Table 1. Characteristics in the four study groups according to responses to weight loss

	Weight Loss Maintenance	Rebound	Slow Weight Loss	Weight Loss Resistance
Subjects (*n*)	37	36	60	21
Age at entry (yr)	35 ± 6	37 ± 6	37 ± 6	37 ± 8
Height (m)	1.73 ± 0.05	1.74 ± 0.04	1.74 ± 0.05	1.72 ± 0.05
BMI (kg/m^2)				
Entry	27.1 ± 1.9	27.7 ± 1.6	27.3 ± 1.7	27.4 ± 2.0
6 months	22.8 ± 1.4†**	22.8 ± 0.8†‖**	25.4 ± 2.0‡	26.6 ± 2.2§
12 months	22.6 ± 2.3†**	23.4 ± 2.2*#	24.9 ± 1.9	26.1 ± 2.9‡
18 months	22.4 ± 2.0†‡**	25.1 ± 2.2#	24.1 ± 2.0*#	26.0 ± 2.3‖#
24 months	22.1 ± 1.5†§**	26.4 ± 2.1‖	23.8 ± 1.9*‡**	26.1 ± 2.2‖#
Total body fat mass (kg)				
Entry	21.5 ± 5.4†‡‖	25.6 ± 4.9*	25.9 ± 6.1	27.8 ± 4.2‡
6 months	17.8 ± 4.7†‡‖#	21.0 ± 3.8*#	22.3 ± 5.2	24.3 ± 4.1‡
12 months	16.5 ± 4.1†‡‖#	21.5 ± 4.1#	20.7 ± 4.9#	22.8 ± 3.8#
18 months	15.6 ± 3.8†§**	22.2 ± 4.7‖#	18.7 ± 5.1*‡**	21.9 ± 4.1‖**
24 months	15.2 ± 4.3†§**	22.5 ± 5.0‖#	17.8 ± 4.9*‡**	21.2 ± 4.7‖**
Waist-to-hip ratio				
Entry	1.16 ± 0.10*‖	1.20 ± 0.11	1.23 ± 0.15	1.23 ± 0.15
6 months	0.98 ± 0.09*‖#	1.03 ± 0.10*‖#	1.15 ± 0.14*‡	1.22 ± 0.09‡‖
12 months	0.95 ± 0.10*‡#	1.05 ± 0.09*#	1.05 ± 0.11*#	1.17 ± 0.13‡‖
18 months	0.92 ± 0.09†§**	1.15 ± 0.20‖	1.03 ± 0.13*‡#	1.12 ± 0.11‖#
24 months	0.90 ± 0.12†§**	1.12 ± 0.14‖	0.98 ± 0.12*‡**	1.16 ± 0.13‖#
Systolic BP (mm Hg)				
Entry	136 ± 12	134 ± 10	137 ± 10	133 ± 12
6 months	135 ± 10	134 ± 9	134 ± 9	134 ± 8
12 months	133 ± 9	136 ± 7	134 ± 8	135 ± 8
18 months	128 ± 10*‡	135 ± 8	132 ± 9	134 ± 6
24 months	128 ± 9#	133 ± 8	130 ± 10#	130 ± 11
Diastolic BP (mm Hg)				
Entry	78 ± 10	77 ± 9	79 ± 9	74 ± 11
6 months	77 ± 9	76 ± 7	76 ± 8	73 ± 7
12 months	76 ± 8	77 ± 6	75 ± 8	73 ± 8
18 months	74 ± 9	77 ± 7	74 ± 9	76 ± 7
24 months	73 ± 8#	76 ± 7	72 ± 10#	72 ± 11
Mean BP (mm Hg)				
Entry	98 ± 12	96 ± 10	98 ± 9	94 ± 14
6 months	97 ± 10	96 ± 7	96 ± 9	94 ± 7
12 months	95 ± 9	97 ± 6	95 ± 8	94 ± 8
18 months	92 ± 10	96 ± 8	94 ± 9	95 ± 6
24 months	92 ± 9#	95 ± 8	91 ± 11#	93 ± 13
Heart rate (beats/min)				
Entry	68 ± 7	70 ± 8	68 ± 8	71 ± 7
6 months	66 ± 6	68 ± 7	67 ± 7	71 ± 6
12 months	65 ± 7	69 ± 7	66 ± 8	69 ± 6
18 months	64 ± 5‡	70 ± 8	66 ± 7	68 ± 7
24 months	62 ± 6‡#	68 ± 7	65 ± 7	67 ± 5
Plasma norepinephrine (pmol/mL)				
Entry	1.53 ± 0.32†‡‖	1.87 ± 0.37*‖	2.25 ± 0.31*‡	2.59 ± 0.53‡‖
6 months	1.31 ± 0.30†¶	1.49 ± 0.41†#	2.01 ± 0.33‡	2.22 ± 0.51§
12 months	1.17 ± 0.31†‡¶#	1.63 ± 0.44*	1.78 ± 0.35#	2.07 ± 0.47‡
18 months	1.11 ± 0.32†‖#	1.40 ± 0.51*#	1.54 ± 0.31**	2.02 ± 0.46‡‖#
24 months	1.09 ± 0.28†‡‖**	1.32 ± 0.37*#	1.41 ± 0.37**	1.87 ± 0.48‡‖**
Plasma leptin (ng/mL)				
Entry	7.9 ± 2.8*‖	8.9 ± 2.9*‖	10.1 ± 3.0‡	11.7 ± 2.7‡
6 months	5.8 ± 2.5†#	6.2 ± 2.4†#	7.8 ± 2.9*	10.2 ± 3.0§‖
12 months	4.5 ± 1.9†‖#	5.8 ± 2.3*#	6.8 ± 2.7*#	8.5 ± 2.4‡‖
18 months	4.0 ± 1.7†‡**	6.1 ± 2.6*#	6.0 ± 2.8*#	8.2 ± 3.0‡‖#
24 months	3.7 ± 1.8†‡**	6.7 ± 2.5‖#	5.1 ± 1.7*‡**	7.1 ± 2.9‖#
HOMA-IR				
Entry	2.3 ± 0.5	2.5 ± 0.5	2.5 ± 0.6	2.7 ± 0.6
6 months	2.0 ± 0.3*	2.0 ± 0.4*#	2.3 ± 0.5	2.5 ± 0.5‡
12 months	1.7 ± 0.5‖	2.0 ± 0.5	2.1 ± 0.4	2.2 ± 0.6
18 months	1.7 ± 0.5*#	2.1 ± 0.6	2.0 ± 0.5#	2.2 ± 0.5
24 months	1.4 ± 0.6*‡‖**	2.1 ± 0.5	1.8 ± 0.5**	2.2 ± 0.4#

Table 1. *Continued*

	Weight Loss Maintenance	Rebound	Slow Weight Loss	Weight Loss Resistance
Caloric intake (×1,000kcal/d)				
Entry	2.3 ± 0.6	2.4 ± 0.5	2.4 ± 0.5	2.3 ± 0.4
6 months	1.5 ± 0.4**	1.5 ± 0.3**	1.6 ± 0.3**	1.6 ± 0.2**
12 months	1.4 ± 0.5**	1.5 ± 0.4**	1.5 ± 0.3**	1.5 ± 0.3**
18 months	1.5 ± 0.4**	1.5 ± 0.5**	1.6 ± 0.5**	1.6 ± 0.2**
24 months	1.5 ± 0.3**	1.5 ± 0.4**	1.5 ± 0.4**	1.5 ± 0.2**
Physical activity (×1,000steps/d)				
Entry	9.7 ± 4.3	10.1 ± 4.8	9.6 ± 3.7	9.8 ± 2.9
6 months	21.5 ± 3.8**	22.3 ± 4.1**	20.9 ± 2.9**	20.5 ± 2.1**
12 months	22.5 ± 2.9**	19.5 ± 4.5#	20.4 ± 3.9**	20.1 ± 2.5**
18 months	21.7 ± 2.3**	20.1 ± 3.2**	19.8 ± 3.4**	20.9 ± 2.5**
24 months	20.7 ± 3.7**	19.7 ± 4.1**	22.3 ± 4.5**	20.7 ± 3.0**

Weight loss resistance indicates the subjects who fail to lose weight significantly during 24 months.
BMI = body mass index; BP = blood pressure.
* $P < .05$, † $P < .01$ compared with values in weight loss resistant subjects; ‡ $P < .05$, § $P < .01$ compared with rebound subjects; ‖ $P < .05$, ¶ $P < .01$ compared with values in slow weight loss subjects; # $P < .05$, ** $P < .01$ compared with values at baseline. Data are mean ± SD. $n = 154$.

Statistical Analyses

Genotype frequencies and the Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean ± SD. All data analyses were performed with SPSS 8.0 for Windows program (Chicago, IL, USA). 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 6, 12, and 24 months were significant within the groups and among the groups compared from baseline. Multiple linear regression analyses were used to examine relations among variables using changes in body weight or in mean BP versus changes in hormonal measurements during weight and BP changes.

Results

Prevalence of Weight Loss Maintenance, Rebound Weight Gain, and Weight Loss Resistance

When significant weight loss was defined as a 10% or more reduction in BMI from baseline, 73 subjects succeeded in achieving weight loss at 6 months. Maintenance of weight loss was noted in 37 subjects and rebound weight gain was found in 36 subjects. Sixty other subjects, who did not have a significant weight loss at 6 months, actually succeeded in significant weight loss at 24 months (slow weight loss group). Thus, a total of 97 subjects succeeded in significant weight loss at 24 months. Fifty-seven subjects failed to have significant weight loss at 24 months, 36 subjects had rebound weight gain, and 21 subjects failed to lose weight during the entire 24-month period. Thus, there were four study groups: subjects who failed to lose weight during 24 months represented the weight loss resistant group ($n = 21$); subjects with maintenance of weight loss represented the weight loss maintenance group ($n = 37$); subjects with weight regain represented the weight rebound group ($n = 36$); and those who failed to lose weight at 6 month but succeeded to lose weight at 24 months represented the slow weight loss group ($n = 60$).

Calorie Intake and Physical Activity

Diet compliance (calorie intake) and physical activity (steps per day) were not significantly different among the four groups (Table 1). Behavior (alcohol intake, cigarette smoking) and socioeconomic status was similar among the four study groups throughout the study. Thus, compliance was considered very good for the 24-month period.

Frequencies of β2- and β3-Adrenergic Receptor Polymorphism

Table 2 shows the frequencies of the genotypes and the alleles of β2- and β3-adrenoceptor genes in the four study groups according to the response in weight loss. The weight loss resistant group, the rebound weight gain group, and the slow weight loss group had a significantly higher frequency of the Gly16 allele of the Arg16Gly of the β2-adrenoceptor compared to the weight loss maintenance group ($\chi^2 = 5.76$, $P = .016$; $\chi^2 = 5.38$, $P = .020$; $\chi^2 = 6.11$, $P = .013$, respectively). The weight loss resistant and slow weight loss groups (both groups failed to lose weight at 6 months) had a higher frequencies of the Glu27 allele of the Gln27Glu of the β2-adrenoceptor compared to a combined group with weight loss maintenance and rebound weight gain group (both groups succeeded in significant weight loss at 6 months) ($\chi^2 = 6.16$, $P = .013$; $\chi^2 = 6.22$, $P = .013$, respectively) (Table 2). The frequency distribution of the Glu27 allele of Gln27Glu was 9.4% and that of the Arg64 allele of Trp64Arg

Table 2. Frequencies of the genotype and the allele of the β2- and β3-adrenoceptor polymorphisms in the four study groups according to the response in weight loss

Groups	Genotype (%)			χ^2 Test Among Four Genotype	Allele (%)		χ^2 Test Among Two Alleles
Arg16Gly of β2-adrenoceptor	Arg16/Arg16	Arg16/Gly16	Gly16/Gly16		Arg16	Gly16	
Weight loss maintenance group	18 (48.6%)	14 (37.8%)	5 (13.5%)		50 (67.6%)	24 (32.4%)	
Rebound weight gain group	8 (22.2%)	18 (50.0%)	10 (27.8%)	$\chi^2 = 13.88$,	34 (47.2%)	38 (52.8%)	$\chi^2 = 11.36$,
Slow weight loss group	13 (21.7%)	32 (53.3%)	15 (25.0%)	$P = .031$	58 (48.3%)	62 (51.7%)	$P = .010$
Weight loss resistant group	3 (14.3%)	12 (57.1%)	6 (28.6%)		18 (42.9%)	24 (57.1%)	
Gln27Glu of β2-adrenoceptor	Gln27/Gln27	Gln27/Glu27	Glu27/Glu27		Gln27	Glu27	
Weight loss maintenance group	33 (91.7%)	4 (10.8%)	0 (0.0%)		70 (94.6%)	4 (5.4%)	
Rebound weight gain group	34 (94.4%)	2 (5.6%)	0 (0.0%)	$\chi^2 = ---$,	70 (97.2%)	2 (2.8%)	$\chi^2 = 9.86$,
Slow weight loss group	44 (73.3%)	16 (26.7%)	0 (0.0%)	$P = ---$	104 (86.7%)	16 (13.3%)	$P = .020$
Weight loss resistant group	14 (66.7%)	7 (33.3%)	0 (0.0%)		35 (83.3%)	7 (16.7%)	
Trp64Arg of β3-adrenoceptor	Trp64/Trp64	Trp64/Arg64	Arg64/Arg64		Trp64	Arg64	
Weight loss maintenance group	26 (70.2%)	10 (27.0%)	1 (2.7%)		62 (83.8%)	12 (16.2%)	
Rebound weight gain group	28 (77.8%)	8 (22.2%)	0 (0.0%)	$\chi^2 = 7.91$,	64 (88.9%)	8 (11.1%)	$\chi^2 = 5.43$,
Slow weight loss group	32 (53.3%)	27 (45.0%)	1 (1.7%)	$P = .245$	91 (75.8%)	29 (24.2%)	$P = .143$
Weight loss resistant group	13 (61.9%)	8 (38.1%)	0 (0.0%)		34 (81.0%)	8 (19.0%)	

The definitions for the 4 study groups according to the response in weight loss are referred to in the Results, Prevalence of weight loss maintenance, rebound weight gain, and weight loss resistance section.

was 18.5%. The frequency distributions for alleles in our subjects were similar to those in previous studies in Japanese cohorts, but lower than studies in whites.[23,24]

Physical Measurements

The mean age, BMI, BP levels, and heart rates at entry were similar among the four groups (Table 1). However, the entry measurements for total body fat mass and waist-to-hip ratio were significantly lower in the weight loss maintenance group versus the other three groups (weight loss resistant, slow weight loss, or rebound weight gain). At 6 months, the weight loss maintenance group had significantly greater weight loss, body fat loss, and a decrease in the waist-to-hip ratio compared to the weight loss resistant and slow weight loss groups. The BP reductions at 24 months were significantly greater in the weight loss maintenance and slow weight loss groups compared to the weight loss resistant group and the rebound weight gain group (Table 1). Only in the weight loss maintenance group did the heart rates decline at 24 months.

The subjects carrying the Gly16 allele had greater total body fat mass and waist-to-hip ratios at entry and throughout the study (Table 3), and the subjects carrying the Glu27 allele had greater total body fat mass (Table 4).

In all subjects, weight loss and mean BP reduction during 24 months were 8.9 ± 4.4 kg (10.8% ± 5.3%) and 4.5 ± 3.1 mm Hg (4.7% ± 3.2%). Mean BP reductions per amount of weight lost were similar among the four study groups (0.4 ± 0.2 mm Hg/kg in the weight loss maintenance group; 0.3 ± 0.1 mm Hg/kg in the rebound weight gain group; 0.5 ± 0.2 mm Hg/kg in the weight loss resistant group; and 0.5 ± 0.3 mm Hg/kg in the slow weight loss group).

Hormone Levels

Plasma NE and leptin levels, and HOMA-IR decreased with weight loss in the four study groups (Table 1). The most significant finding was that plasma NE and leptin levels were substantially greater in the weight loss resistant group compared to the weight loss maintenance group at entry and throughout the study. In the rebound weight gain group, plasma NE level was significantly greater than in the weight loss maintenance group. The slow weight loss group also had higher plasma NE and leptin levels at entry compared to the groups who succeeded in a significant weight loss at 6 months (weight loss maintenance and rebound weight gain groups), but lower values than the weight loss resistant group (Table 1). Plasma NE and leptin levels in the subjects carrying the Gly16 and Glu27 alleles were higher at entry and throughout the study compared to those without the Gly16 or Glu27 allele. The HOMA-IR in the subjects with the Gly16 allele was higher throughout the study, as previously we reported,[25] whereas that in the subjects with the Glu27 allele was similar (Tables 3 and 4).

Table 3. Characteristics of subjects according the genotype of the Gly16 at entry and during a weight loss program

Genotype	Without Gly16 Allele (Arg16Arg)			With Gly16 Allele (Arg16Gly + Gly16Gly)		
	At Entry	At 6 Months	At 24 Months	At Entry	At 6 Months	At 24 Months
Subjects (*n*)	42	42	42	112	112	112
Age (yr)	36 ± 7	37 ± 7§	38 ± 7§	37 ± 6	37 ± 6§	39 ± 6§
BMI (kg/m^2)	27.3 ± 2.0	23.8 ± 2.1	23.6 ± 2.0†§	27.4 ± 1.8	24.5 ± 2.3§	24.6 ± 1.9§
Total body fat mass (kg)	24.0 ± 5.4*	20.2 ± 4.8*‡	17.6 ± 4.5*§	25.5 ± 4.8	21.7 ± 4.3‡	19.2 ± 5.1§
Waist-to-hip ratio	1.16 ± 0.12*	1.05 ± 0.10‡	0.96 ± 0.13*§	1.24 ± 0.10	1.09 ± 0.12‡	1.04 ± 0.14§
Systolic BP (mm Hg)	132 ± 9	130 ± 10	126 ± 9‡	133 ± 10	132 ± 10	131 ± 9
Diastolic BP (mm Hg)	79 ± 9	79 ± 8	75 ± 6‡	79 ± 10	78 ± 9	75 ± 7‡
Mean BP (mm Hg)	97 ± 10	96 ± 10	92 ± 7‡	97 ± 11	96 ± 9	94 ± 9
Heart rate (beats/min)	69 ± 9	67 ± 7	64 ± 7‡	69 ± 7	68 ± 7	66 ± 7
Norepinephrine (pmol/mL)	1.85 ± 0.39*	1.56 ± 0.33*‡	1.26 ± 0.37*§	2.11 ± 0.35	1.80 ± 0.36‡	1.41 ± 0.40§
Leptin (ng/mL)	8.6 ± 2.9*	6.6 ± 2.7*‡	4.9 ± 2.1*§	9.3 ± 3.0	7.3 ± 2.8‡	5.7 ± 2.9‡
HOMA-IR	2.2 ± 0.7*	2.1 ± 0.4	1.8 ± 0.6‡	2.6 ± 0.6	2.3 ± 0.5	2.0 ± 0.5‡

Data are mean ± SD.
BMI = body mass index; BP = blood pressure; HOMA-IR = the homeostasis model assessment of insulin resistance.
* $P < .05$, † $P < .01$ compared with values in subjects with the Gly16 allele; ‡ $P < .05$, § $P < .01$ compared with values at entry.

Relationship With Weight Loss and BP Reduction

Using linear regression analysis, plasma NE levels at entry and at 24 months correlated significantly with mean BP ($r = 0.54$, $P < .001$, $r = 0.42$, $P < .001$, respectively), heart rate ($r = 0.27$, $P < .05$, $r = 0.21$, P = not significant, respectively), BMI ($r = 0.28$, $P < .05$, $r = 0.25$, $P < .05$, respectively), total body fat mass ($r = 0.36$, $P < .001$, $r = 0.35$, $P < .001$, respectively), and plasma leptin level ($r = 0.42$, $P < .001$, $r = 0.37$, $P < .001$, respectively). Changes in heart rate for 24 months did not correlate with changes in plasma NE.

In multiple linear regression analysis, total body fat mass ($P = .043$), plasma NE ($P = .016$) and leptin levels ($P = .020$), but not heart rate, at entry were significant determinant factors for absolute weight changes for 24 months ($R^2 = 0.337$, F = 3.56, $P = .010$). Mean BP ($P = .050$), total body fat mass ($P = .041$), and plasma NE level ($P = .042$) at entry were significant determinant factors for absolute changes in mean BP for 24 months ($R^2 = 0.301$, F = 2.45, $P = .047$). Changes in total body fat mass ($P = .019$), waist-to-hip ratio ($P = .034$), plasma NE ($P = .033$) and leptin levels ($P = .022$) for 2 years were significant determinant factors for absolute changes in mean BP ($R^2 = 0.381$, F = 5.03, $P = .007$).

Discussion

The present study shows that the initial levels of total body fat mass, plasma NE and leptin levels, and the frequency of the Gly16 allele of the Arg16Gly of the β2-adrenoceptor polymorphism are significantly higher in people resistant to weight loss and those who have rebound weight gain compared to those with successful weight loss maintenance. Thus, measurement of these parameters might predict those subjects who will fail to lose weight in both the short and long term or who will regain weight after an initial success in weight loss as determined in a dietary and exercise weight loss program. In addition, the frequency of the Glu27 allele of the β2-adrenoceptor is higher in subjects who are weight loss resistant and in those with slow weight loss, in people who fail to lose weight in the short term (6 months), compared to those with weight loss maintenance or rebound weight gain, who lose weight in the short term. These findings indicate that sympathetic overactivity as reflected by high plasma NE levels associated with the Gly16 and Glu27 polymorphisms might be linked to mechanisms that explain weight loss resistance and rebound weight gain despite adherence to long-term diet and exercise programs.

A number of studies have demonstrated several BP-lowering mechanisms accompanying weight loss.[6,7] In our weight loss program, average percent reduction in body weight and mean BP for 24 months in all subjects were 10.8% and 4.7%, respectively. These results revealed similar values to those levels in the meta-analysis by Neter et

Table 4. Characteristics of subjects according the genotype of the Glu27 at entry and during a weight loss program

Genotype	Without Glu27 Allele (Gln27Gln)			With Glu Allele (Gln27Glu)		
	At Entry	At 6 Months	At 24 Months	At Entry	At 6 Months	At 24 Months
Subjects (*n*)	125	125	125	29	29	29
Age (yr)	36 ± 6	37 ± 6§	38 ± 6§	37 ± 7	37 ± 7§	39 ± 7§
BMI (kg/m^2)	27.4 ± 1.7	24.0 ± 2.1§	24.1 ± 2.0§	27.3 ± 1.8	24.9 ± 2.2‡	24.5 ± 2.1§
Total body fat mass (kg)	24.5 ± 5.7*	20.5 ± 4.5*§	18.0 ± 4.3*§	25.9 ± 6.0	22.4 ± 3.8‡	20.1 ± 4.7§
Waist-to-hip ratio	1.19 ± 0.13	1.09 ± 0.10	0.98 ± 0.12§	1.22 ± 0.11	1.13 ± 0.09	1.03 ± 0.13‡
Systolic BP (mm Hg)	133 ± 9	131 ± 10	125 ± 9*‡	134 ± 10	133 ± 9	131 ± 10
Diastolic BP (mm Hg)	79 ± 9	79 ± 9	75 ± 9‡	79 ± 9	78 ± 7	75 ± 9
Mean BP (mm Hg)	97 ± 10	96 ± 9	91 ± 8*‡	97 ± 11	96 ± 7	94 ± 10
Heart rate (beats/min)	69 ± 8	67 ± 7	65 ± 7	69 ± 7	68 ± 6	65 ± 7
Norepinephrine (pmol/mL)	1.94 ± 0.33*	1.67 ± 0.41*‡	1.29 ± 0.34*§	2.20 ± 0.33	1.92 ± 0.45	1.51 ± 0.42‡
Leptin (ng/mL)	7.1 ± 2.8*	6.9 ± 2.8*	5.1 ± 2.7*‡	9.5 ± 3.1	8.0 ± 3.0‡	5.8 ± 2.8‡
HOMA-IR	2.4 ± 0.4	2.1 ± 0.7	2.0 ± 0.6	2.5 ± 0.6	2.3 ± 0.6	2.0 ± 0.5

Data are mean ± SD.
Abbreviations as in Table 3.
* $P < .05$, † $P < .01$ compared with values in subjects with the Glu27 allele; ‡ $P < .05$, § $P < .01$ compared with values at entry.

al.[7] And, normalization of BP often occurs before obese subjects reach their ideal weight. Therefore, overweight and obese hypertensive patients should be encouraged to lose even a modest amount of weight as it has pronounced beneficial effects on BP levels and other risk factors.

It is established that weight loss is accompanied by reductions in sympathetic nerve activity (SNA), insulin resistance, plasma leptin levels, and BP levels.[1–3,5] However, few investigations have examined how the sympathetic nervous system, insulin resistance, and leptin level are involved in weight loss resistance and rebound weight gain.[26] More than 20 years ago, Tuck et al found significant reductions in SNA and BP during rapid weight loss and weight loss using a very low calorie diet.[2,3,5] In the present study, we note that plasma NE, leptin, and the HOMA-IR levels track with weight changes and in addition that plasma NE and leptin levels at entry are determinant factors for predicting changes in body weight during a weight loss program, thus further demonstrating that SNA (plasma NE levels) and plasma leptin levels are major control factors for changes in body weight.[5,19,27]

In the present study, we used plasma NE levels as an index of SNA. Tuck,[28] Grassi and Esler,[29] and Rahn et al[30] reviewed that there are different results in SNA values in hypertensive patients depending on the method of SNA measurement including: regional NE spillover, muscle sympathetic nerve activity (microneurography), and plasma NE measurements. Spillover methods are considered as the gold standard for SNA measurements, but in humans these are difficult and invasive measurements. Furthermore , Rumantir et al[31] reported different values for regional sympathetic nerve activity between the kidneys and heart in obesity-related hypertensive subjects. Plasma NE levels are more practical for large population studies, [5,15,19,25] but represent several different process (secretion, clearance, and reuptake of NE) making it difficult to determine whether the defect is overproduction or decreased metabolism.

Pathophysiological involvement of genetic abnormalities in the β2- and β3-adrenoceptor system in obesity are well described.[10–15,22] Among β2- and β3-adrenoceptor polymorphisms, amino acid substitutions, Arg16Gly and Gln27Glu of the β2-adrenoceptor and Trp64Arg of the β3-adrenoceptor polymorphism are considered functionally important in the control of body weight.[10–15,22] In the present study, the weight loss maintenance group have a lower frequency of the Gly16 and Glu27 alleles of the β2-adrenoceptor and lower plasma NE levels, suggesting that the Gly16 and Glu27 alleles are related to a blunted β2-adrenoceptor activity and resultant sympathetic overactivity as shown by higher plasma NE levels.[15] Furthermore, the slow weight loss and weight loss resistance groups in our study during a 24-month period have a higher frequency of the Glu27 allele and higher plasma NE levels compared to the groups who succeed in significant weight loss in the short term. We have reported that the individuals carrying the Gly16 and Glu27 alleles have greater weight gain and BP elevations.[15] Taken together,

one could propose that the characteristics of the Gly16 and Glu27 alleles of the β2-adrenoceptor polymorphisms during weight gain may stabilize body weight even with on-going caloric restriction and exercise causing resistance to weight loss.

Kaye et al[32] found a strong relationship between heart rate and the level of cardiac sympathetic nerve activity measured by the spillover method. Our results show that changes in plasma NE do not correlate with changes in heart rate, whereas heart rate correlates with plasma NE at entry. These findings indicate that the limitation that plasma NE level does not always precisely reflect the response of regional (heart) sympathetic nerve activity to weight change, but we could speculate that the subjects carrying the Gly16 or Glu27 alleles who have less reductions in heart rate might have an impaired response of cardiac sympathetic nerve activity to weight loss through the blunted β2-adrenoceptor sensitivity and resultant cardiac risk through resistance to weight loss. However, further studies are needed to evaluate the differences in the sympathetic-mediated thermogenesis in the subjects carrying the β2-adrenoceptor polymorphisms.

In conclusion, greater adiposity and sympathetic overactivity (high plasma NE levels) might predict those obese individuals who have complete resistance to lose weight during the 24-month period and those who will have rebound weight gain after a successful initial weight loss. The sympathetic overactivity in those subjects who have rebound weight gain and in those who have resistance to weight loss may be associated with the polymorphisms in the Gly16 and Glu27 alleles of the β2-adrenoceptor.

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Association Study of Calcitonin-Receptor-like Receptor Gene in Essential Hypertension

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Background: Plasma adrenomedullin (ADM) concentrations increase in patients with hypertension, renal failure, heart failure, essential pulmonary hypertension, myocardial infarction, endotoxin shock, and many other conditions. The ADM receptor is a complex molecule that consists of calcitonin-receptor–like receptor (CRLR) and receptor activity–modifying protein 2 (RAMP2). Because CRLR determines the binding specificity of ADM, the *CRLR* gene is thought to be a susceptibility gene of hypertension. However, studies have not yet defined the relationship between the *CRLR* gene and hypertension. The aim of the present study was to investigate relationships between single-nucleotide polymorphisms (SNP) in the human CRLR gene and essential hypertension (EH) in a Japanese population.

Methods: We selected four SNP in the human *CRLR* gene (rs3771073, rs696574, rs698590, and rs1528233), and we performed a genetic association study in 209 EH patients and 216 age-matched normotensive (NT) individuals.

Results: There was no significant difference in overall distribution of genotypes or alleles of any of the SNP between the EH and NT groups. However, among women, the T allele of the SNP rs696574 (C → T, in intron 6) was significantly more frequent in EH subjects than in NT subjects ($P = .032$).

Conclusion: Our findings suggest that rs696574 can be used as a genetic marker of EH in women. Am J Hypertens 2005;18:403–408 © 2005 American Journal of Hypertension, Ltd.

Key Words: Essential hypertension, calcitonin-receptor–like receptor, single-nucleotide polymorphism, association study.

Adrenomedullin (ADM) was first identified in 1993 as a hypotensive peptide in the adrenal medulla. It exerts strong and persistent hypotensive activity via dilation of the peripheral vascular bed. Adrenomedullin is secreted mainly by vascular endothelial cells and smooth-muscle cells and is involved in the pathophysiology of many cardiovascular diseases.[1,2] Plasma ADM concentrations increase in patients with hypertension, renal failure, heart failure, essential pulmonary hypertension, myocardial infarction, endotoxin shock, and many other conditions.[3–6] The ADM receptor is a complex molecule consisting of calcitonin-receptor–like receptor (CRLR, a G-protein–coupled receptor with seven transmembrane domains) and receptor activity–modifying protein 2 (RAMP2, a protein with a single transmembrane domain). The mechanism of the activation of ADM receptor is unique. The receptor CRLR is transported by RAMP2 from the endoplasmic reticulum to the cell membrane, where it is core-glycosylated to become a receptor for ADM. Because CRLR determines the binding specificity of ADM, the *CRLR* gene is thought to be a susceptibility gene of hypertension.[7–10]

The human CRLR consists of 461 amino acids, and the gene encoding this protein is located at chromosome 2q31 to q32. The gene contains 11 exons interrupted by 10 introns and consists of approximately 100 kilobase pairs (kbp).[1,7,8]

High blood pressure (BP) or hypertension affects 25% of most adult populations and is an important risk factor for death from stroke, myocardial infarction, and congestive heart failure. The main cause of hypertension is a

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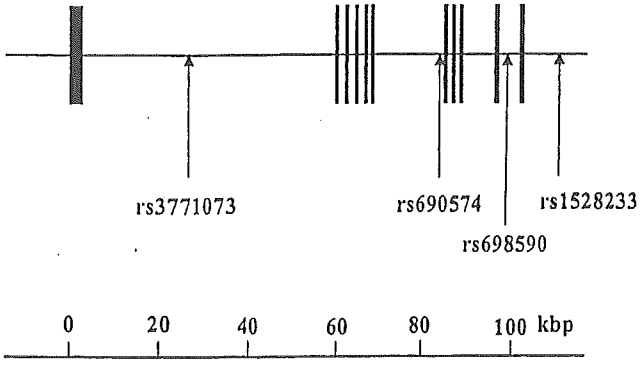

FIG. 1. Organization of the human calcitonin-receptor-like receptor (*CRLR*) gene and location of single-nucleotide polymorphisms (SNP) used for present association study. **Closed boxes** indicate exons and **lines** represent introns.

primary condition known as essential hypertension (EH). It is thought that EH is a multifactorial disease.[11] However, several reports indicate that there are susceptibility genes for EH, including those for angiotensinogen[12] and angiotensin-converting enzyme.[13,14]

Single-nucleotide polymorphisms (SNP) in non-coding sequences can affect phenotype by altering transcriptional regulation (especially when the polymorphic site is located in a promoter region) or by altering a splice site or latent splice site. Furthermore, SNP are useful as polymorphic markers for locating mutations associated with specific diseases.[15]

There have been no previous studies of associations between CRLR and EH. The aim of the present study was to investigate the association between the human *CRLR* gene and EH, using SNP in the human *CRLR* gene.

Methods

Subjects

The EH subjects studied were 209 patients who were diagnosed with EH according to the following criteria: seated systolic BP (SBP) > 160 mm Hg or diastolic BP (DBP) > 100 mm Hg, on three occasions within 2 months after the first medical examination. None of the EH subjects was using antihypertensive medication. Patients diagnosed with secondary hypertension were excluded. The control subjects were 216 normotensive (NT) healthy individuals. None of the control subjects had a family history of hypertension, and all had SBP and DBP < 130 and <85 mm Hg, respectively. Family histories of hypertension were defined as prior diagnosis of hypertension in a grandparent, uncle, aunt, parent, or sibling. Both groups were recruited from the northern area of Tokyo, Japan. Informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University.[16]

Biochemical Analysis

Plasma concentrations of total cholesterol and HDL-cholesterol and serum concentration of creatinine and uric

Table 1. Characteristics of study participants

	Total			Men			Women		
	NT	EH	*P* Value	NT	EH	*P* Value	NT	EH	*P* Value
Number of subjects	217	210		142	130		75	80	
Age (y)	52.4 ± 10.4	50.9 ± 5.4	.068	52.1 ± 6.8	50.9 ± 5.5	.124	53.1 ± 15.0	51.0 ± 5.3	.250
BMI (kg/m^2)	23.6 ± 2.8	23.5 ± 2.9	.842	23.9 ± 2.6	23.3 ± 2.3	.105	22.8 ± 3.2	23.7 ± 3.4	.141
SBP (mm Hg)	112.7 ± 10.8	173.2 ± 20.9	<.001	113.3 ± 10.1	171.0 ± 20.2	<.001	111.5 ± 12.1	177.2 ± 21.5	<.001
DBP (mm Hg)	69.2 ± 8.7	106.1 ± 13.1	<.001	70.3 ± 8.1	107.2 ± 12.7	<.001	67.1 ± 9.4	104.5 ± 13.6	<.001
Pulse (beats/min)	74.3 ± 13.2	77.5 ± 15.6	.053	74.0 ± 14.5	77.2 ± 16.6	.145	75.0 ± 10.3	78.1 ± 14.0	.205
Creatinine (mg/dL)	0.8 ± 0.2	0.8 ± 0.3	.722	0.9 ± 0.2	0.9 ± 0.2	.421	0.7 ± 0.1	0.7 ± 0.2	.974
Total cholesterol (mg/dL)	203.4 ± 42.3	210.3 ± 37.4	.086	199.6 ± 39.8	203.8 ± 34.6	.369	210.4 ± 45.9	220.1 ± 40.0	.163
HDL cholesterol (mg/dL)	55.0 ± 17.3	58.1 ± 17.7	.090	52.1 ± 15.2	54.6 ± 17.0	.248	60.4 ± 19.6	63.5 ± 17.5	.305
Uric acid (mg/dL)	5.5 ± 1.5	5.6 ± 1.7	.517	6.0 ± 1.4	6.1 ± 1.5	.419	4.5 ± 1.3	4.7 ± 1.6	.344
Alcohol consumption (%)	60.8	67.8	.126	72.8	81.6	.066	33.9	38.6	.569
Smoking (%)	40.9	54.5	.004	49.2	64.2	.008	22.8	33.7	.159

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

Table 2. Genotype and allele distribution among normotensive subjects and patients with essential hypertension

	Total Subjects			Men			Women		
	NT	EH	*P* Value	NT	EH	*P* Value	NT	EH	*P* Value
Number of participants	217	210		142	130		75	80	
Variants									
rs3771073									
Genotype									
GG	176 (0.810)	170 (0.809)		114 (0.802)	108 (0.831)		62 (0.827)	62 (0.775)	
GC	39 (0.181)	37 (0.177)		26 (0.183)	21 (0.162)		13 (0.173)	16 (0.200)	
CC	2 (0.010)	3 (0.014)	.886	2 (0.014)	1 (0.008)	.779	0 (0.000)	2 (0.025)	.341
Allele									
G	391 (0.900)	377 (0.897)		254 (0.894)	237 (0.912)		137 (0.913)	140 (0.875)	
C	43 (0.100)	43 (0.103)	.873	30 (0.106)	23 (0.088)	.500	13 (0.087)	20 (0.125)	.274
rs696574									
Genotype									
CC	127 (0.585)	111 (0.528)		78 (0.549)	71 (0.546)		51 (0.68)	40 (0.500)	
CT	80 (0.369)	86 (0.410)		57 (0.401)	50 (0.384)		22 (0.293)	36 (0.450)	
TT	10 (0.046)	13 (0.062)	.456	7 (0.049)	9 (0.069)	.776	2 (0.027)	4 (0.050)	.074
Allele									
C	332 (0.769)	306 (0.732)		213 (0.745)	192 (0.738)		124 (0.827)	116 (0.725)	
T	100 (0.231)	112 (0.268)	.220	71 (0.250)	68 (0.262)	.758	26 (0.173)	44 (0.275)	.032
rs698590									
Genotype									
TT	180 (0.833)	182 (0.867)		118 (0.837)	112 (0.862)		62 (0.827)	70 (0.875)	
AT	36 (0.167)	27 (0.129)		23 (0.163)	18 (0.138)		13 (0.173)	9 (0.1125)	
AA	0 (0.000)	1 (0.005)	.331	0 (0.000)	0 (0.000)	NC	0 (0.000)	1 (0.013)	.358
Allele									
T	396 (0.917)	391 (0.931)		259 (0.918)	242 (0.931)		137 (0.909)	149 (0.932)	
A	36 (0.083)	29 (0.069)	.432	23 (0.082)	18 (0.069)	.588	13 (0.091)	11 (0.069)	.555
rs1528233									
Genotype									
GG	182 (0.839)	184 (0.876)		120 (0.845)	114 (0.877)		62 (0.827)	70 (0.875)	
AG	35 (0.161)	26 (0.124)		22 (0.155)	16 (0.123)		13 (0.173)	10 (0.125)	
AA	0 (0.000)	0 (0.000)	NC	0 (0.000)	0 (0.000)	NC	0 (0.000)	0 (0.000)	NC
Allele									
G	399 (0.919)	394 (0.938)		262 (0.923)	244 (0.938)		137 (0.913)	150 (0.938)	
A	35 (0.081)	26 (0.062)	.288	22 (0.077)	16 (0.062)	.467	13 (0.087)	10 (0.063)	.417

Abbreviations as in Table 1.
NC indicates the statistical result with no calculation of χ^2 in a contingency table, due to inclusion of a cell with no DNA sample.

Table 3. Effect of confounding factors on prevalence of hypertension by multiple logistic regression analysis in women

	df	χ^2	*P* Value	Odds Ratio (95% CI)
rs696574 T/T and C/T genotype	1	5.02	.025	1.23 (1.03–1.47)
Age	1	0.03	.868	1.00 (0.99–1.00)
BMI	1	4.65	.031	1.03 (1.00–1.07)
Creatinine	1	0.40	.527	1.19 (0.67–2.11)
Total cholesterol	1	0.32	.574	1.00 (1.00–1.00)
HDL cholesterol	1	1.65	.199	1.00 (0.95–1.05)
Uric acid	1	0.02	.884	1.00 (1.00–1.01)

CI = confidence interval; other abbreviations as in Tables 1 and 2.

acid were measured using the methods of the Clinical Laboratory Department of Nihon University Hospital.[17]

Genotyping

Using information about allelic frequencies of SNP registered on the website of the National Center for Biotechnology Information (NCBI) and Celera Discovery System–Applied Biosystems, four SNP with minor allele frequencies >20% were selected. Those SNP with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic association studies.

We examined association between EH and four SNP in the human *CRLR* gene. All four SNP were confirmed using the NCBI website; their accession numbers were rs3771073, rs696574, rs698590, and rs1528233 (Fig. 1). Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, NJ) together with TaqMan polymerase chain reaction (Applied Biosystems).[16]

Statistical Analysis

Data are shown as means ± SD. Hardy-Weinberg equilibrium was assessed by χ^2 analysis. The overall distribution of alleles was analyzed using 2 × 2 contingency tables, and the distribution of the genotypes between EH patients and NT control subjects was tested using a two-sided Fisher exact test. To assess the contribution of confounding factors, multiple logistic regression analysis was performed. Statistical significance was established at $P < .05$. Differences in clinical data between the EH and NT groups were assessed by analysis of variance followed by Fisher's protected least-significant difference test.[18]

Results

Table 1 shows the clinical features of the EH patients and NT control subjects. Age, body mass index, pulse rate, serum concentration of creatinine, and plasma concentrations of total cholesterol and uric acid did not differ significantly between the two groups.

Table 2 shows the distribution of genotypic and allelic frequencies of the four SNP in each group. The observed and expected genotypic frequencies of each SNP in the total study group and in the men and women in the NT group were in good agreement with predicted Hardy-Weinberg equilibrium values (data not shown). The overall distribution of genotype in additive model of all four SNP did not significantly differ between the total EH and total NT groups. However, among women, the allelic frequency of the T allele of rs696574 was significantly higher for EH subjects than for NT subjects ($P = .032$). Although the genotype distribution

Table 4. Characteristics of women with each genotype of rs696574

	T/T and C/T	C/C	*P* Value
Number of subjects	67	88	
Age (y)	52.1 ± 9.8	51.9 ± 12.2	.944
BMI (kg/m^2)	23.4 ± 2.7	23.3 ± 3.8	.797
SBP (mm Hg)	152.4 ± 35.6	140.1 ± 37.9	.042
DBP (mm Hg)	90.9 ± 20.2	83.0 ± 23.0	.028
Pulse (beats/min)	78.5 ± 13.4	75.2 ± 11.8	.154
Creatinine (mg/dL)	0.7 ± 0.2	0.7 ± 0.2	.867
Total cholesterol (mg/dL)	216.8 ± 41.2	214.5 ± 44.2	.741
HDL cholesterol (mg/dL)	61.8 ± 17.1	62.1 ± 19.6	.926
Uric acid (mg/dL)	4.5 ± 1.5	4.7 ± 1.4	.579
Alcohol consumption (%)	32.8	40	.177
Smoking (%)	29.2	40	.177

Abbreviations as in Tables 1 and 3.

Table 5. Pairwise LD in *CRLR* gene, evaluated by D′ and r^2

SNP		rs3771073	rs696574	rs698590	rs1528233
rs3771073	D′		0.450	0.912	0.943
	r^2		0.008	0.611	0.611
rs696574	D′			1.000	1.000
	r^2			0.027	0.025
rs698590	D′				1.000
	r^2				0.934
rs1528233	D′				
	r^2				

CRLR = calcitonin-receptor-like receptor; LD = linkage disequilibrium; SNP = single-nucleotide polymorphism. Pairwise LD among the six marker pairs studied in *CRLR* gene were computed, and pairs in LD (D′ > 0.5 or r^2 > 0.5) are shown as gray-shaded values.

of rs696574 did not significantly differ in the additive model of women, T/T and C/T genotype of EH women was significantly higher than that of NT women in the recessive model. The dominant model did not show the significant difference because TT carrier was very few. Multiple logistic regression analysis revealed that the significant association of T/T and C/T with EH (P = .025) remained after adjustment for confounding factors, and the calculated odds ratio was 1.23 (95% confidence interval [CI] = 1.03 to 1.47) (Table 3).

Table 4 shows the clinical data of female subjects for each genotype of rs696574. Both SBP and DBP were higher for the T/T and C/T genotypes than for the C/C genotype (P = .042 and .0278, respectively).

Patterns of linkage disequilibrium in the *CRLR* gene were illustrated by their D′ and r^2 values (Table 5). All SNP of *CRLR* were in tight linkage equilibrium (LD), showing one LD block.

Discussion

Previous animal studies have demonstrated that the ADM gene is associated with abnormalities of vascular function or hypertension. Homozygous ADM knockout mice (ADM−/−) have been found to die frequently from hydrops or cardiovascular abnormalities, and heterozygous ADM knockout mice (ADM+/−) have been found to have higher BP than wild-type mice (ADM+/+).[19–23] The BP is lower in transgenic mice that over-express the ADM gene than in wild-type mice.[10,24] The receptor CRLR was originally identified as an orphan G-protein–coupled receptor with seven transmembrane domains and was recently confirmed to be the specific receptor of ADM. The function of CRLR is modified by receptor activity–modifying protein (RAMP) 2 and 3. These ADM–CRLR systems function as vascular relaxation factors or hypotensive factors and are sometimes dependent on levels of nitric oxide (NO),[25–29] suggesting that the *CRLR* gene is a susceptibility gene of EH. However, there have been no reports about *CRLR* gene knockout mice or mice that over-express the *CRLR* gene. Moreover, there have been no previous reports about the relationship between EH and the *CRLR* gene. Consequently, we conducted the present study of association between the *CRLR* gene and EH using four SNP in the *CRLR* gene.

In the present study, the overall distribution of genotypes and alleles of all four SNP did not significantly differ between the EH and NT groups. However, among women, the allelic frequency of the SNP rs696574 (C → T) in intron 6 significantly differed between EH and NT subjects. There have been four previous studies of ADM knockout mice, but the mice in those studies were not divided according to gender.[10,19–21,24] In the present study, we compared clinical data between women with the C/T and T/T genotypes and women with the C/C genotype. Both SBP and DBP of women with the T allele (T/C and T/T genotypes) were significantly higher than those of women without the T allele (C/C genotype). This suggests that rs696574 can be used as a genetic marker of EH in women.

Some association studies have identified genes associated with gender-specific susceptibility to EH.[30,31] However, the reason for the present finding of a positive association between EH and the SNP rs696574 in women is unclear. Further studies are needed to clarify why there is an association between the *CRLR* gene and EH in women.

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Original Article

Haplotype-Based Case-Control Study Revealing an Association between the Adrenomedullin Gene and Proteinuria in Subjects with Essential Hypertension

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Adrenomedullin (AM) has various physiological actions on the cardiovascular system, including vasodilatation, diuresis, natriuresis, inhibition of aldosterone secretion, and increases of the cardiac output, all of which cause hypotension. Since AM plays a role in the pathophysiology of vascular diseases, genes controlling AM might be involved in the development and etiology of essential hypertension (EH). However, there have been few studies examining the relationship between the AM gene and hypertension. The aims of this study were to genotype some of the genetic markers for the human AM gene in Japanese subjects, and *via* a haplotype-based case-control study, assess the association between and the AM gene and EH or its risk factors, such as hyperlipidemia, renal damage, and proteinuria. We genotyped 205 EH patients and 210 age-matched normotensive (NT) individuals for two single nucleotide polymorphisms of rs4399321, rs7944706 and a microsatellite polymorphism located approximately 5,400 base pairs downstream of the 3′ end of the human AM gene. The overall distribution in each variant and haplotype did not significantly differ between the two groups. However, after dividing the groups into those subjects with and without proteinuria, the haplotype analysis revealed a positive association. In conclusion, a possible mutation linked to the haplotype may indicate a genetic predisposition for proteinuria in EH. (*Hypertens Res* 2005; 28: 229–236)

Key Words: **haplotypes, adrenomedullin, hypertension, essential, polymorphism**

Introduction

High blood pressure or hypertension affects 25% of most adult populations and is an important risk factor for death from stroke, myocardial infarction and congestive heart failure. More than 80% of hypertensive cases are diagnosed as cases of essential hypertension (EH). EH is likely to be a polygenic disorder that results from the inheritance of a number of susceptibility genes. The causal genes identified probably contribute to between 30% and 50% of the variation in blood pressure seen among individuals (*1*). These genetic determinants interact with environmental factors such as dietary salt to produce the final disease phenotype. Despite significant recent progress in genomic and statistical tools, the genetic dissection of human EH remains a major chal-

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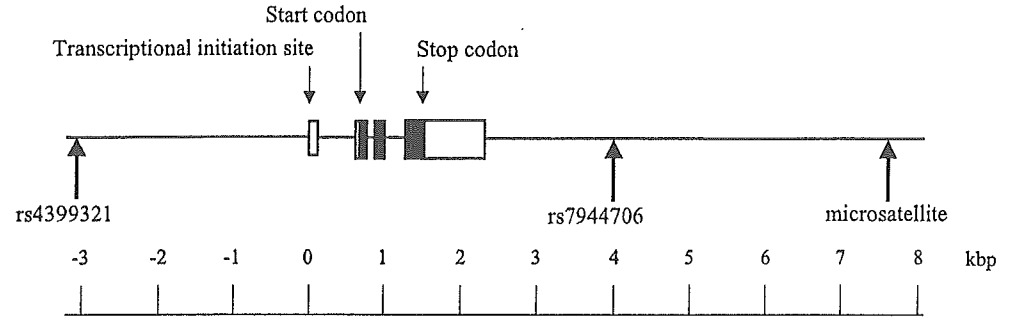

Fig. 1. *Organization of the human AM gene and location of SNPs and the microsatellite based on a haplotype-based case-control study. Boxes indicate exons and lines indicate introns. Closed boxes indicate coding regions.*

Table 1. Characteristics of Study Participants

	Total		*p* value	Men		*p* value	Women		*p* value
	NT	EH		NT	EH		NT	EH	
Number of subjects	210	205		147	120		63	85	
Age (years)	52.5±7.2	51.5±4.8	0.104	52.0±6.7	51.2±5.2	0.272	53.6±8.3	51.9±4.0	0.116
BMI (kg/m^2)	23.2±3.1	23.8±2.9	0.168	23.5±2.9	23.9±2.5	0.278	22.6±3.6	23.7±3.3	0.064
SBP (mmHg)	113.3±10.2	171.6±17.7	<0.001	113.4±9.9	169.1±17.3	<0.001	113.2±11.1	175.2±17.6	<0.001
DBP (mmHg)	70.2±8.0	104.8±12.6	<0.001	70.4±7.8	105.7±13.1	<0.001	69.9±8.5	103.5±11.8	<0.001
Pulse (beats/min)	73.6±10.3	76.4±14.0	0.055	70.4±7.8	75.6±13.7	0.331	73.2±9.0	77.4±14.3	0.073
Creatinine (mg/dl)	0.84±0.23	0.83±0.25	0.470	0.92±0.22	0.93±0.24	0.755	0.67±0.15	0.68±0.16	0.723
Proteinuria (%)	9.8	13.8	0.240	12.4	16.5	0.376	3.5	10	0.156
Total cholesterol (mg/dl)	201.6±43.5	208.7±38.1	0.089	196.7±41.8	201.1±38.7	0.393	213.0±45.7	218.7±35.0	0.397
HDL cholesterol (mg/dl)	56.0±16.6	59.1±17.1	0.086	53.9±15.6	55.7±17.0	0.432	61.0±17.9	63.6±16.3	0.401
Uric acid (mg/dl)	5.9±4.8	5.4±1.6	0.086	6.0±1.4	5.9±1.4	0.737	5.7±8.6	4.7±1.6	0.310
Alcohol consumption (%)	61.4	66.1	0.379	76.1	85.6	0.084	33.3	38.5	0.562
Smoking (%)	39.9	54.2	0.010	50	67.5	0.011	20.8	34.6	0.094

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

lenge (*2*).

Adrenomedullin (AM) is a 52-amino acid peptide that was originally isolated from a human pheochromocytoma in 1993 (*3*). Plasma AM levels are increased in patients with cardiovascular diseases such as myocardial infarction, pulmonary hypertension, heart failure, chronic renal failure and EH (*4*). Because the AM concentration and gene expression levels are quite high in endothelial cells, AM might be secreted mainly by vascular endothelial and smooth muscle cells (*5*). AM has various physiological effects on the cardiovascular system, including vasodilatation (*6*), diuresis, natriuresis, inhibition of aldosterone secretion and increases of the cardiac output (*7*), all of which cause hypotension. Furthermore, AM protects against cardiac hypertrophy, fibrosis, and renal damage, which are known to be complications of hypertension (*8*). Thus AM plays a role in the pathophysiology of vascular diseases, and the genes that control this system may be involved in the development of hypertension (*9*). The human AM gene is located on 11p, spans approximately 2,400 base-pairs (bp) with 4 exons and 3 introns and has TATA, CAAT and GC boxes in the 5′-flanking region (*10*). Although many research groups have investigated the gene expression and regulation of AM and its receptors (*11*), there are few studies that have examined the relationship between the AM gene and EH (*12*). To the best of our knowledge, there have been no reports that have determined the haplotypes of the AM gene or a haplotype-based case-control study for EH.

The aims of this study were to genotype some of the genetic markers of the human AM gene in Japanese subjects, and *via* a haplotype-based case-control study, assess the association between the AM gene and EH or its risk factors, such as hyperlipidemia, renal damage, and proteinuria.

Table 2. Genotype Distribution in NT Subjects and Patients with EH

Variants		NT (210)*	EH (205)*	*p* value
rs4399321	Genotype			
	AA	113 (0.538)	110 (0.53.7)	
	AG	79 (0.376)	78 (0.380)	
	GG	18 (0.086)	17 (0.083)	0.993
	Allele			
	A	305 (0.726)	298 (0.727)	
	G	115 (0.274)	112 (0.273)	0.984
rs7944706	Genotype			
	GG	85 (0.405)	78 (0.381)	
	AG	88 (0.419)	98 (0.478)	
	AA	37 (0.176)	29 (0.141)	0.417
	Allele			
	G	258 (0.614)	254 (0.620)	
	A	162 (0.386)	156 (0.380)	0.877
Microsatellite	Genotype			
	11, 11	18	16	
	11, 13	32	34	
	11, 14	39	42	
	11, 15	1	1	
	11, 19	4	3	
	13, 13	19	13	
	13, 14	44	44	
	13, 15	1	2	
	13, 19	3	6	
	14, 14	42	35	
	14, 19	7	9	
	Allele			
	11	112	112	
	13	118	112	
	14	174	165	
	15	2	3	
	19	14	18	0.914

NT, normotension; EH, essential hypertension. *Number of participants.

Methods

Subjects

A group of 205 patients were diagnosed with EH according to the following criteria: a seated systolic blood pressure (SBP) above 160 mmHg and/or diastolic blood pressure (DBP) above 100 mmHg that occurred on 3 occasions within 2 months after the first medical examination. None of the patients were using any anti-hypertensive medication. Subjects diagnosed with secondary hypertension were excluded. We also included 210 normotensive (NT) healthy individuals as controls. None of the NT participants had any family history of hypertension, and they all had SBP and DBP below 130 and 85 mmHg, respectively. A family history of hypertension was defined as prior diagnosis of hypertension in grandparents, uncles, aunts, parents or siblings. Both groups were recruited from the northern area of Tokyo, and informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University (*13*).

Biochemical Analysis

Plasma concentrations of total cholesterol and high-density lipoprotein (HDL)-cholesterol and serum concentrations of creatinine and uric acid were measured using the methods of the Clinical Laboratory Department of Nihon University Hospital (*14*). Proteinuria (PU) was diagnosed by dipstick analy-

Table 3. Haplotype Distribution in NT Controls and EH Patients

Haplotype	NT (420)	EH (410)
A-A-14	0.267	0.249
A-G-13	0.190	0.185
A-G-11	0.181	0.188
G-G-11	0.081	0.078
G-A-14	0.081	0.098
G-G-13	0.076	0.083
A-G-14	0.055	0.051
A-A-13	0.014	0.005
G-G-19	0.014	0.007
A-A-19	0.012	0.015
G-G-14	0.012	0.005
A-G-19	0.003	0.022
A-A-11	0.002	0.007
A-A-15	0.002	0.005
G-A-11	0.002	0.000
G-A-15	0.002	0.002
G-A-19	0.002	0.000

$\chi^2=13.3$, $p=0.501$. Haplotypes are shown as combined alleles of SNPs and repeat number of the microsatellite. NT, normotension; EH, essential hypertension; SNP, single nucleotide polymorphism.

sis.

Genotyping

Using information about allelic frequencies of single nucleotide polymorphisms (SNPs) registered on the website of the National Center for Biotechnology Information (NCBI) and Celera Discovery System (Applied Biosystems, Branchburg, USA), two SNPs around the human AM gene with minor allele frequencies greater than 20% were selected. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic association studies. These two SNPs were confirmed using the NCBI website, and had the accession numbers rs4399321 and rs7944706 (Fig. 1). The rs4399321 SNP was located at −3053 nucleotides (nt) upstream of the transcription initiation site and is responsible for the changing of adenine to guanine. The rs7944706 was located at +2367 nt downstream of the guanine of the terminal codon and is responsible for the changing of guanine to adenine. Genotypes were determined using Assays-on-Demand kits (Applied Biosystems) together with TaqMan® PCR. When allele-specific fluorogenic probes hybridize to the template during the polymerase chain reaction (PCR), the 5′ nuclease activity of *Taq* polymerase can discriminate alleles. Cleavage results in increased emission of a reporter dye that otherwise is quenched by the dye TAMRA. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe is labeled with a reporter dye (VIC and FAM) at the 5′ end and TAMRA at the 3′ end. Amplification by PCR was performed using TaqMan® Universal Master Mix (Applied Biosystems) with a 25 μl reaction volume that contained a final concentration of 50 ng DNA, 700 nmol/l primer, and 100 nmol/l probe. Thermal cycling conditions consisted of 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 62°C for 1 min and were performed on a GeneAmp 9700® system.

All 96-well plates contained 80 samples of unknown genotype, six known allele 1 homozygotes, six known allele 2 homozygotes, and four reactions with reagents but no DNA. The homozygote and control samples without DNA were required for the SDS 7700® signal processing outlined in the TaqMan® Allelic Discrimination Guide (Applied Biosystems). Direct sequencing, single-stand conformation polymorphism (SSCP), or denaturing high pressure liquid chromatography was used to confirm control sample genotypes. PCR plates were read on the SDS 7700® instrument in the endpoint analysis mode of the SDS version 1.6.3 software package (Applied Biosystems). Genotypes were visually determined by comparison with the dye-component fluorescent emission data shown in the *XY* scatterplot of the SDS software. Genotypes were also automatically determined by the signal processing algorithms in the software. Results of both scoring methods were saved to two output files for later comparison.

The microsatellite polymorphism was located approximately 5,400 bp downstream of the 3′ end of the human AM gene. This microsatellite is a GT repeat that was found in the database of the human genome, although it had not yet been registered in the NCBI and Celera Discovery System. Ishimitsu *et al.* previously reported that this microsatellite is associated with EH (*12*). The nucleotide sequences of the PCR primers were designed to be the same as those of the primers set by Ishimitsu *et al.* (*12*). The 5′ terminal of the forward primer was dye-conjugated with FAM (Applied Biosystems). The reaction mix for PCR was prepared as previously described (*13*). Thermal cycling conditions were 96°C for 3 min, and then 35 cycles of 98°C for 25 s, 60°C for 30 s, and 72°C for 45 s. Thermal cycling was performed on a GeneAmp 9700® system. The PCR products were loaded on an ABI 3700® DNA analyzer (Applied Biosystems) for the genotyping as described previously (*15*). The fluorescent signal was recorded and analyzed by the GeneScan version 2.1 software. Fragments from reactions using each of the different fluorescent dyes were plotted separately, and the sizes of fluorescent peaks were estimated for the base pairs by referencing the in-lane size standard. Marker alleles were classified according to their size using the Genotyper version 2 software. In addition to the automated allele calling, we performed manual surveillance of all genotypes (*15*).

Table 4. Characteristics of Study Participants in Each Genotype

Microsatellite	Subjects without 19-repeat allele	Subjects with 19-repeat allele	*p* value
Number of subjects	383	32	
Age (years)	52.0±6.2	52.4±6.2	0.702
BMI (kg/m^2)	23.5±3.0	23.6±2.6	0.813
SBP (mmHg)	141.9±32.9	144.7±27.9	0.642
DBP (mmHg)	87.0±20.4	90.8±17.5	0.310
Pulse (beats/min)	75.3±12.6	74.2±12.0	0.663
Creatinine (mg/dl)	0.83±0.23	0.89±0.30	0.185
Proteinuria (%)	25.9	10.5	0.016*
Total cholesterol (mg/dl)	205.0±41.5	206.2±36.2	0.876
HDL cholesterol (mg/dl)	57.3±16.6	60.4±20.5	0.352
Uric acid (mg/dl)	5.5±1.5	5.6±1.5	0.686
Alcohol consumption (%)	63.8	68.2	0.682
Smoking (%)	48.2	47.8	0.972

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; NT, normotension; EH, essential hypertension. *Indicates significant difference.

Haplotype Analysis

Based on the genotype data of the three genetic variations, the frequency of the haplotype was estimated using the expectation/maximization (EM) algorithm estimate (*16*). For determining the haplotype, SNPAlyze ver. 4.0 was used (DYNACOM Co., Ltd., Yokohama, Japan), and is available from the website http://www.dynacom.co.jp/products/package/snpalyze/index.html (*17*).

Statistical Analysis

Data are shown as the mean±SD. Differences in clinical data between the EH and NT groups were assessed by analysis of variance (ANOVA) followed by a Fisher's protected least significant difference (PLSD) test.

Hardy-Weinberg equilibrium was assessed by a χ^2 analysis. The overall distribution of SNP alleles was analyzed by 2×2 contingency tables, and the distribution of the SNP genotypes between EH patients and NT controls was tested using a 2-sided Fisher exact test and multiple logistic regression analysis. Statistical significance was established at $p<0.05$.

The overall distribution of microsatellite alleles between EH patients and NT subjects was analyzed by $2\times n$ contingency tables (*18*), and p values less than 0.05 were considered significant. Individual differences in allele frequencies were tested using 2×2 contingency tables for each allele and the combined remaining alleles, and a p value of less than $0.05/n$ was considered significant to correct for the number of comparisons made (Bonferroni correction) (*18*). The threshold value of frequencies of haplotypes included in the analysis was set to $1/2n$ (where n is the number of subjects in each group), as suggested by Excoffier and Slatkin (*19*). All haplotypes below the threshold value were excluded from the analysis. The overall distribution of haplotypes was analyzed using $2\times m$ contingency tables with a value of $p<0.05$ considered to indicate statistical significance. The level of p used for significance was determined by the $0.05/m$ number (*20*). For the permutation method, values of $p<0.05$ were considered to indicate statistical significance (*21*).

Results

Table 1 shows the clinical features of the EH patients and NT controls. The SBP and DBP were significantly higher in the EH than in the NT group. Age, body mass index (BMI), pulse rate, serum concentrations of creatinine, and plasma concentrations of total cholesterol and uric acid did not significantly differ between the two groups.

We performed an association study using three genetic variants. Table 2 shows the distribution of the genotypes and alleles. The overall distribution of genotypes and alleles of rs4399321 and rs7944706 did not significantly differ between the EH and NT groups. The overall distribution of alleles of the microsatellite also did not significantly differ between the EH and NT groups. Table 3 shows the frequencies of 17 observed haplotypes (H1 to H17). The overall distribution of haplotypes did not significantly differ between the EH and NT groups ($\chi^2=13.3$; $p=0.501$).

Ishimitsu *et al.* reported that the frequency of the 19-repeat allele of the microsatellite was significantly higher in EH than NT (*12*). Therefore, we compared the clinical characteristics in subjects with or without the 19-repeat allele (Table 4). Although there were no significant differences in the SBP and DBP between the two groups, surprisingly, the levels of PU were significantly different between the two groups. Therefore, we performed a haplotype analysis on two subgroups, one in which PU was present and one in which it was absent. As seen in Table 5, the overall distribution of haplotypes showed a significant difference between the EH and NT

Table 5. Haplotype Distribution after Division of the Subjects into Groups with or without PU

Haplotype	Non-PU (638)	PU (84)	χ^2 p value	Permutation p value
A-A-14	0.361	0.321	0.481	0.503
A-G-13	0.269	0.250	0.707	0.730
G-G-11	0.260	0.262	0.972	0.907
A-G-14	0.040	0.071	0.182	0.285
A-G-19	0.027	0.083	0.007*	0.009#
A-A-11	0.010	0.000		0.598
A-A-13	0.010	0.000		0.600
G-G-13	0.006	0.012	0.548	0.756
G-G-19	0.004	0.000		0.390
A-G-11	0.003	0.000		0.545
G-A-14	0.002	0.000		0.449
G-G-14	0.002	0.000		0.770
A-A-15	0.000	0.000		0.389

PU, subjects with proteinuria; Non-PU, subjects without proteinuria. Haplotypes are shown as combined alleles of rs4399321, rs7944706 and repeat number of the microsatellite. *Indicates significant difference after a Bonferroni correction. #Indicates significant difference.

groups ($\chi^2=11.8$; $p=0.019$). Furthermore, the A-G-19 haplotype was significantly higher in the PU group by χ^2 test after the Bonferroni correction ($p=0.007$) and by the permutation method ($p=0.009$).

Discussion

Animal model studies have demonstrated that variations in the AM gene affect blood pressure and that the AM gene is directly involved in the development of hypertension. Homozygous AM knockout mice ($AM^{-/-}$) die with hydrops fetalis and cardiovascular abnormalities (*9*). Blood pressure is lower and plasma cGMP is higher in transgenic mice overexpressing AM than in wild-type mice (*22*). Renal perfusion pressure is higher in mice that are heterozygotic for AM ($AM^{+/-}$) than in wild-type mice and lowest in mice overexpressing AM (*23*). Loading with angiotensin and salt causes more coronary vascular fibrosis and hyperplasia, left ventricular hypertrophy, increased urinary secretion of markers for oxidative stress and intimal thickening caused by cuff placement in heterozygotic ($AM^{+/-}$) than in wild-type mice (*24*, *25*). Shindo *et al.* found that arterial blood pressure in unrestrained heterozygous AM knockout mice is significantly higher than that in their wild-type littermates (*26*).

Ishimitsu *et al.* examined the microsatellite DNA polymorphism lying 3′ downstream of the AM gene in normotensives and in patients with EH. They found that among the 4 types of alleles with CA-repeat numbers of 11, 13, 14, and 19, the frequency of the 19-repeat allele was higher in EH than in NT. They hypothesized that the 19-repeat allele is associated with the risk of developing hypertension (*12*). This particular study implies that the AM gene has a close relationship with the development of hypertension. However, no reports have described a relationship between the AM gene and EH when using the haplotype of this gene. Therefore, we tried to establish a haplotype using SNPs and a microsatellite in the gene. In our experiment a 15-repeat allele of the microsatellite was discovered to be a novel allele. However, it was of a low frequency and its discovery was not of significance by itself. Our EH case-control study showed a negative association. The discrepancies in the results of the genetic association studies are not problematic because the results sometimes depend on the sample collection conditions and racial differences. Our criteria for EH and NT were more strict than those in the previous report, which included borderline hypertensives in its NT groups (*12*).

Although the haplotype-based case-control study did not reveal an association with EH, one haplotype was identified as being associated with PU. Ishimitsu *et al.* have also reported that the frequency of the 19-repeat allele of the microsatellite was higher in diabetic patients on hemodialysis than in diabetic patients without nephropathy (*27*). These results are very interesting because they suggest that the 19-repeat alleles may be related to the factor responsible for the development of diabetic nephropathy. Unfortunately, Ishimitsu *et al*'s study did not resolve the mechanisms involved in the development of diabetic nephropathy or any further information on PU. The investigators themselves pointed out that the frequency of the 19-repeat allele of the microsatellite was not increased in hypertensive diabetic patients without nephropathy, which decreases the likelihood that the relationship between the 19-repeat allele and diabetic nephropathy is mediated by a predisposition to hypertension. AM has been reported to play a renoprotective role in chronic hypertensive renal failure (*28*), and the plasma and urinary AM levels in patients with chronic glomerulonephritis have been shown to

reflect the disease activity or glomerular damage represented by the degree of PU (*29*). Overall these results suggest that AM is involved in the pathophysiology of renal function or PU. Our results in this case-control study for PU are consistent with this hypothesis, and this is the first report showing an association between the AM gene and PU. The haplotype showing positive association may be a genetic marker of PU with EH in Japanese subjects, and may be in strong linkage disequilibrium with another functional DNA variant(s) of the AM gene or neighboring genes.

In conclusion, this is the first study to examine the correlation between the human AM gene and EH by using haplotypes. Although this study did not find a relationship between the AM gene and EH, it did reveal a significant association between the AM gene and PU. The data that can be obtained by examining the genetic information of an individual's AM gene may provide a genetic marker for the PU that is found in EH.

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