metsの診断基準はこれまでにいくつか提唱されてきた。代表的なものには，1999年に世界保健機構（WHO）から提唱されたインスリン抵抗性を必須項目とするもの[12]，2001年にUS National Cholesterol Education Program: Adult Treatment Panel Ⅲ（NCEP ATP Ⅲ）の一部として提唱された循環器疾患の予防に焦点をおいたものがあるが[13]，これらに対し，診断基準が複数存在することに対する多岐にわたる評価や診断基準の見直しの必要性も指摘された[14]。2005年に国際糖尿病連合（IDF）から提唱された基準では，ウエスト周囲径による腹部肥満が必須項目とされ，また，従来の項目以外のものも今後研究されるべき項目として考慮されている[15]。2005年に日本内科学会が日本肥満学会・糖尿病学会・動脈硬化学会などの8学会と合同で提唱した日本人のための基準でも，IDFの基準と同様にウエスト周囲径による腹部肥満が必須項目とされている[16]。今後，これらの新しい診断基準を用いた研究が望まれるが，その一方，わが国の過去の健診の多くはウエスト周囲径を項目に含んでいないのが現状である。身長・体重は健診で広く一般に測定され，そこから算出されるBody Mass Index（BMI）は肥満の診断に日常的に使用されている。過去の研究においてNCEP ATP Ⅲの基準のウエスト周囲径による腹部肥満をBMIによる肥満に改変した基準が用いられているが[17]，NCEP ATP Ⅲの基準によると腹部肥満は必須項目ではなく1つの構成因子であり，改変による影響は比較的少ないと思われる。すでに行われた健診のデータを用いてMetsの研究を行う場合には，改変されたNCEP ATP Ⅲの基準を用いるのが現実的な方法と思われる。

著者らは，都市住民を対象に改変したNCEP ATP Ⅲの診断基準を用い，Metsとその構成因子の有病率，Metsに関連する生活習慣を分析し，Metsの予防・改善に役立てることを目的として本研究を行った。

表1　対象者の性・年代別分布

	総数	30歳代	40歳代	50歳代	60歳代	70歳代	80歳代
総数	26 522	2 649	2 697	4 290	9 378	6 055	1 453
男性	8 652	418	504	840	3 649	2 679	562
女性	17 870	2 231	2 193	3 450	5 729	3 376	891

Ⅱ 方　法

（1） 研究の対象

平成16年度吹田市基本健康診査の受診予定者全員（100,885人）にあらかじめ生活習慣問診票を送付し，受診者が記入した問診票は健診の際に医師によって再点検した。健診受診者中の，61,879人の血液検査が同一施設で行われ，このうち30～89歳であり，かつ問診票で有効回答が得られた26,522人（男性8,652人，女性17,870人）を本研究の対象とした。対象者の性・年齢別分布を表1に示す。

（2） 診断基準

NCEP ATP Ⅲ基準の5項目（高血糖［血糖≧110mg/dlまたは治療中］，血圧高値［血圧≧130/85mmHgまたは治療中］，高中性脂肪血症［中性脂肪≧150 mg/dl］，低HDLコレステロール血症［HDLコレステロール　男性40 mg/dl未満・女性50mg/dl未満］，肥満［BMI≧25kg/m^2］）のうち，3項目以上を満たした場合，Metsと診断した[13]。Metsの構成因子とMetsの有病率を性・年代別に求めた。また，Mets対象者についてのMetsの構成因子の有病率を性・年代別に求めた。問診票での食事・運動・睡眠などの30項目の生活習慣のうち，30～49歳・50～69歳・70～89歳のすべての年代で男女共通してMetsと関連する項目を，ロジスティック回帰モデルを用いて年齢調整して求めた。さらに，それらの生活習慣に1つも該当しない対象者とそれらの生活習慣の組み合わせに該当する対象者のMetsの多変量調整オッズ比を，ロジスティック回帰モデルを用いて性・年齢・飲酒・喫煙を調整して求めた。有意水準は$p<0.05$とし，解析にはSPSS ver11.0を用いた。

Ⅲ 結　果

Metsの構成因子とMetsの性・年代別有病率を図1に示す。高血糖あるいは血圧高値の有病率は，男女とも年代と共に上昇傾向にあった。高中性脂肪血症では，男性は40歳代から年代と共に低下傾向，女性は上昇傾向にあった。低HDLコレステロール血症では，男性は年代による大きな変化はなく，女性は年代と共に上昇傾向にあった。肥満では，男性は年代と共に低下傾向，女性は上昇傾向にあった。Metsの有病率は，男性は60歳代で最も高く，女性は年代と共に上昇傾向にあった。30～89歳でのMetsの有病率は，男性19.4%，女性10.7%であった。

Mets有病者のMets構成因子有病率を性・

図1　メタボリックシンドローム（Mets）の構成因子（5項目）とMetsの有病率

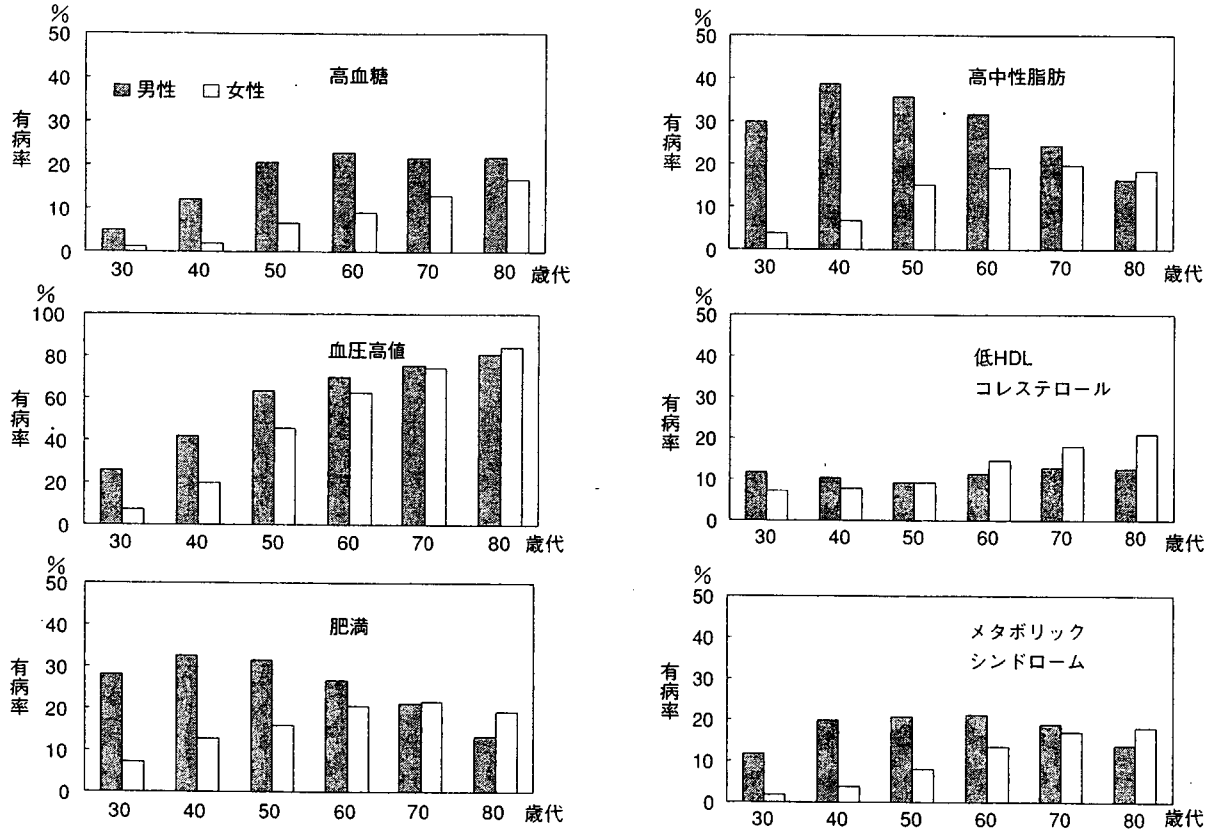

図2　メタボリックシンドローム（Mets）有病者でのMets構成因子（5項目）有病率