Values were expressed as means ± SD unless otherwise indicated.

Uniform resource locators

The JSNP database is available at http://snp.ims.u-tokyo.ac.jp/index.html. The National Center for Biotechnology Information's SNP database is available at http://www.ncbi.nlm.nih.gov/SNP/. The JMDBase is available at http://www.jmdbase.jp. SNPHAP is available at http://www.gene.cimr.cam.ac.uk/clayton/software/

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We acknowledge the outstanding contributions of the SNP Research Center, the Institute of Physical and Chemical Research (RIKEN), the National Cancer Center (NCC) and the International Medical Center of Japan (IMCJ) employees who provided technical and infrastructural support for this work. Above all, we thank the patients and study subjects who made this work possible and who give it value. We thank all the people who continuously support the Suita Study. We also thank Drs Yukio Yamori, Yoshihiro Kokubo, Masakuni Serizawa, Ken Sugimoto, Hiroshi Akasaka, Chikanori Makibayashi and many physicians in Amagasaki Medical Association, Sadahiko Iwamoto, Yoichi Ohno, Mikano Hishiki, Kotoko Kosuge, Noriko Aoi, Akira Haketa, Yoichi Izumi, Toru Izumi, Taishi Sasaoka, Shigeru Hohda and Takeharu Hayashi, and Ms Kaoru Sugama for their contribution to collecting DNA samples and clinical information accompanied; Drs Hiromi Rakugi and Masafumi Kuremura and Ms Akemi Muramoto and Kazuko Iwasa for their advice and assistance.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Grant-in-Aid for Scientific Research on Priority Areas (Millennium Genome Project) from the Ministry of Education, Science, Sports, and Culture of Japan, the grants from the Program for Promotion of Fundamental Studies in Health Sciences of Pharmaceuticals and Medical Devices Agency (PMDA) and a grant for the Core Research for the Evolutional Science and Technology, from the Japan Science Technology Agency (JST).

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Original Article

High Plasma Norepinephrine Levels Associated with β2-Adrenoceptor Polymorphisms Predict Future Renal Damage in Nonobese Normotensive Individuals

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Renal injury is common in obesity and hypertension. In the present study, we examined relationships between renal function alterations, plasma norepinephrine (NE), and β2-adrenoceptor polymorphisms in a longitudinal design over 5 years. In 219 nonobese, normotensive men with entry-normal renal function, we measured serum blood urea nitrogen (BUN), creatinine, creatinine clearance, plasma NE, homeostasis model assessment of insulin resistance (HOMA-IR), body mass index (BMI), total body fat mass, and blood pressure (BP) annually for 5 years. β2 (Arg16Gly, Gln27Glu)-adrenoceptor polymorphisms were determined. The subjects were stable in body weight and BP (<10%) for 5 years. High plasma NE was defined as ≥mean+1 SD at entry. Thirty-seven subjects had entry-high plasma NE and 182 were entry-normal. Entry-high plasma NE subjects had significantly greater total body fat mass and plasma NE and significantly lower creatinine clearance at entry and throughout the study. Increases in BMI, fat mass, BP, plasma NE, BUN, and creatinine, as well as the reduction in creatinine clearance in the 5 years, were significantly greater in entry-high NE subjects. These subjects had significantly higher frequencies of the Gly16 allele of β2-adrenoceptor polymorphisms. Throughout the study, subjects carrying the Gly16 allele had higher plasma NE, HOMA-IR, and fat mass, and significantly greater reductions in creatinine clearance. Plasma NE at entry was a determinant variable for changes in BUN, creatinine, and creatinine clearance over the 5-year period in multiple regression analysis. In conclusion, high plasma NE at entry, associated with the Gly16 allele of the β2-adrenoceptor polymorphisms, predict renal function deterioration (seen in elevations of BUN and creatinine and reduction of creatinine clearance) over a 5-year period accompanying further heightened sympathetic nerve activity and deterioration of insulin resistance. (*Hypertens Res* 2007; 30: 503–511)

***Key Words*: plasma norepinephrine, renal function, blood pressure elevation, weight gain, β2-adrenoceptor polymorphisms**

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Received November 30, 2006; Accepted in revised form January 12, 2007.

Introduction

Renal injury predicts the development of cardiovascular disease (*1*). Hypertension and obesity are currently among the World Health Organization's top 10 global health risks, and are strongly associated with renal injury, chronic renal disease, and end-stage renal disease (*2*, *3*). The incidence of hypertension, one of the primary etiological factors for chronic renal failure, is significantly higher with obesity. Obesity also leads to increases in the incidence of renal disease (*3*), metabolic diseases such as diabetes (*4*), and cardiovascular diseases (*5*). Both diabetes and hypertension, which occur often with obesity, together account for approximately 70% of end-stage renal diseases in the United States and Japan (*6*). Thus, one could speculate that there are strong relationships between hypertension, obesity, and renal injury regardless of its severity. However, most previous studies of these relationships have examined proteinuria/micro-albuminuria as an index of renal injury.

Heightened sympathetic nerve activity is observed in both hypertension and obesity (*7–11*). Further, it has been reported that heightened sympathetic nerve activity predicts incident cardiovascular events (*12*). Many investigations have found that human obesity and hypertension have strong genetic as well as environmental determinants (*13–16*). Several observations show associations of β2-adrenoceptor polymorphisms with hypertension and obesity (*16–18*), but those findings have not been confirmed. Additionally, few investigations have simultaneously taken into account sympathetic nerve activity and renal function as related to adrenoceptor polymorphisms in the same study population followed longitudinally for several years.

Thus, to investigate the relationship between alterations in renal function and certain polymorphisms of the β2-adrenoceptor system that are reportedly associated, with obesity and hypertension (*16*), we created the present study. In this study, we examined renal functions (blood urea nitrogen [BUN], creatinine, and creatinine clearance) accompanying sympathetic nerve activity (plasma norepinephrine [NE]) and the β2-adrenoceptor polymorphisms in nonobese, normotensive subjects with originally normal renal functions in a 5-year longitudinal study, though in previous studies we examined a series of studies (*i.e.*, relationships between the β2-adrenoceptor polymorphisms *vs.* insulin resistance, weight gain, or blood pressure [BP] elevation), in the same cohort (*16*, *19*). To our knowledge, the present study is the first observation regarding the relationships between the β2-adrenoceptor polymorphisms and renal function.

Methods

Subjects

Subjects were recruited from 1,121 men employed at a company in Osaka, Japan, as part of their annual medical evaluation. At study entry, subjects were excluded if they were >50 years of age, obese (body mass index [BMI] ≥25 kg/m^2), or had any of the following: diabetes mellitus (fasting glucose level >100 mg/dL), hypertension (≥140/90 mmHg), renal dysfunction (proteinuria, microscopic hematuria, BUN ≥20.0 mg/dL, creatinine ≥1.3 mg/dL, creatinine clearance <80 mL/min), or hyperuricemia (serum uric acid ≥6.5 mg/dL). Subjects were also excluded if they were taking medications for hypertension, hyperlipidemia, hyperuricemia, or other illnesses. The goal of the present study was to clarify the relationships among the genetic variance in β2-adrenoceptor polymorphisms, sympathetic nerve activity (plasma NE levels), and renal function (proteinuria, BUN, creatinine, and creatinine clearance). Thus, to minimize the influence of changes in body weight or BP levels on renal function and plasma NE levels, only subjects who had been free from significant changes (<10%) in body weight and BP levels over a 5-year period were enrolled in this study (*9–11*, *16*, *19*). After the exclusions, the present study analyzed 219 young, nonobese (BMI <25 kg/m^2), normotensive (<140/90 mmHg) men with normal renal function (no proteinuria, no microscopic hematuria, BUN<20.0 mg/dL, creatinine <1.3 mg/dL, creatinine clearance ≥80 mL/min) who were taking no medications. Informed consent was obtained from each subject, as approved by the Ethics Committee of Osaka University Graduate School of Medicine, Japan.

Measurements

After an overnight fast of >12 h, BMI, total body fat mass, ratio of waist circumference to hip circumference (waist-to-hip ratio), BP, heart rate, and venous sampling for BUN, creatinine, serum uric acid, plasma NE, insulin, leptin, and glucose were sampled after a 30-min rest period in the supine position in a quiet room. Blood samples for the extraction of genomic DNA from leukocytes were also taken. Measurements were made at entry and every year for 5 years. BP and heart rate were measured at each review at least three times in the supine position by an automated sphygmomanometer (TM-2713, A&D Co., Tokyo, Japan) using an adjustable cuff based on arm circumference, which was standardized against a mercury sphygmomanometer, and were averaged. Those who had wide variability in BP and heart rate were asked to return for repeated measurements at least three separate visits to exclude chance variation. The percentage body fat mass was determined with impedance measurements (BF-102, Tanita Co., Tokyo, Japan), and total body fat mass (kg) was calculated according to the following formula: [percentage body fat mass (%)/100] × body weight (kg). Creatinine clearance was calculated according to the following formula established by Cockroft and Gault (*20*): [(140 − age) × body weight] (kg)/[72 × serum creatinine (mg/dL)]. Homeostasis model assessment of insulin resistance (HOMA-IR) was defined as the product of fasting plasma insulin (μU/mL) and

Table 1. Comparisons of Characteristic in Subjects with Normal Plasma Norepinephrine at Entry (<Mean+1 SD) *vs.* High Plasma Norepinephrine at Entry (≥Mean+1 SD)

Variables	Subjects with normal plasma norepinephrine levels			Subjects with high plasma norepinephrine levels		
	At entry	After 5 years	Changes for 5 years	At entry	After 5 years	Changes for 5 years
Subjects (*n*)	182	182	182	37	37	37
Age (years)	39±3	44±3§	5	37±3	42±3§	5
BMI (kg/m²)	21.3±1.4	21.4±2.9	0.1±1.0	21.5±2.4	22.4±2.9	0.9±1.2*
Total body fat mass (kg)	12.9±4.7	13.7±4.4	0.9±3.2	14.3±6.2*	17.1±4.8*,‡	2.8±5.1†
Waist-to-hip ratio	0.84±0.07	0.84±0.09	−0.01±0.06	0.87±0.10	0.93±0.09*	0.05±0.09
Systolic BP (mmHg)	123±7	124±8	1±12	126±9	133±9†	8±7*
Diastolic BP (mmHg)	71±5	73±6	3±4	76±13	79±6*	3±10
Mean BP (mmHg)	88±6	90±6	1±8	93±7	97±7*	5±5*
Norepinephrine (pmol/mL)	1.28±0.27	1.68±0.45	0.40±0.52	1.89±0.25†	2.85±0.46†,§	0.99±0.41†
HOMA-IR	1.53±0.76	2.17±0.93	0.64±0.75	1.71±0.60	2.58±0.75‡	0.87±1.02
Leptin (ng/mL)	3.2±2.0	3.2±2.1	−0.1±1.9	3.4±1.8	3.5±1.0	0.2±1.0
BUN (mg/dL)	10.5±2.9	11.2±3.7	0.6±1.0	10.8±2.3	12.3±2.6*,‡	1.5±0.9*
Creatinine (mg/dL)	0.93±0.12	0.95±0.16	0.02±0.08	0.95±0.09	1.06±0.14*,‡	0.11±0.16*
Creatinine clearance (mL/min)	98.0±19.0	94.9±21.8	−3.1±21.4	85.1±9.9†	72.3±18.9†,§	−12.8±14.8†
Uric acid (mg/dL)	4.3±1.3	4.8±1.5	0.4±1.8	5.0±1.0	5.8±1.5*,‡	0.8±1.5*

BMI, body mass index; BP, blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; BUN, blood urea nitrogen. *$p<0.05$, †$p<0.01$ *vs.* subjects with normal plasma norepinephrine levels; ‡$p<0.05$, §$p<0.01$ *vs.* values at entry. Data are shown mean±SD. N=219.

glucose (mg/dL) divided by 405.

Laboratory Determinations

Plasma NE was measured by high-performance liquid chromatography with a fluorometric method as described previously in detail (*10*). Plasma immunoreactive insulin was measured by a standard radioimmunoassay method as described in detail (insulin RIABEAD II, Dinabott Co., Tokyo, Japan) (*10*). Plasma leptin was measured by radioimmunoassay as described (human leptin RIA kit, Linco Research, St. Charles, USA) (*10*). Serum BUN, creatinine, uric acid, and glucose were measured by an Autoanalyzer (Hitachi-7050, Hitachi Medical Co., Tokyo, Japan).

Genotyping

Genotyping was performed by the TaqMan assay as previously described (*16*, *19*, *21*, *22*). Two polymorphisms in the β2-adrenergic receptors (arginine/glycine substitution, Arg16Gly; glutamine/glutamate substitution, Gln27Glu) of the β2-adrenoceptor genes (*16*, *17*) were evaluated. The probes and primers used in the TaqMan assay were as follows. For Arg16Gly, the probes were CGCATGGCTTCC ATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAG GACGATGAGAGACATGACGAT; for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT (the primers used in this assay were the same as those used for Arg16Gly).

Statistical Analysis

Values shown are means±SD. Changes in measured parameters within each group and differences among groups were examined by two-way analysis of variance. When these differences were significant, the Dunnett test was used to determine whether the differences of the mean measured variables at year 5 were significantly different from baseline within the groups and among the groups. The Mantel-Haenszel χ^2 test was used to compare differences in the genotype frequencies between the groups with and without high plasma NE at the entry period. Multiple linear regression analyses were used to examine relationships among variables using changes in creatinine clearance, serum BUN, or creatinine for 5 years as the dependent variables to evaluate the relationships with plasma NE, insulin sensitivity (HOMA-IR), body weight, total body fat mass, BP levels, and heart rates at entry. The Hardy-Weinberg equilibrium was estimated with the χ^2 test.

Table 2. Comparisons of the Frequencies of Genotype of the β2-Adrenoceptor Polymorphisms between Subjects with and without High Plasma Norepinephrine (Plasma Norepinephrine Levels ≥ Mean+1 SD)

	Genotype frequency				Allele frequency		Normal- *vs.* high-plasma norepinephrine χ^2 test	
							For genotype	For allele
Arg16Gly, β2-adrenoceptor	Arg/Arg	Arg/Gly	Gly/Gly	missing	Arg16 allele	Gly16 allele	χ^2=31.86,	χ^2=58.32,
Normal SNA (*n* (%))	62 (34.1)	93 (51.1)	27 (14.8)	0 (0.0)	217 (59.6)	147 (40.4)	p<0.001	p<0.001
High SNA (*n* (%))	5 (13.5)	11 (29.7)	21 (56.8)	0 (0.0)	21 (28.4)	53 (71.6)		
Gln27Glu, β2-adrenoceptor	Gln/Gln	Gln/Glu	Glu/Glu	missing	Gln27 allele	Glu27 allele	χ^2=—,	χ^2=2.82,
Normal SNA (*n* (%))	156 (86.0)	19 (10.0)	0 (0.0)	7 (4.0)	331 (90.0)	19 (5.2)	p=—	p=0.093
High SNA (*n* (%))	35 (94.6)	0 (0.0)	0 (0.0)	2 (5.4)	70 (94.6)	0 (0.0)		

SNA, sympathetic nervous system measured by plasma norepinephrine.

Table 3. Comparisons of Characteristics between Subjects with and without the Gly16 Allele of Arg16Gly, β2-Adrenoceptor Gene

Variables	Subjects without Gly16 allele			Subjects with Gly16 allele		
	At entry	After 5 years	Changes for 5 years	At entry	After 5 years	Changes for 5 years
Subjects (*n*)	67	67	67	152	152	152
Age (years)	39±3	44±3§	5	39±3	44±3§	5
BMI (kg/m²)	21.7±1.4	21.7±1.7	0.1±1.2	21.2±1.7	21.5±3.0	0.3±2.3
Total body fat mass (kg)	12.7±5.3	13.4±6.7	0.5±4.0	13.4±5.3*	14.7±5.1*,‡	1.4±4.1*
Waist-to-hip ratio	0.83±0.06	0.85±0.08	0.01±0.05	0.87±0.06*	0.91±0.08*,‡	0.04±0.06*
Systolic BP (mmHg)	126±10	126±9	1±6	120±9	124±8	4±7
Diastolic BP (mmHg)	74±6	73±6	−1±7	71±6	75±6	4±8*
Mean BP (mmHg)	91±6	91±7	−1±5	87±6	91±6	4±9
Norepinephrine (pmol/mL)	1.10±0.41	1.69±0.49‡	0.59±0.40	1.50±0.40*	1.98±0.51*,‡	0.47±0.45
HOMA-IR	1.34±0.63	1.95±0.74‡	0.61±1.62	1.67±0.51*	2.44±0.65*,‡	0.77±0.95
Leptin (ng/mL)	3.4±2.6	3.7±2.0	0.3±1.6	3.2±2.9	3.1±3.3	−0.1±4.4
BUN (mg/dL)	10.4±2.6	11.0±2.8	0.6±1.1	10.6±2.9	11.6±3.7	1.1±2.0
Creatinine (mg/dL)	0.92±0.14	0.97±0.14	0.05±0.12	0.94±0.11	0.97±0.16	0.04±0.09
Creatinine clearance (mL/min)	98.2±22.8	95.8±21.6	−2.4±22.5	91.1±21.9	80.3±23.1†	−10.8±20.7†
Uric acid (mg/dL)	4.0±0.7	4.4±1.2	0.4±1.4	4.6±0.5*	5.2±1.3*,‡	0.6±1.5

BMI, body mass index; BP, blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; BUN, blood urea nitrogen. *p<0.05, †p<0.01 *vs.* values in subjects without Gly16 allele, ‡p<0.05, §p<0.01 *vs.* values at entry. Data are mean±SD. N=219.

Results

Characteristics of Entry-Normal (<Mean+1 SD) and Entry-High (≥Mean+1 SD) Plasma Norepinephrine Study Groups

The subjects were subdivided into two groups—one with high plasma NE and the other with normal plasma NE—using the cut-off limit of mean+1 SD (1.34+0.33 pmol/mL) at entry. There were 37 subjects who had entry-high plasma NE and 182 subjects who had entry-normal plasma NE (Table 1). None of the participants had proteinuria or microscopic hematuria throughout the study.

Subjects with entry-high plasma NE had greater total body fat mass and plasma NE levels at entry compared to those with entry-normal plasma NE, although BMI, waist-to-hip ratios, BP, HOMA-IR, BUN, and creatinine levels were similar. Creatinine clearance was significantly lower in subjects with entry-high plasma NE than in those with entry-normal plasma NE at entry and throughout the study. Further, increases in BMI, total body fat mass, BP, plasma NE, BUN, creatinine, and uric acid levels, as well as the reduction in creatinine clearance, over the 5-year period were significantly greater in the subjects with entry-high plasma NE levels (Table 1).

Table 4. Characteristic of the 4 Study Groups According to Entry Plasma Norepinephrine Levels and the Gly16 Allele of the β2-Adrenoceptor Polymorphisms

Variables	Subjects with normal plasma norepinephrine levels				Subjects with high plasma norepinephrine levels			
	Without Gly16 allele		With Gly16 allele		Without Gly16 allele		With Gly16 allele	
	At entry	After 5 years	At entry	After 5 years	At entry	After 5 years	At entry	After 5 years
Subjects (*n*)	62	62	120	120	5	5	32	32
Age (years)	40±4	45±4#	38±3	43±3#	35±6	40±6#	39±3	44±3#
BMI (kg/m²)	20.9±1.3	20.8±2.7	21.5±1.4	21.7±2.8	20.9±2.2	21.8±2.8	21.6±2.0	22.5±2.3
Total body fat mass (kg)	11.9±3.8	12.5±3.9	13.4±4.0	14.3±4.5	12.3±2.8	15.1±4.3	14.9±3.8	17.4±4.5*,§
Waist-to-hip ratio	0.82±0.06	0.80±0.08	0.85±0.09	0.87±0.10	0.83±0.08	0.87±0.05	0.88±0.07	0.94±0.10*,§
Systolic BP (mmHg)	121±6	121±9	124±9	126±7	125±8	127±7	127±8	134±9*,§
Diastolic BP (mmHg)	71±5	71±6	71±6	74±7	76±11	79±9	77±9*	80±9*
Mean BP (mmHg)	88±6	88±6	89±6	91±7	92±5	95±6	94±7*	98±7*
Norepinephrine (pmol/mL)	1.22±0.41	1.54±0.37	1.31±0.35	1.71±0.45§	1.51±0.20	2.40±0.40§	1.95±0.62*	2.92±0.71†,§
HOMA-IR	1.24±0.42	1.42±0.94	1.73±0.65‡	2.55±0.56‡,§	1.39±0.54	1.68±0.75	1.76±0.72	2.57±0.73§
Leptin (ng/mL)	3.0±1.9	3.0±2.0	3.3±2.1	3.4±1.8	3.0±1.5	3.2±1.0	3.4±1.4	3.6±1.5
BUN (mg/dL)	10.1±2.7	10.2±3.3	10.5±2.8	12.1±3.4‡	9.6±2.1	10.4±2.5	11.0±2.3	12.7±2.9
Creatinine (mg/dL)	0.91±0.09	0.93±0.12	0.94±0.12	0.96±0.15	0.90±0.10	1.00±0.13	0.96±0.10	1.07±0.11*,§
Creatinine clearance (mL/min)	98.3±17.9	96.4±22.3	97.9±22.2	91.8±22.2	93.8±10.8	90.1±21.8	84.4±10.0†,‡	70.9±18.8†,‡,#
Uric acid (mg/dL)	4.0±1.3	4.2±1.2	4.5±1.4	5.2±1.5‡	4.4±1.0	5.1±1.4	5.0±1.3	5.9±1.6§

BMI, body mass index; BP, blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; BUN, blood urea nitrogen. *$p<0.05$, †$p<0.01$ *vs.* subjects with entry-normal plasma norepinephrine levels; ‡$p<0.05$ *vs.* subjects without the Gly16 allele; §$p<0.05$, #$p<0.01$ *vs.* value at the entry period.

Comparisons of the Frequencies of the β2-Adrenoceptor Polymorphisms According to Plasma NE Levels

Subjects with high plasma NE levels had significantly higher frequencies of the homozygous Gly16 genotype and the Gly16 allele of the Arg16Gly, the β2-adrenoceptor polymorphism. However, the frequencies of the Glu27 allele of the Gln27Glu were similar between subjects with and without high plasma NE levels (Table 2).

Comparisons of Characteristics between Subjects with and without the Gly16 Allele of the Arg16Gly β2-Adrenoceptor Polymorphism

The finding that subjects with high plasma NE had higher frequencies of the Gly16 allele of the Arg16Gly, but not of the Glu27 allele, indicates that the Gly16 allele is closely linked to high plasma NE levels. Thus, we compared the parameters between the subjects with and without the Gly16 allele.

Subjects carrying the Gly16 allele had greater total body fat mass, waist-to-hip ratio, plasma NE, HOMA-IR, and uric acid at entry and after 5 years compared to those without the Gly16 allele, but BUN and creatinine levels throughout the study were similar. Creatinine clearance at entry was similar between those with and without the Gly16 allele, but subjects with the Gly16 allele had significantly lower creatinine clearance after 5 years and significantly greater reductions in creatinine clearance over the 5-year period compared to those without the Gly16 allele. Subjects with the Gly16 allele also had significantly greater increases in total body fat mass and waist-to-hip ratio over the 5-year period (Table 3).

Comparisons of the Characteristics of the Study Groups According to Entry Plasma Norepinephrine Levels and the Gly16 Allele of β2-Adrenoceptor Polymorphisms

When we compared the four study groups according to the entry plasma NE levels and the Gly16 allele of the β2-adrenoceptor polymorphisms, total body fat mass, waist-to-hip ratio, plasma NE, HOMA-IR, BUN, creatinine, and uric acid levels at entry were greatest in the group that had both entry-high plasma NE and the Gly16 allele. BP elevations over the 5-year period and increases in total body fat mass, waist-to-hip ratio, plasma NE, HOMA-IR, BUN, creatinine, and uric acid were also greatest in the group that had both entry-high plasma NE and the Gly16 allele (Table 4).

Further, subjects with both entry-high plasma NE and the Gly16 allele had the lowest creatinine clearance rate throughout the study period, as well as the greatest reduction in creatinine clearance over the 5-year period. Only in subjects with entry-high plasma NE, creatinine clearance was significantly lower in subjects carrying the Gly16 allele than in those with-

Table 5. Multiple Linear Regression Analyses

Independent variables	Dependent variables		
	ΔCreatinine clearance	ΔBUN	ΔCreatinine
A: At entry			
Plasma NE	$p=0.011$	$p=0.008$	$p=0.018$
Plasma leptin	n.s.	n.s.	n.s.
HOMA-IR	n.s.	n.s.	n.s.
Mean BP	n.s.	n.s.	n.s.
Heart rate	n.s.	n.s.	n.s.
BMI	n.s.	n.s.	n.s.
Total body fat mass	n.s.	n.s.	n.s.
Creatinine clearance	$p<0.001$	$p=0.035$	$p=0.027$
Serum BUN	n.s.	n.s.	$p=0.025$
Serum creatinine	n.s.	n.s.	$p=0.041$
	$F=8.842$	$F=2.750$	$F=2.18$
	$p<0.001$	$p=0.033$	$p=0.032$
	$r^2=0.630$	$r^2=0.271$	$r^2=0.325$
B: Change over a 5-year period			
ΔPlasma NE	$p=0.028$	$p=0.013$	$p=0.038$
ΔPlasma leptin	$p=0.048$	$p=0.024$	n.s.
ΔHOMA-IR	n.s.	$p=0.014$	$p=0.029$
ΔMean BP	$p=0.025$	$p=0.030$	n.s.
ΔHeart rate	$p=0.037$	n.s.	n.s.
ΔBMI	n.s.	n.s.	n.s.
ΔTotal body fat mass	$p=0.011$	$p=0.018$	$p=0.049$
ΔCreatinine clearance	—	$p=0.022$	$p=0.011$
ΔSerum BUN	n.s.	—	$p=0.018$
ΔSerum creatinine	$p=0.012$	$p=0.018$	—
	$F=3.838$	$F=1.866$	$F=4.830$
	$p=0.011$	$p=0.049$	$p=0.002$
	$r^2=0.348$	$r^2=0.172$	$r^2=0.475$

NE, norepinephrine; HOMA-IR, homeostasis of model assessment of insulin-resistance; BP, blood pressure; BMI, body mass index; BUN, blood urea nitrogen; Δ, change over a 5-year period.

out the Gly16 allele. However, in subjects with entry-normal plasma NE, creatinine clearance was similar between those with and without the Gly16 allele (Table 4).

Multiple Regression Analysis Using Changes in Creatinine Clearance, BUN, or Creatinine over the 5-Year Period as a Dependent Variable

Multiple linear regression analyses using changes in creatinine clearance, serum BUN, or serum creatinine over the 5-year period as dependent variables demonstrated that plasma NE level at entry was a significant determinant variable for changes in creatinine clearance, BUN, or creatinine (Table 5, A). Change in plasma NE and change in total body fat mass over the 5-year period were also significant determinant variables for changes in creatinine clearance, serum BUN, or serum creatinine (Table 5, B).

Discussion

The present study shows that subjects carrying baseline high plasma NE have significantly greater increases in total body fat mass, BP levels, plasma NE, BUN, and creatinine, as well as greater reductions in creatinine clearance, over the 5-year period. Subjects with high plasma NE have significantly higher frequencies of the Gly16 of the Arg15Gly, the β2-adrenoceptor polymorphism. Significant deterioration in renal function (shown in creatinine clearance, serum creatinine, and BUN) is observed especially in subjects who carry both the Gly16 allele and entry-high plasma NE. Multiple regression analyses demonstrate that the plasma NE level at entry is a significant determinant of changes in BUN, creatinine, and creatinine clearance over the 5-year period. These findings demonstrate that high plasma NE levels associated with the Gly16 allele of a β2-adrenoceptor polymorphism are

related to the deterioration in renal function (BUN, creatinine, and creatinine clearance) over the 5-year period in originally normal renal function, nonobese, normotensive Japanese men.

Mortality caused by cardiovascular disease is more than three times higher in subjects with renal dysfunction than in those with normal renal function (*23*). Many investigators have reported that plasma NE and sympathetic nerve overactivity predict survival and incident cardiovascular events (*12, 24*). Recently, Joles and Koomans have reported that sympathetic nerve stimulation contributes to the progression of renal disease (*25*). The 40-min infusion of NE into the renal artery of dogs produces a reversible ischemic model of acute renal failure (*26*). Another study demonstrates renal protection by β-adrenergic receptor blockade in a nephrectomized rat experiment without any BP changes (*27*). The findings that high plasma NE levels at entry are closely linked to future renal function (BUN, creatinine, and creatinine clearance) could also indicate that sympathetic nerve overactivity (high plasma NE) might be a cause of renal injury. These investigations demonstrate that direct effects of sympathetic nerve overactivity may lead to proteinuria and renal injury (*25*), and that renal dysfunctions associated with sympathetic overactivity is related to much higher mortality compared to that in individuals with normal renal function.

Previous epidemiological and clinical studies have used micro-albuminuria as a marker of renal injury (*1*). However, it should be noted that we used BUN, creatinine levels, and creatinine clearance, which are more direct markers of renal function relative to micro-albuminuria, and that those are deteriorated over the 5-year period in association with further heightened sympathetic nerve activity. Prolonged sympathetic nerve overactivity (high plasma NE) can induce changes in intrarenal blood vessels. Catecholamines induce proliferation of smooth muscle cells and adventitial fibroblasts in the vascular wall (*28*). The findings that reductions in creatinine clearance were significantly greater in subjects carrying entry-high plasma NE, the Gly16 allele, or both indicated that subjects carrying high sympathetic nerve activity (entry-high plasma NE), the β2-adrenoceptor polymorphisms, or both are related to deterioration of renal function. Further, the finding that, in subjects with high plasma NE, the deterioration in renal function is significantly greater in subjects carrying the Gly16 allele, suggests that the Gly16 allele might have additional effects on future renal injury, especially in subjects who carry heightened sympathetic nerve activity. The novel findings in the present study are that the Gly16 allele of the β2-adrenoceptor associated with entry-high plasma NE might be related to future renal injury. To our knowledge, this is one of the first investigations into the relationships between the β2-adrenoceptor polymorphisms and renal injury.

Hypertension and obesity are also risk factors for progressive renal function loss in patients with known renal disease and may damage the kidneys in otherwise healthy subjects (*29*). Typically, significant hypertension initially affects the renal vasculature, resulting in hyaline thickening of small arteries and arterioles. At an earlier stage, hypertension and atherosclerosis may be intimately linked through their effects on endothelial function. A dysfunctional endothelium allows adhesion of lipid-filled macrophages and consequent chemotaxis and aggregation of inflammatory cells. In large vessels, hypertension favors atherosclerosis progression primarily by accelerating the conversion of fatty streaks to atheroma (*30*).

Several investigators have reported that cardiac sympathetic nerve activity is not substantially elevated in obese humans, whereas sympathetic nerve activity is increased in the kidneys (*3, 31*). Their observations show that the kidneys play an important role in heightened sympathetic nerve activity in obesity. Obesity can also produce renal injury by early up-regulation of numerous pro-inflammatory (*e.g.*, leptin, interleukins, adiponectin, tumor-necrosis factor-α) and growth-promoting (*e.g.*, angiotensin II, TGF-β, leptin) factors, leading to mesangial matrix production and thickening of the glomerular and tubular basement membrane lesions that may precede glomerulosclerosis (*29*). Obesity can lead to hypertension by increasing renal tubular sodium reabsorption, impairing pressure natriuresis, and inducing volume expansion, as well as by physical compression of the kidneys. With prolonged obesity, there is increasing urinary protein excretion and a gradual loss of nephron function that worsens with time and exacerbates hypertension (*3*). Further, obesity is considered the phenotypic hallmark of metabolic syndrome (insulin resistance). Patients with metabolic syndrome have a high prevalence of micro-albuminuria, which is considered an early marker of renal endothelial dysfunction and chronic kidney disease as well as generalized endothelial dysfunction. With the worsening of metabolic syndrome and the development of type II diabetes in some obese patients, kidney damage progresses much more rapidly and seriously. Thus, aggressive obesity-lowering therapies are needed to ameliorate renal disease progression (*3, 32*). Importantly, both the pathogenesis of hypertension and obesity are associated with sympathetic nerve overactivity, as previously documented.

It should be noted that subjects with entry-high plasma NE in part associated with the Gly16 allele of the Arg16Gly polymorphism in the present study had greater increases in total body fat mass and BP elevations accompanied by greater increases in plasma NE, even though we tried to minimize (<10% in changes in BMI or BP levels over 5 years) the influence of weight gain (obesity) or BP elevation (hypertension) on renal function. Further, those subjects who had greater increases in adiposity and BP levels had more deterioration in renal function. We previously reported the associations of the Gly16 allele of the Arg16Gly, a β2-adrenoceptor polymorphism, with weight gain and BP elevations (*16*). These observations suggest that BP elevation and weight gain determined by the Gly16 allele of Arg16Gly (even though the degree of those elevations was very small [<10%]), might contribute to renal function accompanied by sympathetic

nerve overactivity (high plasma NE). Thus, one could speculate on the possibility that weight gain and BP elevation associated with the Gly16 allele of the Arg16Gly might lead to high plasma NE levels at entry, greater increases in plasma NE over 5 years, and resultant renal injury. Subjects carrying entry-high plasma NE had greater increases in plasma leptin levels over the 5-year period associated with greater increases in fat mass, suggesting that greater increases in plasma leptin (adiposity) might lead to further heightened sympathetic activity (*33*).

There is an association between the Gly16 allele of Arg16Gly and insulin resistance (*19*). The close relationships between plasma NE (sympathetic nerve activity) and insulin (insulin resistance) were also observed in this cohort of nonobese, normotensive subjects with a cross-sectional design (*34*). Further, we have reported that heightened sympathetic nerve activity (high plasma NE) precedes hyperinsulinemia in nonobese, normotensive individuals (*10, 11*). Taken together with previous observations, in nonobese, normotensive individuals, heightened sympathetic nerve activity associated with the Gly16 allele might play a primary role in renal injury, and hyperinsulinemia linked to the Gly16 allele as well as high plasma NE might be an ancillary mechanism.

In conclusion, the findings demonstrate that baseline plasma NE in part associated with the Gly16 allele of Arg16Gly, a β2-adrenoceptor polymorphism, could predict future renal injury (BUN and creatinine elevation, and reduction in creatinine clearance) accompanied by increases in abdominal obesity (waist-to-hip ratio and total body fat mass), BP elevation, and insulin resistance over 5 years in nonobese, normotensive male individuals. Thus, we would propose that plasma NE levels, a β2-adrenoceptor polymorphism (Arg16Gly), and relative abdominal obesity might be useful for predicting future renal injury.

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AJH 2007; 20:527–532

Genetics

Association Between the Calcitonin-Related Peptide α (CALCA) Gene and Essential Hypertension in Japanese Subjects

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Background: Calcitonin-related peptide α (CALCA) is a neuropeptide that is a very potent vasodilator. It has been reported that CALCA knockout mice have a significantly elevated systolic blood pressure (BP). The aims of this study were to discover novel polymorphisms or mutations in the 5′ flanking region of the human CALCA gene in Japanese subjects and to assess the association between this gene and essential hypertension (EH).

Methods: Japanese patients with EH (50.1 ± 6.6 years old, $n = 274$) and age-matched Japanese subjects without EH (51.1 ± 6.6 years old, $n = 225$) were recruited. The 5′ flanking region of the human CALCA gene was searched to identify novel polymorphisms in the 20 EH patients using polymerase chain reaction (PCR) and a direct sequencing method. These novel polymorphisms, as well as the known single nucleotide polymorphisms (SNPs), were used for genotyping.

Results: We discovered a novel 2-bp microdeletion polymorphism in intron 1. The only three participants with 2-bp microdeletion polymorphism were found in the EH group. None of the subjects without EH had a 2-bp microdeletion polymorphism. The genotype and allele distribution of the 4 SNPs were not significantly different between the groups. All five polymorphisms were located in one haplotype block. The haplotype was constructed using, in order, rs1553005, 2-bp microdeletion polymorphism, and rs5241. There was a significant association between EH and the C-AGins-A haplotype ($P = .00031$).

Conclusions: A novel 2-bp microdeletion polymorphism was discovered in the CALCA gene. Based on the results of the haplotype-based case control study, the CALCA gene could be the susceptibility gene for EH. Am J Hypertens 2007;20:527–532 © 2007 American Journal of Hypertension, Ltd.

Key Words: Calcitonin-related peptide α (CALCA), polymorphism, SNP, haplotype, association study.

Calcitonin-related peptide α (CALCA) is a 37-amino-acid vasoactive neuropeptide. In mammals, CALCA is distributed in the central and peripheral nervous systems. The calcitonin/calcitonin gene-related peptide gene complex encodes a small family of peptides: calcitonin, katacalcin, and CALCA. CALCA is produced by tissue-specific alternative splicing of the primary transcript of the calcitonin/calcitonin gene-related peptide gene complex and is synthesized almost exclusively in neuronal tissues.[1] CALCA is a potent vasodilator, which is from 100 to 1000 times more potent than other vasodilators, such as adenosine, substance P, or acetylcholine.[2] The systolic blood pressure (BP) of CALCA knockout mice has been reported to be significantly elevated.[3]

Hypertension affects 25% of most adult populations and is a major risk factor for death from stroke, myocardial infarction, and congestive heart failure.[4–8] The largest group of hypertensive patients has essential hypertension (EH). Essential hypertension is considered to be a multifactorial disease.[9] Several reports have shown that there are EH susceptibility genes, including the genes for angiotensinogen[10] and angiotensin-converting enzyme.[11]

The human CALCA gene is located on chromosome 11p15.2-p15.1, spans approximately 3.8 kb, and contains five exons (Fig. 1). In the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/projects/SNP/), 28

Received April 6, 2006. First decision May 24, 2006. Accepted June 6, 2006.

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This work was supported financially by a grant from the TORAY Co., Ltd., and the Ministry of Education, Culture, Sports, Science and Technology of Japan (High-Tech Research Center, Nihon University).

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0895-7061/07/$32.00
doi:10.1016/j.amjhyper.2006.06.008

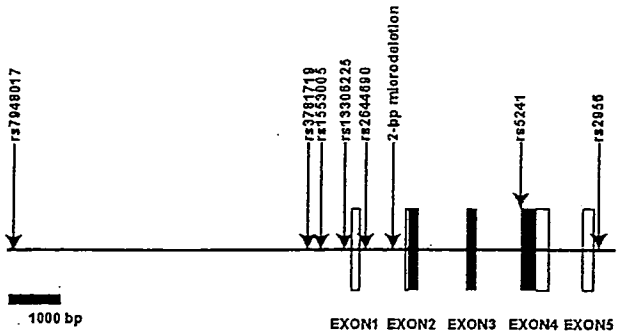

FIG. 1. The structure of the human calcitonin-related peptide α gene. This gene consists of five exons separated by four introns. Boxes indicate exons and lines indicate introns and intergenic regions. Filled boxes indicate coding regions. Arrows mark the locations of polymorphisms. The bar marks the length of 1000 base pairs.

SNPs have been recorded (accessed on April 6, 2005). In the coding region, four mutations have been recorded: two missense mutations (rs5241 and rs13306224 located on exon 4) and two silent mutations (rs5239, located on exon 3, and rs2644689, located on exon 4). Nonsense mutations in the coding region were not recorded.

A 16-bp microdeletion polymorphism on intron 1 has been reported in a family with multiple cases of unipolar or bipolar depressive disorder; however, this polymorphism has not been formally registered.[12] There have been no reports dealing with the relationship between the CALCA gene variants and EH.

The aims of this study were to discover novel polymorphisms or mutations in the 5′ flanking region of the CALCA gene in Japanese subjects and to assess the association between this gene and EH using novel genetic markers and known SNPs.

Methods

Subjects

From 1993 to 2003, subjects diagnosed as having EH were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo. There were 274 EH patients (male/female ratio = 1.84). Essential hypertension was diagnosed based on the following criteria: seated systolic BP >160 mm Hg or diastolic BP >100 mm Hg on three occasions within 2 months after the first BP reading. None of the EH patients used antihypertensive medications. Patients diagnosed as having secondary hypertension were excluded. A total of 225 normotensive (NT) age-matched healthy individuals (male/female ratio = 2.6) served as control subjects. None of the control subjects had a family history of hypertension, and all had systolic BP <130 mm Hg and diastolic BP <85 mm Hg. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.

Genotyping

We sequenced from −1 to −2000 nucleotides upstream ATG as the start codon of the human CALCA gene to find novel polymorphisms or mutations in the 20 EH patients using the polymerase chain reaction (PCR) and the direct sequencing method.[13] In addition, based on the information obtained from the NCBI SNP database and the Applied Biosystems–Celera Discovery System (ABI-CDS, http://www.appliedbiosystems.com) database, SNPs that had a minor allele frequency of more than 20% or had a known function were selected.

Blood samples were collected from all participants and genomic DNA was extracted from the peripheral blood leukocytes by extraction with phenol and chloroform.[14]

Genotyping was done using the TaqMan SNP Genotyping Assay (Applied Biosystems Inc., Foster City, CA), PCR and single-stranded conformation polymorphism (PCR-SSCP) analysis, and PCR and electrophoresis.

The TaqMan SNP Genotyping assays were done using the method of *Taq* amplification.[15] In the 5′ nuclease assay, discrimination occurs during PCR, as the allele-specific fluorogenic probes, when they are hybridized to the template, are cleaved by the 5′ nuclease activity of *Taq* polymerase. The probes contain a 3′ minor groove-binding group (MGB) that hybridizes to single-stranded targets with increased sequence specificity compared to ordinary DNA probes. This reduces nonspecific probe hybridization and results in low background fluorescence during the 5′ nuclease PCR assay (TaqMan, ABI). Cleavage results in the increased emission of a reporter dye. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe was labeled with two reporter dyes at the 5′ end. In this study, VIC and FAM were used as the reporter dyes. The primers and probes in the TaqMan SNP Genotyping Assay (ABI) were chosen from the information available on the ABI web site (http://myscience.appliedbiosystems.com).

The PCR amplification was done using 6 μL of TaqMan Universal Master Mix, No AmpErase UNG (2×) (ABI) in 12-μL final reaction volumes, with 2 ng of DNA, 0.22 μL of TaqMan SNP Genotyping Assay Mix (20× or 40×) containing 900 nmol/L primers, and a 200-nmol/L final concentration of the probes. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and finally 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system.

Each 96-well plate contained 80 samples of an unknown genotype and 4 samples with no DNA but with reagents (control). The control samples without DNA were necessary in the Sequence Detection System (SDS) 7700 for signal processing as outlined in the TaqMan Allelic Discrimination Guide (ABI). These plates were read on the SDS 7700 instrument using the end point analysis mode of the sequence detection system (SDS) version 1.6.3 software package (ABI). The genotypes were deter-

mined visually, based on the dye component fluorescent emission data depicted in the X-Y scatter plot of the SDS software. The genotypes were also determined automatically by the signal processing algorithms of the software. The results of each scoring method were saved in two separate output files for later comparison.[16]

Genotyping of the 2-bp microdeletion polymorphism was performed using PCR and electrophoresis. The PCR primer was designed as follows: sense primer, 5′-CCCAGAAGAGGAGGACAGCTCTGGGT-3′; and antisense primer, 5′-AGAGCTGGAGGAGCGATCCTAGAGGGA-3′. The PCR conditions included initial denaturation at 96°C for 3 min, followed by 60 cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were separated on 6% polyacrylamide sequencing gel.[17] A 184-bp fragment and a 182-bp fragment were distinguished.

Genotyping of rs13306225 was done using PCR-SSCP analysis. The PCR primer was designed as follows: sense primer, 5′-CAGGTTCTGGAAGCATGAGGGTGACGC-3′; and antisense primer, 5′-CGACTGCTCTTATTCCCGCCGCTGT-3′. The PCR conditions were initial denaturation at 96°C for 3 min, followed by 60 cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were analyzed on 5% nondenaturing acrylamide gels.[17,18]

Biochemical Analysis

The plasma total cholesterol concentration and the serum creatinine concentration were measured using standard methods in the Clinical Laboratory Department of Nihon University Hospital.

Statistical Analysis

All continuous variables are expressed as mean ± standard deviation. Continuous variables were assessed using Mann-Whitney's U test. Categorical variables were assessed with Fisher's exact test. The genotype and allele distributions were examined with Fisher's exact test. The contingency table including zero was not examined. Multiple logistic regression analyses were done to assess the contribution of confounders (gender, body mass index [BMI], history of diabetes, and hyperlipidemia). Differences in continuous variables between groups were analyzed by one-way ANOVA or t test after normal distribution and homogeneity of variance were confirmed. Based on the genotype data of the genetic variations, a linkage disequilibrium (LD) analysis and a haplotype-based case-control study were done using the expectation maximization (EM) algorithm[19] of the SNPAlyze software program, version 3.2 (DYNACOM Co., Ltd., Yokohama, Japan). A pair-wise LD analysis was done using four SNP pairs. D′ values >0.5 were used to assign SNP locations to one haplotype block. Tagged SNPs were selected by omitting one SNP from an SNP pair showing an r^2 >0.5 for each haplotype block. In this haplotype-based case-control study, haplotypes with a frequency <0.03 were excluded. The distribution of the haplotypes' frequency was calculated using the χ^2 test. Statistical significance was established at $P < .05$. Statistical analyses were done using SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL).

Results

We discovered a novel 2-bp microdeletion polymorphism GenBank accession number AB259617 located in intron 1 and −374 nucleotides upstream ATG as the start codon. The four SNPs were already registered and were named rs3781719 (−1784 T/C), rs1553005 (−1750 C/G), rs13306225 (−1218 C/T), and rs2644690 (−1036 G/A). rs3781719 and rs1553005 were completely linked. The T allele of rs3781719 was linked to the C allele of rs1553005, and the C allele of rs3781719 was linked to the G allele of rs1553005. In addition, we added three SNPs, rs7948017 (A/C), rs5241 (C/A), and rs2956 (A/T), that were referenced in the database. rs7948017 was located at −7644 bp upstream from the start codon. rs5241, which was a missense mutation, was located on exon 4. rs2956 was located at 120 bp from the end of exon 5. Thus, in total, we genotyped six SNPs and one microdeletion in the human CALCA gene to examine genetic differences between EH patients and control subjects.

Table 1 shows the clinical features of the two study populations. The BMI, systolic and diastolic BP, pulse, and the rate of hyperlipidemia were significantly higher in the EH group than in the control group. The total cholesterol plasma concentration and the rate of diabetes were significantly higher in the total EH group and the male EH group.

rs2644690 was excluded because there was no heterogeneity; all of the participants were genotyped as A/A. rs13306225 was also excluded because the genotype distribution of rs 13306225 did not agree with the Hardy-Weinberg equilibrium. In the present study, rs7948017 was labeled as SNP1, rs1553005 as SNP2, rs5241 as SNP3, rs2956 as SNP4, and the 2-bp microdeletion polymorphism was labeled as DEL. The genotype distribution of each polymorphism did not show a statistically significant difference from the Hardy-Weinberg equilibrium values (data not shown).

Table 2 shows the distribution of the genotypes and the alleles of the four SNPs and the 2-bp microdeletion polymorphism. There were no statistically significant differences between the groups using Fisher's exact test and multiple logistic regression analysis (the results of multiple logistic regression analysis are not shown). The three subjects with DEL were all from the EH group. In the EH group, there was a significant difference in the diastolic BP between the two genotypes of DEL using Student t test (with DEL, 124.3 ± 23.8; without DEL, 105.5 ± 14.0; $P = .023$).

Table 1. Characteristics of study participants

	Total			Men			Women		
	EH patients	Control subjects	*P*	EH patients	Control subjects	*P*	EH patients	Control subjects	*P*
Age (y)	50.1 ± 6.6	51.1 ± 6.6	.291	49.5 ± 7.2	51.4 ± 6.2	.053	51.5 ± 4.7	50.4 ± 7.5	.228
BMI (kg/m^2)	24.6 ± 3.8	22.7 ± 3.1	<.001	24.8 ± 3.6	22.9 ± 3.2	<.001	24.4 ± 4.1	22.4 ± 3.0	.002
SBP (mm Hg)	173.2 ± 20.0	112.7 ± 10.5	<.001	171.2 ± 19.4	112.8 ± 10.4	<.001	177.5 ± 20.8	112.5 ± 10.8	<.001
DBP (mm Hg)	105.7 ± 14.2	69.8 ± 8.2	<.001	106.1 ± 14.4	69.9 ± 8.0	<.001	104.9 ± 13.9	69.5 ± 8.6	<.001
Pulse (beats/min)	78.2 ± 15.0	73.2 ± 14.5	<.001	78.1 ± 15.4	73.4 ± 16.3	.002	78.3 ± 14.1	72.6 ± 8.6	.016
Creatinine (mg/100 mL)	0.85 ± 0.25	0.84 ± 0.22	.818	0.94 ± 0.24	0.90 ± 0.21	.339	0.68 ± 0.18	0.69 ± 0.16	.437
Total cholesterol (mg/100 mL)	212.1 ± 38.4	201.1 ± 41.0	.001	208.8 ± 38.6	197.4 ± 38.8	.009	218.8 ± 37.3	210.8 ± 45.2	.129
HDL cholesterol (mg/100 mL)	56.2 ± 17.3	56.2 ± 16.6	.863	53.1 ± 16.4	54.5 ± 16.0	.323	62.5 ± 17.3	60.6 ± 17.4	.496
Uric acid (mg/100 mL)	5.7 ± 1.6	5.5 ± 1.5	.307	6.2 ± 1.5	5.8 ± 1.4	.053	4.6 ± 1.2	4.6 ± 1.4	.612
Hyperlipidemia	56%	39%	<.001	53%	37%	.003	63%	43%	.021
Diabetes	9%	4%	.045	10%	4%	.042	6%	3%	.700
Drinking	70%	74%	.318	84%	83%	.884	40%	51%	.189
Smoking	58%	61%	.522	69%	68%	.908	36%	44%	.399

BMI = body mass index; DBP = diastolic blood pressure; EH = essential hypertension; HDL = high density lipoprotein; SBP = systolic blood pressure.

Continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as percentage. The *P* value of continuous variables was calculated by Mann-Whitney's U test. The *P* value of categorical variables was calculated by Fisher's exact test.

The patterns of linkage disequilibrium in the CALCA gene are shown with their D′ and r^2 values (Table 3). All polymorphisms were located in one haplotype block. Because the r^2 of SNP1–SNP2–SNP4 was large, we constructed a haplotype-based association study using SNP2, DEL, and SNP3. Three possible haplotypes (H1, H2, and H3) were predicted, and each had a frequency of more than 0.03 (Table 4). The overall distribution of these haplotypes was statistically significantly different between the EH group and the control group (χ^2 = 16.13, *P* = .00031). There was also a statistically significant difference in the H3 haplotype (C-AGins-A) between the groups (χ^2 = 23.4, *P* = .000059).

Table 2. Genotype and allele distributions in patients with EH and control subjects

	EH patients	Control subjects	*P*
SNP1 rs7948017			
Genotype			
A/A	149	125	.711
A/C	97	82	
C/C	28	18	
Allele			
A	395	332	.568
C	153	118	
SNP2 rs1553005			
Genotype			
G/G	149	125	.711
G/C	97	82	
C/C	28	18	
Allele			
G	395	332	.568
C	153	118	
DEL			
Genotype			
AGins/AGins	271	225	–
AGins/–	3	0	
–/–	0	0	
Allele			
AGins	545	450	–
–	3	0	
SNP3 rs5241			
Genotype			
C/C	253	214	–
C/A	21	11	
A/A	0	0	
Allele			
C	527	439	.279
A	21	11	
SNP4 rs2956			
Genotype			
A/A	162	134	.979
A/T	91	75	
T/T	21	16	
Allele			
A	415	343	.882
T	133	107	

DEL = 2-bp microdeletion polymorphism; EH = essential hypertension.

The *P* values of genotypes and alleles were calculated by Fisher's exact test.

Table 3. Pairwise linkage disequilibrium (D'below diagonal and r^2 above diagonal) for the five polymorphisms

		r^2									
		EH Patients					Control Subjects				
		SNP1	SNP2	DEL	SNP3g	SNP4	SNP1	SNP2	DEL	SNP3	SNP4
D'	SNP1		1.000	0.001	0.103	0.810		1.000	0.000	0.071	0.878
	SNP2	1.000		0.001	0.103	0.810	1.000		0.000	0.071	0.878
	DEL	0.248	0.248		0.011	0.002	0.000	0.000		0.000	0.000
	SNP3	1.000	1.000	0.279		0.006	1.000	1.000	0.000		0.008
	SNP4	0.989	0.989	0.933	0.700		1.000	1.000	0.000	1.000	

EH = essential hypertension.
Values of D' >0.5 and values of r^2 >0.5 are shaded.

Discussion

CALCA has been well documented as one of the major vasodilators.[2] It has been reported that plasma immunoreactive CALCA levels are significantly higher in spontaneously hypertensive rats (SHR).[20] In addition, neuronal levels of CALCA mRNA have been reported to be increased in rats with mineralocorticoid salt-induced hypertension.[21] Furthermore, the systolic BP of CALCA gene knockout mice was significantly higher than in wild-type mice, in both male mice (knockout, 160 ± 6.1 *v* wild, 125 ± 4.8 mm Hg) and female mice (knockout, 163 ± 4.8 *v* wild, 135 ± 33 mm Hg).[3] Furthermore, the renin-angiotensin system was found to be activated in CALCA gene knockout mice.[22] Based on this information, we concluded that the human CALCA gene could be a candidate gene for EH.

In the present study, we genotyped seven polymorphisms of the CALCA gene in Japanese subjects and then assessed the association between the CALCA gene and EH. There were no significant differences between any of the CALCA gene polymorphisms and EH. However, all of the participants with a 2-bp microdeletion polymorphism (DEL) belonged to the EH group. Given the small number of subjects, it was not possible to assess the relationship between DEL and EH using statistical techniques. In the EH group, there was a significant difference in diastolic BP between the two genotypes of DEL. Given this finding, a study with more subjects needs to be done to further assess the relationship between DEL and EH.

Since the draft sequence of the human genome was completed in 2001, the methodology and strategy of doing genetic research have changed dramatically. The SNPs are now used for the positional cloning of susceptibility genes by doing whole genome-wide scanning.[23] Most of all, haplotype analysis has changed. Recent studies have shown that the human genome has a haplotype block structure that can be divided into discrete blocks of limited haplotype diversity. In each block, a small fraction of SNPs, referred to as tag SNPs, can be used to distinguish a large fraction of the haplotypes. These tag SNPs have the potential to be extremely useful for association studies, as they could make it unnecessary to genotype all of the SNPs. Haplotype-based analysis is considered to be much more powerful than a marker-by-marker analysis.[24] In genes with multiple susceptibility alleles, in particular when the LD between the polymorphisms is weak, a

Table 4. Haplotype frequency estimates

Haplotype	Frequency			
SNP2- DEL-SNP3	EH patients	Control subjects	χ^2	*P*
H1 Mj-Mj-Mj G-AG-C	0.721	0.755	1.36	.24
H2 Mn-Mj-Mj C-AG-C	0.242	0.245	0.02	.90
H3 Mn-Mj-Mn C-AG-A	0.037	<0.001	16.13	.000059*

EH = essential hypertension; Mj = major allele; Mn = minor allele.
Overall distribution of these haplotypes was statistically significant (χ^2 = 16.2, *P* = .00031).
Haplotypes with frequencies more thanr 0.03 were estimated using SNPAlyze software.
Mj and Mn indicate haplotypes with major and minor frequencies, respectively.
* $P < .05$.

haplotype-based association study has advantages over an analysis based on individual polymorphisms.[25] In the present study, the haplotypes were constructed using three polymorphisms (SNP2, DEL, and SNP3), based on the results of the LD analysis. The H3 haplotype (C-AGins-A) was statistically significantly more frequent in the EH group than in the control group. This result was not found by using a haplotype-based association study but only by using an association study that examined each polymorphism. The frequency of the H3 haplotype in the EH group was only 3.7%. However, this is not surprising given the low frequency of the susceptibility haplotype, as EH is thought to be a multifactorial disorder.

In conclusion, a novel 2-bp microdeletion polymorphism was discovered in intron 1 of the CALCA. The haplotype-based case-control study showed that the CALCA gene could be the susceptibility gene of EH. Further studies are needed to clarify the causal/susceptibility mutation of the CALCA gene or neighboring genes in EH.

Acknowledgments

We thank Naoyuki Sato and Kaoru Sugama for their excellent technical assistance.

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International Journal of Medical Sciences
ISSN 1449-1907 www.medsci.org 2007 4(3):146-152

Research Paper

A Novel Variable Number of Tandem Repeat of the Natriuretic Peptide Precursor B gene's 5'-Flanking Region is Associated with Essential Hypertension among Japanese Females

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Received: 2006.12.04; Accepted: 2007.05.15; Published: 2007.05.16

Background: Brain natriuretic peptide (BNP) acts primarily as a cardiac hormone; it is produced by the ventricle and has both vasodilatory and natriuretic actions. Therefore, the BNP gene is thought to be a candidate gene for essential hypertension (EH). The present study identified variants in the 5'-flanking region of natriuretic peptide precursor B (*NPPB*) gene and assessed the relationship between gene variants and EH.

Methods: The polymerase chain reaction-single strand conformation polymorphism method and nucleotide sequencing were used to identify variants.

Results: A novel variable number of tandem repeat (VNTR) polymorphism in the 5'-flanking region (-1241 nucleotides from the major transcriptional initiation site) was discovered. This VNTR polymorphism is a tandem repeat of the 4-nucleotide sequence TTTC. There were 8 alleles, ranging from 9-repeat to 19-repeat. An association study was done involving 317 EH patients and 262 age-matched normotensive (NT) subjects. The 11-repeat allele was the most frequent (88.2%); the 16-repeat allele was the second most frequent (10.5%) in the NT group. The observed and expected genotypes were in agreement with the predicted Hardy-Weinberg equilibrium values (P=0.972). Among females, the overall distribution of genotypes was significantly different between the EH and NT groups (p=0.039). The frequency of the 16-repeat allele was significantly lower in the female EH group (6.5%) than in the female NT group (12.2%, p=0.046).

Conclusions: The 16-repeat allele of the VNTR in the 5'-flanking region of *NPPB* appears to be a useful genetic marker of EH in females.

Key words: Brain natriuretic peptide, essential hypertension, variable number of tandem repeat, association study

1. Introduction

Natriuretic peptides constitute a family of three structurally related molecules: atrial natriuretic peptide (ANP) [1], brain natriuretic peptide (BNP) [2], and C-type natriuretic peptide (CNP) [3]. ANP and BNP act mainly as cardiac hormones and are produced primarily by the atria and ventricles, respectively, whereas C-type natriuretic peptide is expressed mainly in the brain [4,5].

BNP, which was originally isolated from the porcine brain [6], shows an amino acid sequence homology to ANP. BNP has central and peripheral actions that are similar to those of ANP. The heart has the highest concentrations of BNP, and BNP acts as a cardiac hormone. Intravenous injection of BNP causes a significant decrease in blood pressure [7]. Transgenic mice that overexpress BNP have a measurable reduction in blood pressure [8]. Mukoyama *et al.* showed that plasma BNP levels are higher in individuals with essential hypertension (EH) than in normotensive (NT) individuals [7]. These findings suggest that the BNP gene is a candidate gene for EH. EH is thought to be a multifactorial disorder; several studies have shown that an association analysis with genetic variants can be used to identify susceptibility genes for EH [9].

The aim of the present study was to identify mutations or polymorphisms in the 5'-flanking region of the *NPPB* gene and to assess the relationship between variants of the gene and EH.

2. Subjects and Methods

Subjects

The EH group consisted of 317 patients (mean age, 49.7 ± 7.8 years) with EH diagnosed based on sitting systolic blood pressure (SBP) greater than 160

mmHg and/or diastolic blood pressure (DBP) greater than 100 mmHg measured on three separate occasions within 2 months after the initial BP measurement (Table 1). These patients were not being treated with antihypertensive drugs. All EH subjects had a positive family history of hypertension; patients diagnosed with secondary hypertension were excluded from the study. We also enrolled 262 healthy, normotensive (NT) subjects (mean age, 51.2 ± 10.8 years). None of the NT subjects had a family history of hypertension; all had an SBP less than 130 mmHg and a DBP less than 85 mmHg. A family history of hypertension was defined as having grandparents, uncles, aunts, parents, or siblings who had been diagnosed with hypertension. Written informed consent was obtained from each subject based on a protocol approved by the Ethics Committee of the Nihon University School of Medicine and the Clinical Studies Committee of Nihon University Hospital [10].

Table 1. Characteristics of study participants

	Total			Males			Females		
	NT	EH	p Value	NT	EH	p Value	NT	EH	p Value
No. of subjects	262	317		184	193		78	124	
Age (years)	51.2±10.8	49.7±7.8	0.052	50.1±9.4	48.5±8.4	0.072	53.6±13.2	51.5±6.5	0.135
BMI (kg/m²)	22.6±4.0	24.6±4.4	<0.001	22.9±3.4	24.7±4.7	<0.001	21.6±5.1	24.3±3.9	<0.001
SBP (mmHg)	113±11	173±20	<0.001	114±10	171±19	<0.001	113±11	175±23	<0.001
DBP (mmHg)	70±8	107±13	<0.001	70±8	108±13	<0.001	69±9	105±14	<0.001
Pulse (beats/min)	74±12	78±15	<0.001	73±12	78±16	0.002	75±12	78±14	0.110
Creatinine (mg/dl)	0.85±0.22	0.85±0.26	0.928	0.92±0.20	0.94±0.26	0.302	0.69±0.18	0.69±0.18	0.766
Total cholesterol (mg/dl)	197±44	212±43	<0.001	193±42	209±46	0.001	208±46	217±38	0.141
HDL cholesterol (mg/dl)	57±17	56±17	0.256	56±16	52±15	0.022	61±19	62±17	0.972
Uric acid (mg/dl)	5.8±4.4	6.0±7.4	0.610	5.8±1.3	6.1±1.4	0.018	5.6±8.0	5.9±11.8	0.894

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; NT, normotensives; EH, essential hypertension

Biochemical analysis

Plasma total cholesterol concentrations, as well as serum creatinine and uric acid concentrations, were measured using standard methods in the Clinical Laboratory Department of Nihon University Hospital [11]. Plasma BNP levels were measured in NT subjects and EH patients. A total of 76 subjects (43 in the NT group and 33 in the EH group) who did or did not have the 16- repeat allele were selected randomly. Plasma BNP levels were measured using a highly sensitive immunoradiometric assay (Shiono RIA BNP assay kit, Shionogi Co., Ltd., Tokyo, Japan) as described previously [12].

Polymerase chain reaction - single strand conformation polymorphism (PCR-SSCP)

Genomic DNA was extracted from peripheral blood leukocytes using a standard method [13]. To screen for mutations, two oligonucleotide primers (sense, 5'- AAGGAGGCACTGGGAGAGGGGAAAT -3' (bases -1323 to -1299 from the major transcriptional initiation site) and antisense, 5'-AATTAGCTGGGCATGGTGGCAGGCG-3' (bases -1075 to -1051)) that recognize part of the 5'-flanking region of the *NPPB* gene were designed, since this region has been reported to be a major promoter region (Fig. 1A) [14]. PCR-SSCP was done (GenePhor System; Amersham Biosciences Corp, Piscataway, NJ, USA) [15]. PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Branchburg, NJ, USA) with the following amplification conditions: initial denaturation at 96°C for 3 min followed by 35 cycles of 98.5°C for 25 s, 65°C for 30 s, 68°C for 30 s, and a final extension of 68°C for 10 min. PCR products were separated by electrophoresis on 10% precast polyacrylamide gels (Amersham Biosciences Corp) at 5°C for 80 min and then subjected to silver staining (Dai-ichi Kagaku, Tokyo, Japan). The electrophoresis parameters were set according to the manufacturer's protocol.

Sequencing analysis

Two oligonucleotides (sense, 5'-AAGGAGGCACTGGGAGAGGGGAAAT-3' (bases -1323 to -1299) and antisense, 5'-CCCCACCAAGCCAACACAGGATGGA -3' (bases -919 to-895) were used to amplify a 429-bp product from genomic DNA (Fig. 1A). The PCR products were purified using a Microcon 100 column (U.S. Amicon Inc. Beverly, MA,USA). The resulting products were ligated to pCR2.1™ vectors and cloned (TA Cloning Kit, Invitrogen, San Diego, CA, USA). The ligation of the products was confirmed by direct DNA sequencing (ABI PRISM 310 Genetic Analyzer) [16].

Genotyping

Genotyping was done using fragment analysis, and the sequencing primers were used. PCR amplification consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 98.5°C for 25 sec, 63°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 10 min. Then, the PCR products were analyzed using an automatic electrophoresis system (Agilent 2100 bioanalyzer system™; Agilent Technologies, Waldbronn, Germany).

Statistical analysis

Data are presented as the mean ± SD. The Hardy-Weinberg equilibrium was assessed by doing chi-square (χ^2) analysis. Differences in the clinical data between the EH and NT groups were assessed by analysis of variance (ANOVA). The distributions of the genotypes or alleles between EH patients and NT subjects were tested using a two-sided Fisher's exact test. Multiple logistic regression analyses were done to assess the contribution of confounders (gender, BMI) [17]. A value of $P < 0.05$ was considered statistically significant.

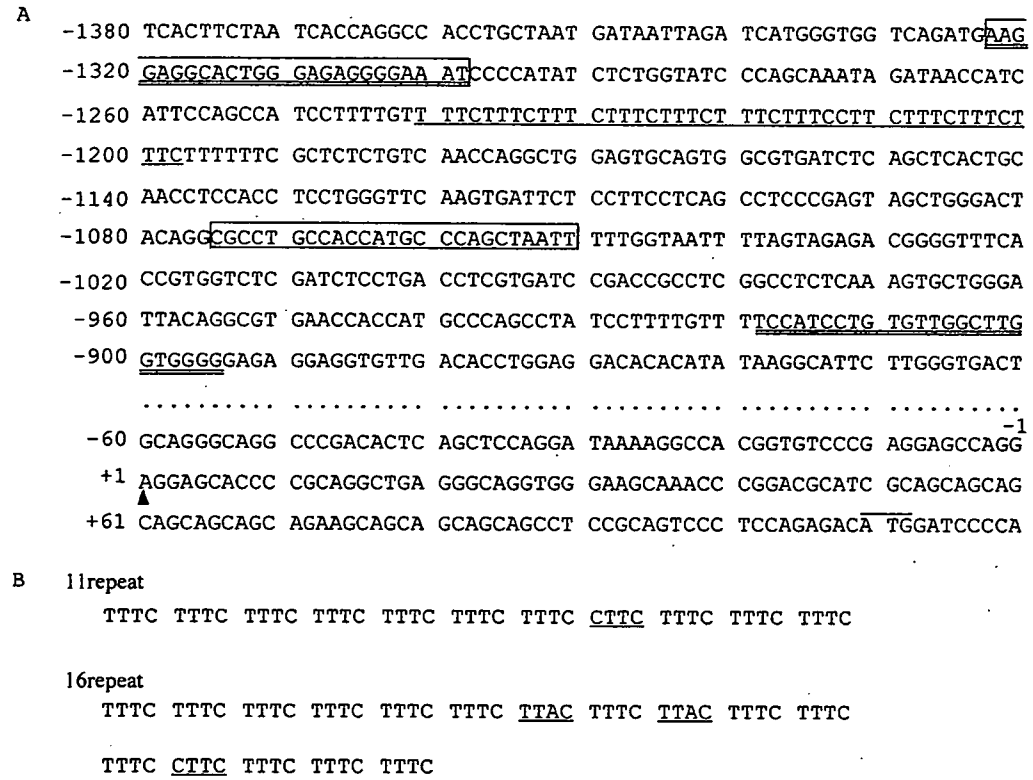

Figure 1 A: Nucleotide sequence of the 5'-flanking region of the human *NPPB* gene. The nucleotide sequences are numbered (left) with respect to the major transcription start site (designated +1; *closed triangle*). *Boxes*, the primers (PCR-SSCP); *double underlining*, the primers (sequence and genotyping); *overlining*, ATG initiation codon; *Underlining*, the variable number of tandem repeat (VNTR) consists of the 4 nucleotides (TTTC). This figure shows the 11 repeat types. **B**: Nucleotide sequence of the 11 and 16 repeat allele.

3. Results

We discovered a novel variable number of tandem repeat (VNTR) polymorphism consisting of a 11-nucleotide repeat of 4 base pairs (bp) in the 5'-flanking region (-1241 nucleotides from the major transcriptional initiation site) (GenBank accession number AB265677). This VNTR polymorphism is a tandem repeat of the 4-nucleotide sequence TTTC (Fig. 1A). There were 8 alleles of this VNTR polymorphism, ranging from 9-repeat to 19-repeat (Fig. 1B).

The association study showed that the 11-repeat allele was most frequent in the NT group (88.2%). The 16-repeat allele was second most frequent in the NT group (10.5%). Furthermore, in the NT group, the observed and expected genotypes were in good agreement with the predicted Hardy-Weinberg equilibrium values (P=0.972). Of note, the overall distribution of genotypes in females was significantly different between the EH and NT groups (p=0.039); the frequency of the 16-repeat allele was significantly lower in the EH group (6.5%) than in the NT group (12.2%, p=0.046) (Table 2).

On multiple logistic regression analysis, a significant association between allele 16 (p=0.034) and female gender was noted, even after adjustment for confounding factors; the calculated odds ratio was 1.18 (95%CI: 1.07-1.20).

The clinical data of each genotype were assessed. There were no significant differences in SBP and DBP levels, or in the pulse of subjects with or without the 16 repeat allele (Table 3).

The plasma BNP level was significantly higher in the EH group than in the NT group (p=0.0203). The plasma BNP level in each genotype with or without the 16 repeat allele was determined (Table 4); there were no significant differences among the groups. It was impossible to perform this analysis in EH females, as none of them had the 16 repeat allele.

All subjects were classified into 3 groups based on their BMI levels (lean, BMI<18.5; normal, 18.5<BMI<25; obese, BMI >25). There was no associa-