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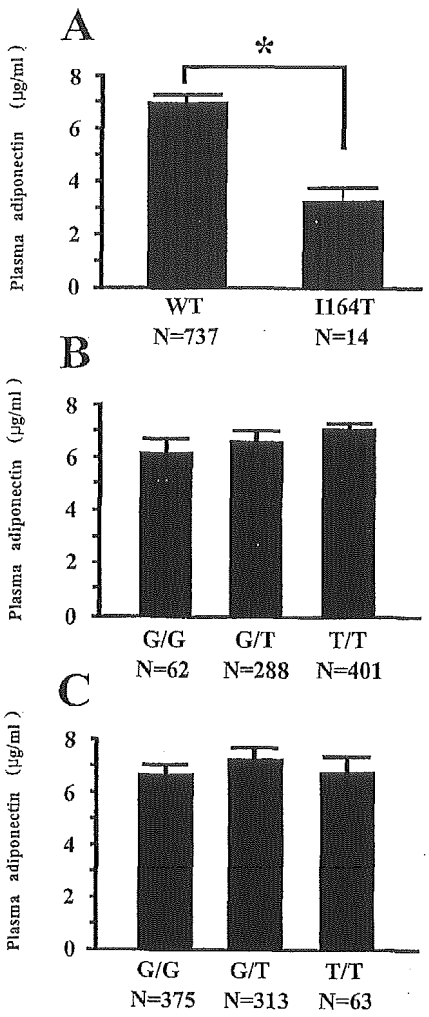

Figure 1. Association of I164T mutation, SNP94, and SNP276 with plasma adiponectin concentrations. (A) Plasma adiponectin levels in the subjects with wild type (WT) or I164T mutation in adiponectin gene. (B) Relationship between SNP94 genotypes and plasma adiponectin levels. (C) Relationship between SNP276 genotypes and plasma adiponectin levels. Columns and vertical bars denote mean and SE of the indicated sample numbers. *$p < 0.05$ vs. WT.

significant difference was observed in BMI between the subjects with and without I164T mutation (24.4 ± 1.2 vs. 24.0 ± 0.1 kg/m^2). The plasma adiponectin levels in subjects with the mutation were markedly low in both CAD and control groups (2.9 ± 0.6 vs. 4.3 ± 1.2 μg/ml, respectively), and did not correlate with BMI. The negative correlation between plasma adiponectin levels and BMI was observed in subjects without the mutation (data not shown). These data indicated that hypoadiponectinemia in subjects with the mutation was independent of BMI. The plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP94 were 6.2 ± 0.6, 6.6 ± 0.2, and 7.1 ± 0.2 μg/ml, respectively (Fig. 1B). The plasma adiponectin level in the subjects having G allele at SNP94 tended to be lower, but it was not statistically significant. On the other hand, no differences were observed in plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP276 (6.6 ± 0.2, 7.2 ± 0.2, and 6.7 ± 0.5 μg/ml, respectively) (Fig. 1C).

As shown in Table 3, all subjects carrying I164T had at least one risk factor including diabetes mellitus, hypertension, and dyslipidemia. Six (case 4 to 8, and 11) of the 11 CAD patients with the I164T mutation and 75 of 372 wild type CAD patients had all three metabolic abnormalities, which is a key feature of the metabolic syndrome. The percentage of the subjects with all three metabolic abnormalities was significantly higher in I164T mutation (54.5%) than that in wild type (20.2%) ($p < 0.01$). Nine (case 4 to 8 and 11 to 14) of 14 subjects with I164T mutation had diabetes mellitus, and cases 13 and 14 had received insulin treatment. However, except three cases (3, 4, and 8), six diabetic I164T patients had no apparent insulin resistance assessed by HOMA-insulin resistance (IR) compared with CAD patients (n = 383, HOMA-IR; 2.4 ± 0.2). In addition, there were no differences in HOMA-IR levels between nondiabetic I164T subjects (case 1 to 3, 9, and 10) and control subjects (n = 368, HOMA-IR; 1.8 ± 0.1).

DISCUSSION

In the present study, we found that the I164T mutation of adiponectin gene was associated with CAD prevalence and hypoadiponectinemia in the Japanese population. In contrast, the genotypes of SNP94 and SNP276, which were reported to be present in type 2 diabetes, influenced neither the prevalence of CAD nor the plasma adiponectin level.

Table 3. Clinical Profile of the Subjects With I164T Mutation

Case Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Age, yrs	53	65	78	52	59	59	61	69	71	72	65	67	70	73
Gender	M	M	F	M	M	M	M	M	M	M	F	F	F	F
Plasma adiponectin, μg/ml	2.7	6.7	3.7	0.4	2.7	2.8	2.6	3.7	3.5	0.9	4.4	2.0	1.6	7.2
BMI, kg/m^2	23.6	ND	24.0	27.0	25.4	29.2	25.6	21.7	19.2	23.8	34.1	25.0	19.0	19.2
FPG, mmol/l	3.5	4.7	5.3	7.6	16.6	5.8	8.2	8.6	5.2	5.4	6.3	6.1	4.8	7.5
FIRI, μU/ml	8.0	4.0	5.0	13.0	5.7	6.0	3.0	10.9	6.2	4.8	6.4	10.2	3.5	2.3
HOMA-IR	1.8	0.8	1.2	3.3	4.2	1.5	2.3	4.2	1.4	1.2	1.4	2.8	0.7	0.8
Number of risk factors*	1	2	2	3	3	3	3	3	1	1	3	2	2	2
Coronary artery disease	-	-	-	AP	AP	AP	AP	MI	AP	MI	AP	AP	AP	AP

*Risk factors: diabetes mellitus, hypertension, and dyslipidemia.
AP = angina pectoris; BMI = body mass index; FIRI = fasting immunoreactive insulin; FPG = fasting plasma glucose; HOMA-IR = homeostatis model assessment of insulin resistance; MI = myocardial infarction.

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Abbreviations and Acronyms

BMI	= body mass index
CAD	= coronary artery disease
HbA1C	= hemoglobin A1C
HDL-chol	= high-density lipoprotein cholesterol
HOMA	= homeostasis model assessment
PCR	= polymerase chain reaction
SNP	= single nucleotide polymorphism
T-chol	= total cholesterol
TG	= triglyceride
TNF	= tumor necrosis factor

(SNP94) in the Japanese population (19,20). Among these mutations, the I164T mutation correlated with type 2 diabetes (19); SNP94 was reported to be associated with type 2 diabetes and obesity (21,22). A weak association was observed between SNP94 and plasma adiponectin levels in French Caucasians, although no significant association was found in the Japanese population (23). Recently, SNP at position 276 (SNP276) was reported to be associated with type 2 diabetes (21); SNP276 was associated with plasma adiponectin levels in French Caucasians and only in obese Japanese subjects (21,23). In addition, the haplotype identified by SNP94 and SNP276 was related with obesity and other features of the insulin resistance syndrome in Caucasians (24). A susceptibility locus for type 2 diabetes was mapped on chromosome 3q27, which harbors the adiponectin gene (25). A genome-wide scan for CAD replicated linkage with the metabolic syndrome on the region 3q27, suggesting that adiponectin might be one of the candidate genes susceptible for the metabolic syndrome-linked CAD (26). Although the metabolic syndrome includes insulin resistance, it is very important to elucidate the genetic contribution of adiponectin in the development of CAD.

In the present study, we investigated the frequency and the clinical significance of I164T, SNP94, and SNP276 of adiponectin gene in consecutive CAD patients and age- and body mass index (BMI)-matched non-CAD subjects.

METHODS

Study subjects. Consecutive 383 CAD patients were recruited from the inpatients who were admitted to Osaka University Hospital. The criteria for CAD were a 75% ≤ organic stenosis of at least one segment of a major coronary artery confirmed by coronary angiogram. The control subjects were selected from people who received medical check in Osaka University Hospital or our affiliated hospitals. In these latter subjects, it was unethical to perform coronary angiography to rule out the presence of asymptomatic CAD. Therefore, the following inclusion criteria were used: no history of angina or other atherosclerotic vascular diseases, and normal exercise electrocardiogram stress testing. They were matched with CAD patients for age and BMI. All patients and subjects enrolled in this study were Japanese and gave written informed consent. This study was approved by the Ethics Committee of Osaka University.

Laboratory methods. Venous blood was drawn from all patients and control subjects after an overnight fast. Plasma samples were kept at −80° centigrade for subsequent assay. Plasma concentration of adiponectin was evaluated by a sandwich ELISA system (Adiponectin ELISA Kit, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) as previously reported (27). Serum total cholesterol (T-chol) and triglyceride (TG) concentrations were determined by an enzymatic method. High-density lipoprotein cholesterol (HDL-chol) was also measured by an enzymatic method after heparin and calcium precipitation. Plasma glucose was measured by a glucose oxidase method. The value of hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography. Insulin resistance was assessed by homeostasis model assessment (HOMA) (insulin resistance index = [fasting glucose (mmol/l) × fasting insulin (U/ml)]/22.5 (28). Body mass index was calculated as weight/height^2.

Definitions of risk factors. Diabetes mellitus was defined according to World Health Organization criteria, and/or having received treatment for diabetes mellitus (29). Dyslipidemia was defined as a T-chol concentration >5.69 mmol/l, a TG concentration >1.69 mmol/l, an HDL-chol concentration <1.03 mmol/l, and/or having received treatment for dyslipidemia. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or having received treatment for hypertension. We did not exclude the subjects under medical treatment for diabetes mellitus, dyslipidemia, and hypertension.

DNA extraction and genotyping. Genomic DNA was prepared from frozen whole blood with the use of a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, California). We determined the missense mutation I164T and the SNP276 of adiponectin gene by the TaqMan (Roche Molecular Systems Inc., Pleasanton, California) polymerase chain reaction (PCR) chemistry method as previously described (30). The TaqMan probe is a fluorogenic probe that consists of an oligonucleotide labeled with both a fluorescent reporter dye and a quenched dye. The fluorescent reporter dye, such as VIC and FAM (Applied Biosystems Inc., Foster City, California), is covalently linked to the 5′ end of the nucleotide. Each of the reporters is quenched by minor groove binder, typically located at the 3′ end. The following primers were used for the missense mutation I164T: a forward primer, 5′-AACATTCCTGGGCTGTACTACTTTG-3′; a reverse primer, 5′- GGCTGACCTTCACATCCTTCATA-3′; a T-allele-specific probe, 5′-VIC-ACCACATCA-CAGTCTA-MGB-3′; a C-allele-specific probe, 5′-FAM-CCACACCACAGTCT-MGB-3′. The following primers were used for the G/T SNP at position 276: a forward primer, 5′-AGAATGTTTCTGGCCTCTTTCATC-3′; a reverse primer, 5′- TTCTCCCTGTGTCTAGGCCTTAGT-3′; a G-allele-specific probe, 5′-FAM-CTATATGAAGGCAT-TCATTA-MGB-3′; T-allele-specific probe, 5′-VIC-

Table 1. Clinical Characteristics of Control Subjects and CAD Patients

	Control Subjects (n = 368)	CAD Patients (n = 383)	p Value
Age, yrs	62.3 ± 0.6	63.0 ± 0.4	NS
Gender, M/F	240/128	270/113	NS
Adiponectin, μg/ml	7.7 ± 0.2	6.1 ± 0.2	< 0.001
BMI, kg/m^2	23.8 ± 0.2	24.1 ± 0.2	NS
Family history of diabetes mellitus, n (%)	(15.8)	(18.5)	NS
Diabetes mellitus, n (%)	58 (10.3)	71 (48.0)	< 0.001
FPG, mmol/l	38 ± 0.04	184 ± 0.14	< 0.001
	5.40	6.67	
HbA1c, %	5.11 ± 0.04	6.09 ± 0.08	< 0.001
Dyslipidemia, n (%)	179 (48.6)	259 (67.6)	< 0.001
T-chol, mmol/l	5.23 ± 0.05	5.29 ± 0.05	NS
TG, mmol/l	1.57 ± 0.05	1.77 ± 0.06	<0.05
HDL-chol, mmol/l	1.52 ± 0.03	1.19 ± 0.02	< 0.001
Hypertension, n (%)	272 (73.9)	264 (68.9)	NS
SBP, mm Hg	134.6 ± 1.0	132.9 ± 0.9	NS
DBP, mm Hg	80.1 ± 0.7	75.4 ± 0.8	< 0.001

Data represent means ± SE.

BMI = body mass index; CAD = coronary artery disease; DBP = diastolic blood pressure; FPG = fasting plasma glucose; HbA1c = hemoglobin A1C; HDL-chol = high-density lipoprotein cholesterol; SBP = systolic blood pressure; T-chol = total cholesterol.

AAACTATATGAAGTCATTCATTA-MGB-3′. The fluorescence level of PCR products was measured with the ABI PRISM 7200 Sequence Detector (Applied Biosystems, Inc.). We determined the SNP94 in exon 2 of adiponectin gene by a PCR-based assay for the analysis of restriction fragment length polymorphism as previously described (20).

Statistical methods. For continuous variables, results are presented as mean ± SE. Differences in continuous parameter, such as BMI, between two groups were calculated by the Student *t* test, and differences in continuous parameter, such as plasma adiponectin level, among more than three groups were evaluated by analysis of variance. Because plasma adiponectin level, HOMA, and TG were skewed, these three parameters were log-transformed before analysis, and the parameters presented were back-transformed. Categorical variables were presented using frequency counts, and intergroup comparisons were analyzed by chi-square test. A level of $p < 0.05$ was accepted as statistically significant. All calculations were performed using a standard statistical package (JMP for Macintosh, version 4.0, SAS Institute Inc., Cary, North Carolina).

RESULTS

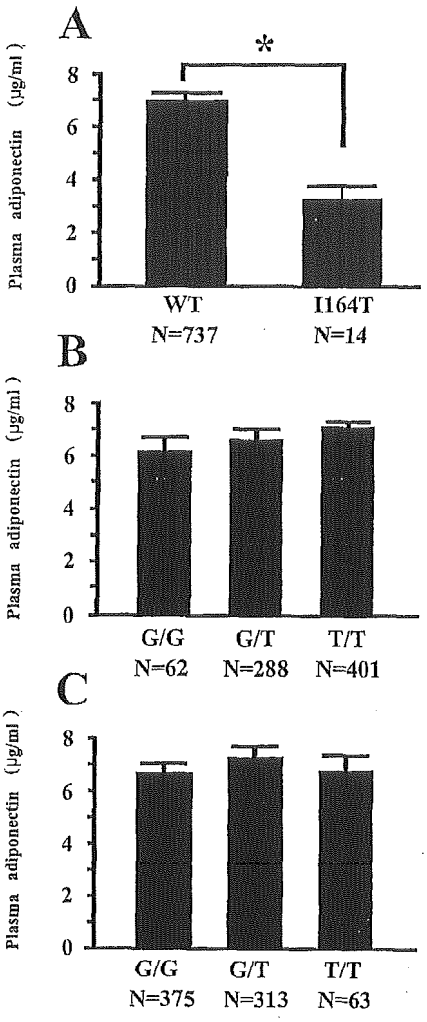
The clinical characteristics of CAD patients and non-CAD control subjects are shown in Table 1. The mean plasma adiponectin level in CAD patients was significantly lower than the control ($p < 0.001$), as we described previously (10). Patients with CAD had significantly higher levels of fasting plasma glucose, HbA1c, TG, numbers of diabetes mellitus, dyslipidemia, and lower levels of HDL-chol and diastolic blood pressure than the control group. There were no significant differences in age, gender, BMI, number of family history for diabetes, T-chol, systolic blood pressure, and number of hypertension between the two groups.

The frequency of I164T mutation in CAD patients (11 [2.9%] of 383) was significantly higher than that in non-CAD subjects (3 [0.8%] of 368, $p < 0.05$) (Table 2). All subjects with the mutation were heterozygotes. In contrast to this mutation, no significant differences in the distribution of SNP94 and SNP276 genotypes were observed between the two groups. The plasma adiponectin levels in subjects carrying the I164T mutation (3.2 ± 0.5 μg/ml) were significantly lower than in subjects without the mutation (6.9 ± 0.2 μg/ml, $p < 0.0001$) (Fig. 1A), although no

Table 2. Frequency of Mutation and Polymorphism in Adiponectin Gene

		Control Subjects	CAD Patients	
n		368	383	p Value
I164T, n (%)		3 (0.8)	11 (2.9)	<0.05
	G/G	29 (7.9)	33 (8.6)	
SNP94, n (%)	G/T	148 (40.2)	140 (36.6)	NS
	T/T	191 (51.9)	210 (54.8)	
	G/G	190 (51.6)	185 (48.3)	
SNP276, n (%)	G/T	149 (40.5)	164 (42.8)	NS
	T/T	29 (7.9)	34 (8.9)	

CAD = coronary artery disease; SNP = single nucleotide polymorphism.



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significant difference was observed in BMI between the subjects with and without I164T mutation (24.4 ± 1.2 vs. 24.0 ± 0.1 kg/m^2). The plasma adiponectin levels in subjects with the mutation were markedly low in both CAD and control groups (2.9 ± 0.6 vs. 4.3 ± 1.2 μg/ml, respectively), and did not correlate with BMI. The negative correlation between plasma adiponectin levels and BMI was observed in subjects without the mutation (data not shown). These data indicated that hypoadiponectinemia in subjects with the mutation was independent of BMI. The plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP94 were 6.2 ± 0.6, 6.6 ± 0.2, and 7.1 ± 0.2 μg/ml, respectively (Fig. 1B). The plasma adiponectin level in the subjects having G allele at SNP94 tended to be lower, but it was not statistically significant. On the other hand, no differences were observed in plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP276 (6.6 ± 0.2, 7.2 ± 0.2, and 6.7 ± 0.5 μg/ml, respectively) (Fig. 1C).

As shown in Table 3, all subjects carrying I164T had at least one risk factor including diabetes mellitus, hypertension, and dyslipidemia. Six (case 4 to 8, and 11) of the 11 CAD patients with the I164T mutation and 75 of 372 wild type CAD patients had all three metabolic abnormalities, which is a key feature of the metabolic syndrome. The percentage of the subjects with all three metabolic abnormalities was significantly higher in I164T mutation (54.5%) than that in wild type (20.2%) ($p < 0.01$). Nine (case 4 to 8 and 11 to 14) of 14 subjects with I164T mutation had diabetes mellitus, and cases 13 and 14 had received insulin treatment. However, except three cases (3, 4, and 8), six diabetic I164T patients had no apparent insulin resistance assessed by HOMA-insulin resistance (IR) compared with CAD patients (n = 383, HOMA-IR; 2.4 ± 0.2). In addition, there were no differences in HOMA-IR levels between nondiabetic I164T subjects (case 1 to 3, 9, and 10) and control subjects (n = 368, HOMA-IR; 1.8 ± 0.1).

DISCUSSION

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Age, yrs	53	65	78	52	59	59	61	69	71	72	65	67	70	73
Gender	M	M	F	M	M	M	M	M	M	M	F	F	F	F
Plasma adiponectin, μg/ml	2.7	6.7	3.7	0.4	2.7	2.8	2.6	3.7	3.5	0.9	4.4	2.0	1.6	7.2
BMI, kg/m^2	23.6	ND	24.0	27.0	25.4	29.2	25.6	21.7	19.2	23.8	34.1	25.0	19.0	19.2
FPG, mmol/l	3.5	4.7	5.3	7.6	16.6	5.8	8.2	8.6	5.2	5.4	6.3	6.1	4.8	7.5
FIRI, μU/ml	8.0	4.0	5.0	13.0	5.7	6.0	3.0	10.9	6.2	4.8	6.4	10.2	3.5	2.3
HOMA-IR	1.8	0.8	1.2	3.3	4.2	1.5	2.3	4.2	1.4	1.2	1.4	2.8	0.7	0.8
Number of risk factors*	1	2	2	3	3	3	3	3	1	1	3	2	2	2
Coronary artery disease	-	-	-	AP	AP	AP	AP	MI	AP	MI	AP	AP	AP	AP

*Risk factors: diabetes mellitus, hypertension, and dyslipidemia.
AP = angina pectoris; BMI = body mass index; FIRI = fasting immunoreactive insulin; FPG = fasting plasma glucose; HOMA-IR = homeostatis model assessment of insulin resistance; MI = myocardial infarction.

Importantly, all subjects carrying I164T in the present study including CAD and non-CAD subjects had at least one or more metabolic disorders including diabetes mellitus, hypertension, and dyslipidemia. Among CAD patients, the prevalence of the metabolic syndrome was significantly higher in I164T mutation than that in wild type. These findings suggest that the I164T mutation of adiponectin gene is associated with the development of the metabolic syndrome-linked CAD. Importantly, the severe hypoadiponectinemia in subjects with the I164T mutation was independent of BMI. Recently, we have demonstrated that intimal thickening was accelerated in mechanically injured arteries of adiponectin knockout mice, and that adenovirus-mediated supplement of adiponectin completely abolished the enhanced neointimal formation (15). These results suggest that hypoadiponectinemia directly contributes to abnormal vascular remodeling. Therefore, the I164T mutation plays a pivotal role in the development of atherosclerosis.

We have reported that the plasma adiponectin levels were significantly low in subjects with obesity (27), diabetes mellitus (31), and hypertension (32). In addition, we reported that plasma adiponectin level was predictive of the development of type 2 diabetes in the Pima Indian population (33). These observations suggest that the plasma adiponectin levels might be closely associated with the development of the metabolic syndrome. In adiponectin knockout mice, glucose metabolism was normal under standard diet, and severe insulin resistance, hyperglycemia, and hypertension were developed after two weeks' feeding of atherogenic diet (18,34). In the present study, all subjects carrying I164T had at least one or more coronary risk factors. However, HOMA-IR levels of nondiabetic I164T mutation were no different than those of control subjects. These results suggest that the hypoadiponectinemia caused by I164T mutation might lead to diabetes mellitus, hypertension, and atherosclerosis only under overnutrition in the modern industrialized countries.

A recent study demonstrated that the I164T mutation was not found in the type 2 diabetic and obese French Caucasian subjects and that the genotypes of SNP94 and SNP276 affected plasma adiponectin levels (23). Higher plasma adiponectin levels were associated with the T allele of SNP94 and the G allele of SNP276 in Caucasians (23). We and others demonstrated that the I164T mutation was observed in the Japanese population (19,21). In the present study, the G allele of SNP94 tended to be associated with lower plasma adiponectin levels, and SNP276 did not correlate with plasma adiponectin levels in CAD and non-CAD Japanese subjects whose mean BMI were approximately 24 kg/m^2. Recently, the genotypes of SNP276 were reported to be associated with plasma adiponectin levels only in the obese subgroup of Japanese subjects (21). These differences between the French and Japanese populations may be due to ethnic background, although a larger population study is required to elucidate the discrepancy.

In the current study, three of the 14 subjects with the I164T mutation did not suffer from CAD, although they had at least one coronary risk factor and markedly low plasma adiponectin level. The follow-up study will be necessary to clarify whether the non-CAD subjects with I164T mutation develop CAD in the future.

In summary, we demonstrated that the I164T mutation of adiponectin gene affects CAD prevalence and the clustering of multiple risk factors for atherosclerosis. Our results indicate that screening the common genetic background of hypoadiponectinemia is helpful in evaluating the risk of the metabolic syndrome and CAD.

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Reprints requests and correspondence: Dr. Shinji Kihara, Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: kihara@imed2.med.osaka-u.ac.jp.

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G2736A polymorphism of thiazide-sensitive Na-Cl cotransporter gene predisposes to hypertension in young women

Akiko Matsuo, Tomohiro Katsuya, Kazuhiko Ishikawa, Ken Sugimoto, Yoshio Iwashima, Koichi Yamamoto, Mitsuru Ohishi, Hiromi Rakugi and Toshio Ogihara

Objective The thiazide-sensitive Na-Cl cotransporter (TSC) is located in the distal renal tubules. Several mutations of the TSC gene cause Gitelman's syndrome, which is an autosomal recessive disease characterized by low blood pressure and hypokalemia. Recently, an association between TSC gene polymorphisms (Arg904Gln, G2736A; Thr465Thr, C1420T; Gly264Ala, G816C) and essential hypertension has been reported in Sweden. We examined the genetic involvement of the TSC gene in essential hypertension in Japanese.

Design Participants were recruited from outpatients of Osaka University Hospital. We investigated 386 hypertensive and 371 normotensive subjects.

Methods Genotypes of TSC polymorphisms (G2736A, C1420T, G816C) were determined by the TaqMan polymerase chain reaction (PCR) method, and statistical significance was examined using JMP 5.0.1J (SAS Institute Inc., Cary, North Carolina, USA). The allele frequency of A2736 and T1420 was 6.0 and 3.0%, respectively, whereas we could not detect the G816C polymorphism in this study. Only the G2736A polymorphism was significantly associated with the prevalence of hypertension ($P < 0.04$), and the estimated odds ratio was 1.8 (95% confidence interval, 1.1–3.0) in A2736 allele carriers. The odds ratio for hypertension in A2736 carriers was increased to 2.2 (1.1–4.9) in women ($n = 413$), and further to 3.3 (1.4–8.0) in women with early onset of hypertension (≤ 50 years old). In addition, all subjects with the homozygous A2736 allele in this study ($n = 2$) and the Swedish study ($n = 5$) were hypertensive.

Conclusion G2736A polymorphism of the TSC gene is a genetic predisposing factor for essential hypertension in Japanese women. *J Hypertens* 22:2123–2127 © 2004 Lippincott Williams & Wilkins.

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Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita, Japan.

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Correspondence and requests for reprints to Tomohiro Katsuya, MD, PhD, Assistant Professor of Medicine, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, #B6, Suita 565-0871, Japan.
Tel: +81 6 6879 3852; fax: +81 6 6879 3859;
e-mail: katsuya@geriat.med.osaka-u.ac.jp

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Introduction

Essential hypertension is a common disease that is affected by both environmental and genetic factors. Classically, aging, obesity, excess salt intake, ethnicity (e.g. African-American) and sex (male) are known to be risk factors for hypertension [1], and recent genetic investigations have revealed several genetic variants that are candidates in the pathogenesis of hypertension [2–4]. Genetic variants of the epithelial sodium channel or mineral corticoid receptor have not only been shown to cause monogenic hypertension [5], but also to be candidates for genetic predisposing factors for essential hypertension. For example, the T594M polymorphism in the beta-subunit of the epithelial sodium channel (ENaC) gene is associated with hypertension in black populations [6].

Gitelman's syndrome [7–10] is an autosomal recessive disease that shows similar clinical characteristics to Bartter's syndrome [11,12] and is characterized by low blood pressure due to renal sodium wasting, hypokalemia, hypomagnesemia, and hypocalciuria from a young age. It is reported that Gitelman's syndrome is caused by novel homozygous mutations of the thiazide-sensitive Na-Cl cotransporter (TSC) gene, which leads to loss of TSC function and thus reduced renal sodium reabsorption [10]. TSC is a member of the electroneutral cation-chloride-coupled cotransporter gene family

(SLC12: solute carrier family 12), which encompasses two major branches, one of which includes two bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporters and TSC (SLC12A3) [13]. TSC, which is located in the distal renal tubules [14], is a target of the diuretic effect of thiazides [5], which are known to be useful for patients with hypertension who are salt sensitive, such as African-Americans [15]. Melander *et al.* [16] recently investigated some polymorphisms of the TSC gene, and showed that homozygous A2736 and T1420 alleles were significantly associated with hypertension in the Swedish population. In the present study, we investigated the association of these polymorphisms with essential hypertension in the Japanese population.

Methods

Study population

Patients with essential hypertension and control subjects were recruited from in- and outpatients of Osaka University Hospital. All cases and controls were Japanese and gave informed consent before participating in the research protocol, which was approved by the Hospital Ethics Committee. All cases ($n = 386$) had a family history of hypertension in first-degree relatives, and were diagnosed as having primary hypertension (those with secondary hypertension or apparent ischemic heart disease were excluded). The criteria for hypertension were defined as systolic blood pressure higher than 140 mmHg and/or diastolic blood pressure higher than 90 mmHg, or receiving antihypertensive therapy. Controls ($n = 371$) without a history of hypertension were recruited from the same population. To enhance detection of the genetic effect on hypertension, we excluded subjects under 50 years old from the control group, because blood pressure generally tends to increase as people get older. Subjects completed a standard questionnaire on their personal medical history and family history of hypertension. Blood pressure was measured twice with the subjects seated after 5 min of rest.

Genotype determination of TSC gene polymorphisms

To determine TSC genotype, we employed the TaqMan polymerase chain reaction (PCR) method. A fluorescent reporter dye, such as 6-carboxy-fluorescein (FAM) or (VIC), was linked covalently to the 5′ end of the nucleotide. For the present investigation, we prepared two probes and two primers for each fragment, as follows: 5′-VIC-CCA CCC TCT CCT CT-MGB-3′, 5′-FAM-CCA CTC TCT CCT CTG-MGB-3′, CCA AAT CCC CAC AGA CCA T (forward primer), GTC ATC TCG ACC CCT TTC TGC (reverse primer) for C1420T; 5′-VIC-CCC TCG GGC TGA G-MGB-3′, 5′-FAM-AGA ACC CTC AGG CTG-MGB-3′, CCA CAT CCT CCC TGA CAT CAA (forward primer), GAA GCC CCA AAA CAG AAC TTA CTG (reverse primer) for G2736A; 5′-VIC-ATC ATT GGC GTG GTC-MGB-3′, 5′-FAM-ATC ATT GCC GTG GTC-MGB-3′, TCG TGG ACC CCA TTA ACG A (forward primer), TGG CCA GCA GCA CAG TGA (reverse primer) for G816C.

PCR was carried out using a Gene Amp 9700 (Applied Biosystems, Foster City, California, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 60 s. During the PCR cycles, two TaqMan probes hybridize competitively to a specific sequence of the target DNA, and the reporter dye separates from the quencher dye, resulting in an increase of fluorescence of the reporter. The fluorescence level of PCR products was measured using an ABI PRISM 7200 or 7900 Sequence Detector (Applied Biosystems), resulting in clear identification of two polymorphisms (C1420T and G2736A) of TSC. Genotyping data of all minor allele carriers and representative subjects ($n = 15$) with homozygous major allele were confirmed by sequencing.

Thiazide-loading test

To examine the gain of function of TSC due to the G2736A polymorphism, we carried out the thiazide-loading test in healthy volunteers with homozygous GG ($n = 3$) or heterozygous GA ($n = 3$), with their informed consent. Before and after administration of 50 mg hydrochlorothiazide (HCT), we collected urine and peripheral blood using a Tempus Blood RNA Tube (Applied Biosystems), calculated the sodium excretion rate and quantified the expression level of the TSC gene by real-time reverse transcription PCR (RT-PCR) in comparison with 18sRNA. Urinary sodium excretion rate (UNaV) (net; mmol/150 min) was calculated by the following formula: UNaV (cumulative) (total sodium excretion from 30 to 180 min after HCT administration, mmol/150 min) $-$ 150 $\times$ UNaV (basal) (sodium excretion per minute for 1 h before administration of HCT, μmol/min).

Statistical analysis

All statistical analyses were conducted using JMP 5.0.1J (SAS Institute Inc., Cary, North Carolina, USA). Difference in genotype or allele frequency between normotensives and hypertensive subjects were examined by chi-squared analysis. The association between TSC polymorphisms and clinical variables was examined by one-way ANOVA. We assessed the quantitative effects of covariates by multiple logistic regression analysis with JMP.

Results

Clinical features of participants

There were significant differences in sex, age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and triglyceride level (TG), but not in total cholesterol level and prevalence

of diabetes between hypertensives and normotensive subjects (Table 1).

Association between TSC polymorphisms and hypertension

The genotype distributions of the C1420T and G2736A variants were not significantly deviated from Hardy–Weinberg's expectation. In this Japanese population, there was no C816 allele of the G816C polymorphism. Neither the allele or genotype frequency of C1420T were significantly different between hypertensive and normotensive subjects (Table 2). In contrast, the G2736A polymorphism was significantly associated with the prevalence of hypertension ($P < 0.04$) (Table 2). The calculated odds ratio for hypertension in A2736 allele carriers was 1.8 [95% confidence interval (CI): 1.1–3.0] after adjustment for confounding factors of sex, age, BMI and TG. Although no significant association between the A2736 allele and hypertension was observed in men, female A2736 allele carriers were significantly ($P < 0.04$) predisposed to hypertension (odds ratio: 2.2, 95% CI: 1.1–4.9) (Table 3). Furthermore, the significance of the association with hypertension was enhanced in female subjects with early-onset ($\leq$ 50 years old) hypertension ($P < 0.01$, odds ratio: 3.3, 95% CI: 1.4–8.0) (Table 4).

Table 1 **Clinical features of study participants**

	HT ($n = 386$)	NT ($n = 371$)	*P* value
Male/female	225/161	188/183	0.04
Age (years)	61 ± 11	67 ± 7	< 0.0001
BMI (kg/m^2)	24 ± 3	22 ± 3	< 0.0001
Systolic BP (mmHg)	170 ± 26	111 ± 10	< 0.0001
Diastolic BP (mmHg)	101 ± 17	69 ± 7	< 0.0001
Total cholesterol (mg/dl)	201 ± 36	202 ± 35	NS
TG (mg/dl)	147 ± 95	114 ± 67	< 0.0001
Diabetes (%)	15.8	19.5	NS

Variables are mean ± SD. HT, hypertension; NT, normotension; BMI, body mass index; TG, triglyceride; NS, not significant.

Thiazide-loading test

Baseline TSC gene expression level in the peripheral blood in heterozygous patients (GA) (0.17 ± 0.10) was slightly but not significantly higher than that in those homozygous for GG (0.12 ± 0.03). The sodium excretion rate after HCT administration [UNaV (net)] was higher in heterozygous subjects (26.6 ± 15.9) than in those homozygous for GG (11.7 ± 19.6), but there was no significant difference between them.

Discussion

The current study suggested a positive association between the TSC G2736A polymorphism and essential hypertension in younger women. G2736A (Arg904Gln) is located in the intracellular region close to the C terminus, and the Arg to Gln amino acid substitution leads to a change in the protein from electronically positive to negative. However, the role of this site is not known, and the amino acid substitution is not conserved between rats and humans. Loss of function in TSC causes a decrease of sodium reabsorption and leads to Gitelman's syndrome [10,17], so we speculate that G2736A is a gain of function polymorphism. As an example, gain of function in the amiloride-sensitive epithelial sodium-channel gene causes hypertension (Liddle's syndrome) [18,19], whereas loss of function in the same gene causes hypotension [20] (pseudohypoaldosteronism type I). Furthermore, subjects with heterozygous mutations that cause Gitelman's syndrome in the homozygous state have lower blood pressure than subjects without mutations [21]. Even though we could not clearly show proof of gain of function due to G2736A polymorphism, our preliminary investigation using thiazide loading suggested an increase of TSC gene expression and sodium excretion after HCT administration in the subjects with the A2736 allele.

In the present study, the G2736A polymorphism was positively associated with hypertension in younger

Table 2 **Genotype and allele distribution of C1420T and G2736A polymorphisms in all subjects ($n = 757$)**

C1420T	C allele	T allele	*P* value	Odds ratio (95%CI)	CC	CT	TT	*P** value	Odds ratio (95% CI)
HT	753	19	NS	0.6	367	19	0	NS	0.5***
%	97.5	2.5		(0.3–1.1)	95.1	4.9	0		(0.6–1.6)***
NT	713	29			344	25	2		
%	96.1	3.9			92.7	6.7	0.5		
G2736A	G allele	A allele	*P* value	Odds ratio (95%CI)	GG	GA	AA	*P*** value	Odds ratio (95%CI)
HT	716	56	0.02	1.7	332	52	2	< 0.04***	1.8***
%	92.8	7.2		(1.1–2.6)	86.0	13.5	0.5		(1.1–3.0)***
NT	709	33			338	33	0		
%	95.6	4.4			91.1	8.9	0		

*CC versus CT + TT. **GG versus GA + AA. ***Adjusted by sex, age, body mass index (BMI) and triglyceride level (TG). HT, hypertension; NT, normotension; NS; not significant; 95% CI, 95% confidence interval.

Table 3 Genotype and allele distribution of G2736A polymorphism in male ($n = 413$) and female ($n = 344$) subjects

	G allele	A allele	*P* value	Odds ratio (95% CI)	GG	GA + AA	*P* value	Odds ratio (95% CI)
Male								
HT	425	25	NS	1.1	200	25	NS	1.5*
(%)	(94.4)	(5.6)		(0.6–2.0)	(88.9)	(11.1)		(0.7–3.1)*
NT	357	19			169	19		
(%)	(94.9)	(5.1)			(89.9)	(10.1)		
Female								
HT	291	31	< 0.01	2.7	132	29	< 0.04*	2.2*
(%)	(90.4)	(9.6)		(1.4–5.1)	(82.0)	(18.0)		(1.1–4.9)*
NT	352	14			169	14		
(%)	(96.2)	(3.8)			(92.4)	(7.6)		

*Adjusted by age, body mass index (BMI) and triglyceride level (TG). HT, hypertension; NT, normotension; NS, not significant; 95% CI, 95% confidence interval.

Table 4 Distribution of G2736A polymorphism in females with onset of hypertension at ≤ 50 years of age ($n = 256$)

	G allele	A allele	*P* value	Odds ratio (95% CI)	GG	GA + AA	*P* value	Odds ratio (95% CI)
HT	131	15	< 0.01	2.9 (1.4–6.1)	59	14	< 0.01*	3.3* (1.4–8.0)*
(%)	(89.8)	(10.2)			(80.8)	(19.2)		
NT	352	14			169	14		
(%)	(96.2)	(3.8)			(92.3)	(7.7)		

*Adjusted by body mass index (BMI) and triglyceride level (TG). HT, hypertension; NT, normotension; 95% CI, 95% confidence interval.

women. This could be explained as follows. First, female hormones, such as estrogen, accelerate sodium and water retention in young premenopausal women. Hurwitz *et al.* [22] reported that the highest systolic salt sensitivity (SSS) was observed in premenopausal women with low renin activity, and Verlander *et al.* [23] reported that estrogen enhances TSC density in the distal convoluted tubule. In addition, it has been known that estrogen reduces renal sodium excretion [24]. Another feasible explanation of this result is that the effect of environmental risk factors, such as smoking, drinking or excessive eating, on hypertension was dominant, and masked the genetic effect of the TSC polymorphism in men and/or postmenopausal women, whereas the effect of the polymorphism on hypertension was significant and relatively major in young women who were less exposed to environmental risks.

Previously, a Swedish group reported a borderline association of the G2736A polymorphism with hypertension ($P = 0.05$) [16], but their analysis did not divide the subjects by sex. Furthermore, Asian populations, such as the Japanese, are known to be more salt sensitive than Caucasian people [25]. So these results of two genetically different populations seem to be reasonable. Interestingly, there were two A2736 homozygous subjects in our population, who were both hypertensive, and five AA homozygous subjects in the Swedish study, were also hypertensive [16]. In contrast, another polymorphism, the T1420 allele, was positive in Caucasians but not in Japanese. Even though the allele frequency of T1420 was too low to discuss the significance of the difference between Japanese and Caucasian populations, the frequency of T1420 was lower in hypertensive than normotensive subjects in the present study. Furthermore, C1420T was a synonymous polymorphism and not in linkage disequilibrium with G2736A, suggesting that genetic determination of G2736A is worthwhile in the risk estimation for hypertension rather than C1420T.

There were some study limitations. We do not show plasma potassium and renin activity, because the lack of data in many subjects could lead to ambiguous results. We analyzed the available data of renin activity and potassium, but they were approximately the same in subjects with G2736 and A2736. It is known that salt-sensitive patients with hypertension have low renin activity [26]. However, some patients had a moderate level of renin activity in a previous report [27].

Even though we examined TSC function using the thiazide-loading test, the examined number was too small to discuss the significance of the association, and the results of TSC expression were obtained from peripheral blood and not from distal tubules. Furthermore, we only examined heterozygotes (GA subjects) but not homozygotes (AA subjects), so we could not exclude the possibility that subjects with AA clearly show a gain of function of TSC.

Salt-sensitive hypertension is a relatively clear category of essential hypertension, and administration of diuretics is reasonable and effective therapy; however, it takes much effort to distinguish these subjects in the present clinical situation. In the future, determination of the TSC gene polymorphism may contribute to identifying patients with salt-sensitive hypertension and the choice of antihypertensive medication. In conclusion, the G2736A genotype of the TSC gene may be a risk factor for essential hypertension in younger Japanese woman.

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Review

Salt Sensitivity of Japanese from the Viewpoint of Gene Polymorphism

Tomohiro KATSUYA, Kazuhiko ISHIKAWA, Ken SUGIMOTO,
Hiromi RAKUGI, and Toshio OGIHARA

Excess salt intake is an important environmental risk for the predisposition to essential hypertension. Previous physiological studies have shown that salt sensitivity is associated with insulin resistance, enhancement of sympathetic nerve activity and decrease of blood pressure decline at night. We have been examining the genetic importance of candidate gene polymorphisms of salt-sensitive hypertension using several populations. The angiotensinogen gene (*AGT*) is a thrifty gene which increases the risk for common disease with growth of civilization *via* sodium and body fluid retention. The *CC* genotype of the *AGT/T+31C* polymorphism, which is in complete linkage disequilibrium with the *TT* genotype of the *M235T* polymorphism, was associated with a decrease of blood pressure decline at night in the Ohasama Study. On the other hand, the *Gly460Trp* genotype of the α-adducin gene (*ADD1*) is associated with erythrocyte sodium transport and increases tubular sodium reabsorption and risk for hypertension. We also revealed in the Ohasama Study that the *Trp460* allele of *ADD1* is associated with hypertension in young subjects with low renin activity. In addition to these polymorphisms, the *T(−344)C* polymorphism in the promoter of the aldosterone synthase gene (*CYP11B2*) and the *C825T* polymorphism of the G-protein β3 subunit gene (*GNB3*) are considered candidates for the genetic risk of salt-sensitive hypertension. We compared the allele frequency of five candidate genes between Japanese and Caucasians; the results showed that the frequencies of all alleles were significantly higher in Japanese than in Caucasians. This interesting finding might suggest a feasible explanation for the huge interracial differences in the frequency of salt-sensitive hypertension. (*Hypertens Res* 2003; 26: 521–525)

***Key Words*: genetics, non-dipper, insulin resistance, essential hypertension, lacunar infarction**

Introduction

Blood pressure is a quantitative phenotype and has a continuous distribution. Multiple genetic and environmental factors determine one's blood pressure level, and "essential hypertension" is merely the upper end of the distribution (*1*). Subjects with essential hypertension are those who happen to inherit an aggregate of genes related to hypertension and/or who are exposed to exogenous factors that predispose them to hypertension. Whereas young adults with a familial predisposition to hypertension and those without such a predisposition both show a pressor response to high sodium intake, only the former show a depressor response to a high potassium intake (2). Garay *et al.* found a defect in the furosemide-sensitive Na-K cotransfer mechanism in red cells of patients

From the Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita, Japan.
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Address for Reprints: Tomohiro Katsuya, M.D., Ph.D., Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2–2 #B6, Yamada-oka, Suita 565–0871, Japan. E-mail: katsuya@geriat.med.osaka-u.ac.jp
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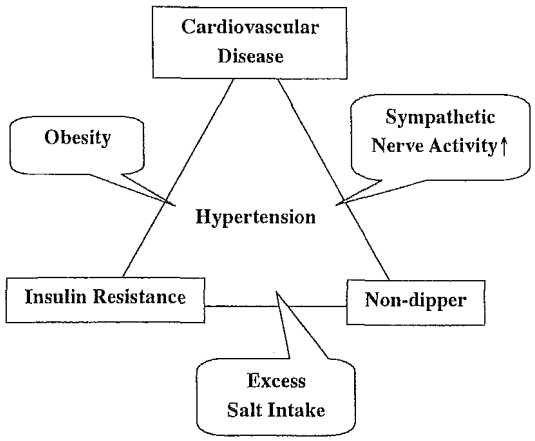

Fig. 1. *The "deadly trio" creating a predisposition to hypertension, and the "three evils" exacerbating hypertension.*

with essential hypertension and in some of their normotensive relatives (*3*). The same defect is found in several strains of experimental animals bred for high susceptibility to salt-induced hypertension or spontaneous hypertension.

It has been reported that NaCl loading blunted the nocturnal decline in blood pressure in salt-sensitive patients with essential hypertension but not in their salt-resistant counterparts (*4*). Takakuwa *et al.* reported that a non-dipper pattern is common in patients with an aldosterone-producing adenoma on a normal salt intake, and that, under such conditions, volume expansion plays a major role in the impairment of nocturnal blood pressure reduction (*5*). These results suggest that subjects with an impairment of nocturnal blood pressure reduction, known as "non-dippers," are predisposed to salt-sensitive hypertension. In addition to salt sensitivity, sympathetic nerve activity involved in nocturnal blood pressure elevation may differ between dipper and non-dippers (*6*), and the non-dipper profile appears to be of prognostic significance because it is associated with increased target-organ damage and a worsened cardiovascular outcome (*7*).

Considering hypertension from the vantage point of obesity, a variety of endocrine, genetic, and metabolic mechanisms have been linked to each other. These include insulin resistance and hyperinsulinemia, increased serum aldosterone levels, salt sensitivity and expanded plasma volume in the presence of increased peripheral vascular resistance, a genetic predisposition, and possibly increased leptin levels. Presence of pressure and volume overload lead to a mixed eccentric-concentric form of left ventricular hypertrophy and increase the predisposition to congestive heart failure. To put these confounding factors together, we propose a "deadly trio" of factors predisposing for hypertension—*i.e.*, cardiovascular disease, insulin resistance, and non-dipper status—as well as a deadly trio of factors which exacerbate hypertension: excess salt intake, accentuation of sympathetic nerve activity, and obesity (Fig. 1). Our recent investigation, which revealed that endothelin 1 and β2-adrenoceptor gene polymorphisms increased genetic predisposition to hypertension only in obese subjects, suggested the possibility that several gene polymorphisms may be involved in the "deadly trio" *via* modulation of the interaction between genes and the environment. In this review, we verified the effect of candidate genes involved in the deadly trio, with a special emphasis on salt sensitivity.

Table 1. Angiotensinogen Gene Polymorphism and Clinical Features in Our Previous Investigations

Clinical features	Association	References
Hypertension	positive	(*9*)
Family history of hypertension	positive	(*10*)
Coronary heart disease	positive	(*11*)
Lacunar infarction	positive	(*12*)
Type II diabetes mellitus	negative	(*13*)
Stroke	negative	(*14*)
Sarcoidosis	negative	(*15*)
Pneumonia (in elderly)	negative	(*16*)

Angiotensinogen Gene Polymorphism and Cardiovascular Disease

A strong genetic predisposition to hypertension and target organ damage appears to be correlated with African ancestry, referred to as "the African gene." Sub-Saharan Africans have endured the selective pressure of extreme heat for thousands of generations. Polymorphisms in the renin-angiotensin system may predispose them to hypertension and related disorders because they confer genetic advantage for survival when resources are scarce, but increase the prevalence of lifestyle-related diseases, such as hypertension, when resources are prevalent and overconsumption rampant (*8*). Our previous investigations revealed that several clinical features that are mainly related to hypertension are associated with genetic variants of the angiotensinogen gene (*AGT*) (Table 1) (*9–16*). The *T235* allele in exon 2 or *C+31* allele in exon 1 of *AGT* has been associated with increased risk for hypertension (*9*), positive family history of hypertension (*10*), coronary heart disease (*11*) and lacunar infarction (*12*).

To elucidate the detailed relation between blood pressure and *AGT* polymorphism, we recently assessed the genetic involvement of the *T+31C* polymorphism on circadian rhythm of blood pressure variation in the Ohasama Study (*17*). After gaining approval from the ethical committees of Tohoku and Osaka University, we recruited 802 subjects aged 40 years or older from a rural Japanese community; all of them gave their informed written consent for monitoring of their ambulatory blood pressure and genetic analysis. Although there was no significant difference in 24-h and daytime ambulatory blood pressure values, the nighttime blood pressure was significantly lower in the subjects with the *TT* genotype of *AGT/T+31C*, resulting in a greater decline of

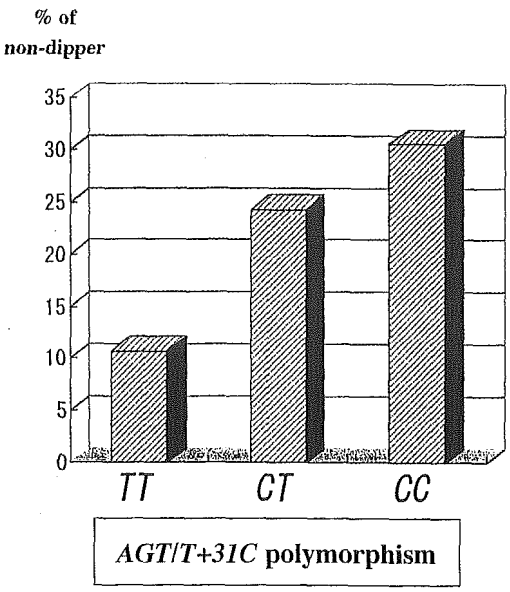

Fig. 2. *Prevalence of non-dipper status and* AGT/T+31C *polymorphism. Non-dippers were defined as those with a nocturnal decline <10%. There was a significant difference (*p*=0.024) among genotypes of the* AGT/T+31C *polymorphism.*

nocturnal systolic ($p=0.090$) and diastolic ($p=0.025$) blood pressure in subjects with *TT*. The prevalence of non-dipper status was significantly higher in the order of *CC*> *CT*>*TT* (Fig. 2). Even though we did not directly examine the volume of sodium intake and excretion in the Ohasama Study, the positive association between *AGT* polymorphism and non-dipper (*17*) or lacunar infarction (*12*) suggests that angiotensinogen might be involved in the pathogenesis of salt-sensitive hypertension.

α-Adducin Gene Polymorphism and Low Renin Hypertension in Japanese

Two membrane skeleton heterodimetric proteins, α- and β-adducin, promote the spectrin-actin interaction. Genetic analysis of the Milan hypertensive strain of rats (MHS), a model of salt-sensitive and renal hypertension, revealed that a mutation at position 316 of the rat adducin gene accounts for up to 50% of the blood pressure difference between the MHS and Milan normotensive strain of rats (MNS), and that a polymorphism of β-adducin only modulates the effect of the α-unit (*18*). In a study on humans, Cusi *et al.* identified an amino-acid substitution (*Gly460Trp*) in the human α-adducing gene (*ADD1*) and showed a significant association between the *Trp460* allele of *ADD1* and hypertension ($p=0.0003$) in Italian and French populations (*19*). The human *Trp460* allele may be considered a putative hypertension-favoring allele because it may affect blood pressure by increasing renal tubular reabsorption through the activation of Na,K-ATPase. Basal plasma renin activity (PRA) was lower and the decline in blood pressure after diuretic therapy was more pronounced ($p<0.01$) in hypertensives with the *Trp460* allele than in those with the *Gly460* allele homozygote (*20*). To examine the precise association between *Gly460Trp* polymorphism of *ADD1* and blood pressure, we carried out an association study using the Ohasama Study data. The baseline characteristics (age, body mass index, systolic blood pressure, diastolic blood pressure, frequency of antihypertensive medication use, prevalence of hypertension, *etc.*) of all subjects did not differ significantly among the various genotypes of the *Gly460Trp* polymorphism of *ADD1*. However, in the younger subjects (age<60 years) with a low level of PRA (<1.0 ng/ml/h), the ambulatory blood pressure (ABP) and home blood pressure (HBP) were significantly higher in the carriers with the *Trp460* allele than in those with the *Gly460* allele homozygote (*21*). Our findings suggested that the *Gly460Trp* polymorphism of *ADD1* may be associated with lower renin hypertension. Because younger subjects have less chance to be exposed to environmental factors than older ones, genetic factors have a relatively greater influence on younger subjects than older ones. Since PRA decreases with age, elderly subjects with low renin activities are quite common, and the positive association between low renin hypertension and the *Trp460* allele suggests that *ADD1* is also genetically involved in the salt-sensitive hypertension in Japanese. In support of this hypothesis, Williams and coworkers reported that the *Trp460* allele of *ADD1* is associated with hypertension in the low renin state (*22*).

Aldosterone Synthase Gene Polymorphism in Japanese

The aldosterone synthase gene (*CYP11B2*), which is located on 8q21 and encodes steroid 18-hydroxylase, is strikingly different from that of the *CYP11B1*, although the sequences of their exons are 93% identical. *CYP11B2*, which plays a critical role in the biosynthesis of aldosterone, is expressed in both adrenal fasciculata and glomerulosa. There is a polymorphism in the 5′-flanking region of the *CYP11B2*, *T(−344)C*, which has been reported to be associated with hypertension and plasma aldosterone levels in a Caucasian population (*23*). We examined the genetic involvement of *T(−344)C* polymorphism of *CYP11B2* in two large general populations in Japan, those of the Ohasama Study (*24*) and the Suita Study (*25*). In the Ohasama Study, the frequencies of the *CC*, *CT*, and *TT* genotypes were 0.14, 0.44, and 0.42, and the frequency of the *T(−344)* allele (0.64) was higher than that in Caucasians. Although there was no significant difference in 24-h ambulatory blood pressure levels among the genotypes, the nocturnal blood pressure decline was significantly greater in the *CC* homozygous subjects than in the other subjects ($p=0.0065$ for systolic and $p=0.031$ for diastolic decline in nocturnal blood pressure), suggesting that

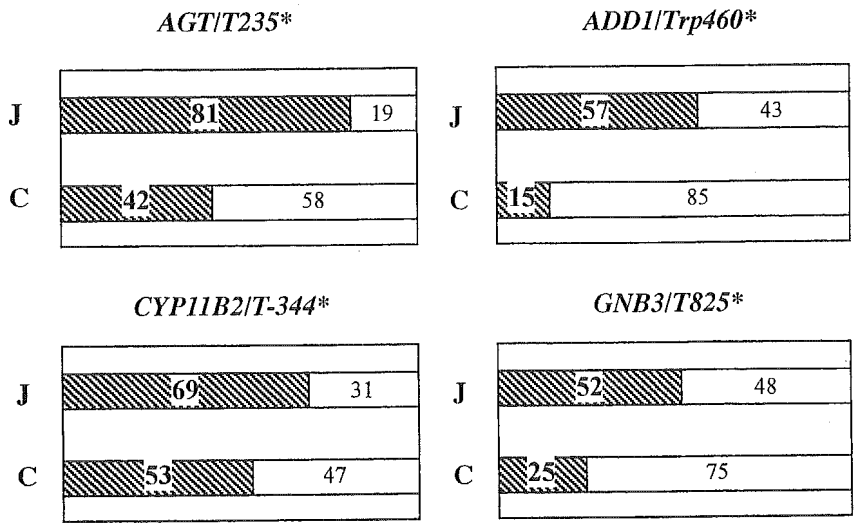

Fig. 3. *High frequency of the salt-sensitive allele in Japanese. Numbers indicate the frequency (%) of the major (left, bold) and minor (right, plain) allele. J, Japanese; C, Caucasians.* * $p<0.001$. *The significant difference in genotype frequency between Japanese and Caucasians was examined by* χ^2 *analysis. The number of examined subjects are shown below.* AGT/T235*:* T235 *allele of* M235T *polymorphism of the angiotensinogen gene. J:* n=*4,013; C:* n=*10,720.* ADD1/Trp460*:* Trp460 *allele of* Gly460Trp *polymorphism of the α-adducin gene. J:* n=*1,490; C:* n=*799.* CYP11B2/T−344*:* T(−344) *allele of* T(−344)C *polymorphism of the aldosterone synthase gene. J:* n=*4,049; C:* n=*673.* GNB3/T825*:* T825 *allele of* C825T *polymorphism of the G-protein β3 subunit gene. J:* n=*762; C:* n=*853.*

non-dipper status is significantly more prevalent in *T(−344)* allele carriers. The prevalence of previous cardiovascular disease was significantly higher in *T(−344)* allele carriers than in the subjects with the *CC* genotype, although age, body mass index, gender, smoking, use of alcohol, and antihypertensive medication did not differ among the three genotypes. In our trial using the Suita Study data, we compared the *T(−344)*C genotype distribution between hypertensive subjects (n=1,535) and normotensive subjects (n=2,514). There was no significant difference between hypertensive and normotensive subjects in either men (frequency of *C(−344)* allele: 0.30 *vs.* 0.31, p=0.48) or women (0.32 *vs.* 0.32, p=0.93), but the frequency of the *T(−344)* allele in the Suita Study was also significantly higher than in Caucasians. Thus the data obtained from two large Japanese general populations suggested that the *T(−344)* allele of *CYP11B2* is frequent among Japanese individuals and increases the prevalence of non-dipper status and previous cardiovascular disease.

High Frequency of Salt-Sensitive Alleles in Japanese

In addition to *AGT*, *ADD1*, and *CYP11B2*, the gene encoding the G-protein β 3 subunit, *GNB3*, is also a candidate for genetic risk for salt sensitive hypertension, and its polymorphism, *C825T*, has been associated with enhanced G-protein activation and Na^+–H^+ exchanger activity in cells from hypertensive patients. After attaining approval from the ethical committees of Tohoku and Osaka University, we examined the association between *GNB3/C825T* and blood pressure and other parameters in the Ohasama Study and the Handai Study, which consists of 762 cases and controls from outpatients at the Osaka University Medical School. Though the *GNB3* genotype distribution did not differ significantly between normotensives and hypertensives in either of the two studies, the frequency of the *T825* allele of *GNB3* was significantly higher in Japanese (0.52) (*26*) than that in Caucasians (0.25) (*27*).

Following these results, we compared the allele frequency of five candidate genes for salt-sensitive hypertension between Japanese and Caucasians. The *AGT/T235* (*10*, *28*), *ADD1/Trp460* (*19*, *21*), *CYP11B2/T(−344)* (*23*, *25*), and *GNB3/T825* (*26*, *27*) alleles were significantly higher in Japanese than in Caucasians (Fig. 3). None of these alleles were directly associated with an increased risk for hypertension, but collateral evidence obtained from our previous results suggested that the high salt sensitivity in Japanese might be attributed to the higher frequency of one or more of these alleles.

In conclusion, *AGT/T235*, *ADD1/Trp460*, *CYP11B2/T(−344)*, and *GNB3/T825*, which are candidate gene polymorphisms responsible for salt-sensitive hypertension, are significantly more frequent in Japanese than in Caucasians. Even though the direct effect of these polymorphisms on blood pressure is not strong, their genetic analysis might be useful for estimating their impact on salt sensitivity in various subgroups of essential hypertension, such as subjects with low PRA, non-dipper-type circadian blood pressure variation, or lacunar infarction. Because Japan has a geneti-

cally salt-sensitive constitution, low sodium diets should be encouraged as a means of decreasing the prevalence of hypertension and thereby preventing cardiovascular disease.

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ORIGINAL ARTICLE

Genetic risk factors for cerebral infarction using data from a large-scale genetic epidemiological study: the Ohasama Study

Tomohiro Katsuya,[1] Ken Sugimoto,[1] Atsushi Hozawa,[2] Takayoshi Ohkubo,[2] Koichi Yamamoto,[1] Akiko Matsuo,[1] Kazuhiko Ishikawa,[1] Mitsunobu Matsubara,[3] Hiromi Rakugi,[1] Ichiro Tsuji,[2] Yutaka Imai[3] and Toshio Ogihara[1]

[1]Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, [2]Public Health and [3]Department of Clinical Pharmacology and Therapeutics, Tohoku University Graduate School of Medicine and Pharmaceutical Science, Sendai, Miyagi, Japan

Background: With the imminent advent of an extremely aged society, there will be an increasing requirement for the prediction, early detection and treatment of cerebral infarction. Involved in the etiological mechanisms of cerebral infarction are a number of complex genetic and environmental factors related to the onset and progression of hypertension and arteriosclerosis. Elucidation of the significance of the various risk factors will require definite identification of phenotypes using large numbers of subjects.

Methods: The present study was conducted as part of a cohort study with subjects from the general population of a rural community (the Ohasama Study). Blood pressure (BP) patterns were assessed through random home-based and clinical measurements, as well as 24 h ambulatory BP monitoring. Magnetic resonance imaging of the brain was carried out in some subjects in order to detect asymptomatic cerebral infarcts, the maximum intima–media thickness was determined by carotid high-resolution ultrasonography, and cognitive function was assessed using the mini mental state examination. Correlation analysis of these parameters and the candidate hypertensive genotypes was then performed.

Results: Significant associations were seen between (i) gene polymorphisms in the renin–angiotensin system and asymptomatic cerebral infarction and the non-dipper pattern of circadian blood pressure variation; and (ii) endothelial nitric oxide synthase (eNOS) gene polymorphism and arterial pressure, lacunar score and cognitive function. An association was seen between the endothelin-1 polymorphism and hypertension, but only in obese subjects.

Conclusion: There are interactions between genes and the environment in the etiology of cerebral infarction.

Keywords: essential hypertension, genetics, genetic susceptibility, renin–angiotensin system, single nucleotide polymorphisms.

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Introduction

One of the greatest problems faced by Japan, where the aging of society is proceeding even more rapidly than in Western countries, is the increasing economic, as well as medical and welfare, cost to society of cardiovascular disease. Hypertension is a well-known risk factor for

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Correspondence: Tomohiro Katsuya, MD, PhD, 2-2 B6, Yamada-oka, Suita, Osaka 565-0871, Japan. Email: katsuya@geriat.med.osaka-u.ac.jp

cardiovascular disease, and through the introduction of effective antihypertensive medications and lifestyle interventions such as a reduction in salt intake, there has been a definite reduction in the incidence of cerebral hemorrhage and infarction. However, despite the widespread adoption of these measures, the mortality from cardiovascular diseases in the present rapidly aging society has again begun to increase, and further elucidation of the causes, as well as new approaches to prevention and treatment, of cerebral infarction will be required.

In order to elucidate the causes of essential hypertension, which accounts for more than 90% of all cases of hypertension, and the risk factors for progression to end-organ disease, we have previously performed genome screening on spontaneously hypertensive rats and also conducted analyses of genetic susceptibility to hypertension in human populations. These efforts failed to isolate what could be called a definite 'hypertensive' gene. The aims of the present study, part of the Ohasama Study, a large-scale cohort study using the general population of a rural community, are to examine the significance of both genetic factors and the traditional environmental risk factors, and also to examine the interaction between environmental and genetic factors in cardiovascular disease.

Rationale for examination of genetic susceptibility for hypertension

If we plot the frequency of blood pressure (BP) values, it will resemble the normal distribution, indicating that BP has a quantitative character and is derived from a number of different factors. The onset of hypertension is therefore not related to an abnormality of a single gene, but rather it is reasonable to assume that hypertensive patients possess several genes that tend to raise BP (i.e. hypertension susceptibility genes). The number of such genes can be extrapolated from the BP histogram of the general population, and statistical analysis suggests the existence of no less than 10 separate genes. A gene that exerts a particularly great effect on the phenotype (BP) is called a main effective gene, but there have to date been no reports of any single gene increasing BP by 10 mmHg or more. Unlike the situation in which a single mutation in one gene determines the pathology in its entirety, the role of genes in a multifactorial condition can be understood only through the interaction of a number of genetic and environmental factors. Base pair (bp) substitutions are known as gene polymorphism, a typical form being the single nucleotide polymorphisms (SNP), and are seen at a certain level in the general population in a state of equilibrium. This means that they do not confer any extreme survival disadvantage in the absence of any major environmental change, and that the influence they have on the phenotype in question is small; indeed, they may have a beneficial effect on a different phenotype. Carriers of the homozygous deletion polymorphism (*DD*) in the angiotensin-converting enzyme gene (*ACE*), for example, with a homozygous 287 bp deletion at intron 16, are known to have a high susceptibility to ischemic heart disease,[1] but at the same time many are long-lived, with a low incidence of Alzheimer's disease. We must therefore recognize that the significance of gene polymorphism can change with time (i.e. age and the passage of time) and situation (i.e. environment).

Methods

Investigation of genetic factors in the Ohasama Study

The subjects consisted of 1490 residents of Ohasama City in Iwate Prefecture, aged 40 years and over, who consented to participate in the study and undergo genetic analysis. Blood was taken from subjects, and after separation of the white cells, DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA). As well as BP measurements at the time of medical consultations (casual BP (CBP)), subjects underwent 24 h ambulatory BP monitoring (ABPM), with measurements taken every 30 min using the ABPM-630 (Colin Corporation, Japan). The mean daytime and night-time BP were then calculated. Subjects were also asked to measure their BP at home (HBP), using the HEM-401C (Omron Life Science, Kyoto, Japan), for 4 weeks within 1 h of both waking and retiring, and the mean BP for that 4 week period was calculated from these measurements. Some of the subjects underwent magnetic resonance imaging of the brain and the following indices of asymptomatic cerebral infarction were calculated: lacuna score, a count of areas 3–10 mm in diameter with low intensity on T_1-weighted images and high intensity on T_2-weighted images; and periventricular hyperintensity (PVH) grade, scoring the periventricular areas of low intensity. A selected group of subjects also underwent carotid Doppler imaging to measure the maximum intima–media thickness (max IMT), and assessment of cognitive function using the mini mental state examination (MMSE).

The TaqMan polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) methods were used to determine the *M235T* polymorphism of the angiotensinogen gene (*AGT*), the insertion/deletion (*I/D*) polymorphism of *ACE*, the *A1166C* polymorphism of the angiotensin II type 1 receptor gene (*AT1*), the *C3123A* genotype of the angiotensin II type 2 receptor gene (*AT2*), the *Gly460Trp* (*G/T*) polymorphism of the α-adducin gene (*ADD1*), the *Lys198Asn* (*G/T*) genotype of the endothelin 1 gene (*ET1*), the *Glu298Asp* polymorphism of the endothelial nitrous oxide gene (*eNOS*), and the UCSNP-*43G/A* polymorphism of the

Table 1 Association between α-adducin Gly460Trp polymorphism and hypertension in younger subjects with low renin activity (< 1.0 ng/mL per h)

ADD1 genotype	*GG* (mean ± SEM)	*GG + TT* (mean ± SEM)	*P*
24 h ABPM ($n = 90$)			
n	46	144	
Daytime BP			
Systolic BP (mmHg)	127.8 ± 2.0	131.8 ± 1.5	< 0.04
Diastolic BP (mmHg)	77.7 ± 1.3	79.7 ± 0.9	0.09
Night-time BP			
Systolic BP (mmHg)	110.5 ± 1.9	114.0 ± 1.3	< 0.05
Diastolic BP (mmHg)	65.6 ± 1.1	67.0 ± 0.8	0.17
Home BP ($n = 235$)			
n	55	180	
Systolic BP (mmHg)	121.5 ± 1.7	124.7 ± 1.2	< 0.05
Diastolic BP (mmHg)	77.0 ± 1.3	78.2 ± 0.9	0.37

ADD1, α-adducin gene; BP, blood pressure; ABPM, ambulatory BP monitoring.

calpain-10 gene (*CAPN10*). Associations between blood pressure readings and genotype were examined using ANOVA, and those between these data and genotype with the χ^2 test using contingency table analysis.

Results

The *AGT/M235T* polymorphism was significantly associated ($P < 0.05$) with the scores for the brainstem, basal ganglia and cerebral lacuna. The *AT1/A1166C* polymorphism was also associated in younger subjects with the basal ganglia and cerebral lacuna scores, and with the PVH grade. Multivariate analysis confirmed the significance of these associations, suggesting that these genotypes are independent risk factors for asymptomatic cerebral infarction.[2] Examination of the relationship between diurnal variation in BP on ABPM, and the *AGT/T + 31C*, which is in the highest linkage dysequilibrium with *M235T* polymorphism, revealed a significant tendency to a higher incidence of the non-dipper pattern, with a reduced nocturnal drop in BP, in response to an increase in the number of *C + 31* alleles (= *T235* allele).

If we limited our analyses to the elderly group, aged 65 or over, of the Ohasama subject population, we found a relative weakening of the effect of genetic factors in comparison to the younger group. No influence on BP was seen for *ADD1* polymorphism in the entire population or the elderly group but when we examined its influence in younger subjects with low renin activity, known to be closely related to salt sensitivity, we found that, as in Caucasian subjects, BP was significantly elevated in subjects with the *Trp640* allele (Table 1).[3]

We examined the relationships between the lacuna score, PVH grade and max IMT, as the risk factors for asymptomatic cerebral infarction, and each genotype. No significant associations were found, apart from the basal ganglia lacunae and border regions in the *ACE/DD* and *AT1/C1166* allele carriers. The prevalence of hypertension was associated significantly ($P < 0.015$) with the *AT1/C1166* allele but with no others, and no associations were seen between the HBP or mean BP measurements during 24 h ABPM and any polymorphisms. Because the G allele carriers of *eNOS* polymorphism increased the prevalence of hypertension, BP tended to rise, and the MMSE score decreased significantly (Fig. 1; $P < 0.006$). The effect of this polymorphism on cognitive function in the elderly is particularly of note

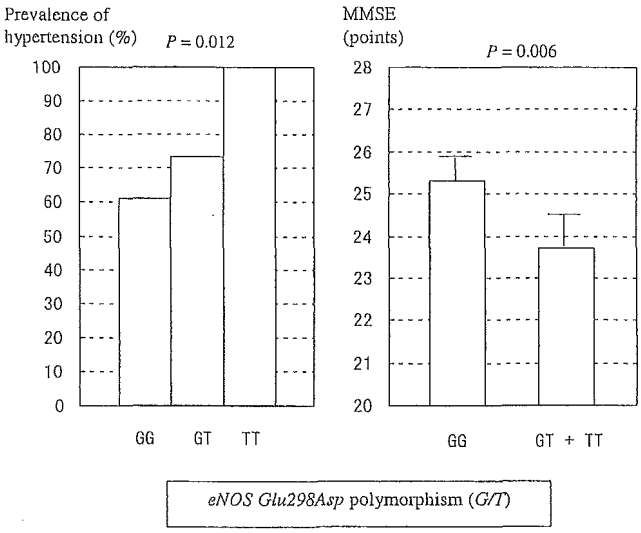

Figure 1 Association between endothelial nitric oxide synthase gene (*eNOS*) polymorphism and hypertension and cognitive function in the elderly.

with the increasing emphasis on the consequences of increased BP.

Discussion

Interactions between genes and the environment, and the advent of 'tailor-made' therapies

The 'thrifty gene', thought to aid survival in times of famine, is known to cause lifestyle illnesses such as hypertension in times of abundance. Possession of the *AGT/T235* allele, useful for retaining water and salt in the body, held a survival advantage for peoples living in the African savanna or making long ocean voyages, but for those living in cold European climates with a comfortable lifestyle, this genotype is transformed into a risk factor for cardiovascular disease.[4] It has been known for some time that the non-dipper pattern, with high nocturnal BP, is common in salt-sensitive hypertensives, and the high incidence of the non-dipper pattern in carriers with the *AGT/T235* allele has interesting implications for the production of 'tailor-made' therapies. In a large-scale cohort study conducted in the USA into the effects of 3 years of weight loss and salt restriction in normotensive subjects, the risk of developing hypertension was definitely higher in carriers with the *AGT/T235* allele, but the interventions were also more effective.[5] This indicates that the risk of developing hypertension can be effectively reduced in carriers with the *AGT/T235* allele through intensive lifestyle modification.

The results of the present study also demonstrated that, solely in overweight subjects with a body mass index (BMI) = 25 kg/m^2, the *Lys198Asn* (*G/T*) polymorphism of the *ET1* significantly increased susceptibility to hypertension,[6] which agrees with results obtained from Caucasian subjects, indicating that *ET1* polymorphism carries a risk of hypertension only in the pathological state of obesity. In other Japanese study populations, different β2 adrenergic receptor genotypes have also affected the raised BP associated with obesity and it can therefore be expected that intensive dietary advice and exercise regimens will be effective in such patients.

A person's genotype is unchanged throughout life, and if it becomes clear that certain genotypes modify the influence of environmental exposure, then it may become possible to reduce the likelihood of developing some diseases through stringent control of the relevant environment factors. These tailor-made therapies will therefore consist not only of choosing the appropriate medication for the individual patient's constitution, but will also include ancillary therapies based on the results of genetic analysis. In fact, the nationwide Millenium Project in Japan is now in progress, and the stated final aims of the Council on High Blood Pressure are (i) the discovery of at least 30 genes associated with diseases and drug responsiveness; (ii) improved therapeutic results through the selection of the most appropriate medication for the individual patient (i.e. tailor-made therapy); and (iii) development of landmark new drugs, with the aim of reducing estimated admissions for cardiovascular diseases by 20% and the requirement for treatment of stroke by 20%.

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