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H. 知的財産権の出願・登録状況

なし

別添６

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Original Article

Combined Analysis of Polymorphisms in Angiotensinogen and Adducin Genes and Their Effects on Hypertension in a Japanese Sample: The Shigaraki Study

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We examined the interactions between lifestyle and polymorphisms of salt-sensitive genes and their effects on hypertension in a general Japanese sample (The Shigaraki Study). The study group consisted of 2,902 subjects who underwent a medical examination in 1999 in Shigaraki, a suburban area in Shiga. Among 1,647 subjects not receiving antihypertensive medication, in a combined analysis of angiotensinogen (AGT) and adducin (ADD1) polymorphisms, double homozygosity of 235Thr or 460Trp was not found to be associated with hypertension. A multiple logistic regression analysis showed that age (odds ratio [OR]: 1.07, 95% confidence interval [95% CI]: 1.06–1.08), body mass index (BMI) (OR: 1.18, 95% CI: 1.13–1.23), alcohol consumption (OR: 1.39, 95% CI: 1.16–1.66), family history of hypertension (OR: 1.57, 95% CI: 1.18–2.07), and combined AGT M235T Thr/Thr and ADD1 Trp/Trp polymorphisms (OR: 1.37, 95% CI: 1.03–1.82) were associated with hypertension. However, there was no interaction between eating salty food and combined AGT and ADD1 polymorphisms. Furthermore, eating salty food was not associated with hypertension in a multivariate analysis. Therefore, a combination of the AGT and ADD1 polymorphisms appears to be associated with hypertension. However, a simple questionnaire regarding salt intake was not sufficient to confirm the relationship between salt intake and hypertension and/or salt-sensitive genes. (*Hypertens Res* 2005; 28: 645–650)

Key Words: angiotensinogen M235T polymorphism, adducin Gly460Trp polymorphism, hypertension, lifestyle

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Introduction

The pathophysiological mechanisms related to salt-sensitive essential hypertension are not completely understood. Excess salt intake is an important environmental risk factor for the predisposition to essential hypertension. Therefore, polymorphisms that might increase the formation of angiotensin II (such as the angiotensinogen [AGT] polymorphism) are relevant in the context of sodium sensitivity. The AGT M235T (the substitution of threonine [Thr] for methionine [Met] at codon 235) polymorphism is associated with an increased risk of hypertension (*1*, *2*) and has also been evaluated in relation to salt sensitivity, with controversial results (*3*, *4*). The Gly460Trp genotype of adducin (ADD1) (the substitution of tryptophan [Trp] for glycine [Gly] at codon 460) is also associated with erythrocyte sodium transport, increases in tubular sodium reabsorption, and risk for hypertension (*5–7*). One epidemiologic study showed that the ADD1 Trp/Trp genotype was associated with higher systolic blood pressure (sBP) among men with a high sodium intake (*8*).

The purpose of this study was to elucidate the relationship between AGT, ADD1, both genotypes combined, and hypertension in a general Japanese sample. Moreover, we examined the effects of salt intake and polymorphisms of salt-sensitive genes on hypertension, and we conducted a statistical analysis of the interactions between these factors after adjusting for other lifestyle factors.

Methods

Study Population

The Shigaraki Study was based on a medical examination undertaken in 1999 at Shigaraki, a farming community near Kyoto, in western Japan (*9–11*). A total of 2,902 subjects underwent the examination, of whom 2,395 were enrolled in this genetic study after receiving a full explanation and providing informed consent. Of these subjects, 748 were excluded for the following reasons: undetermined genotype, $n=41$; already taking antihypertensive agents, $n=431$; a serum GOT or GPT level of over 100 IU/l, $n=13$; and/or a history of transient ischemic attack, stroke, angina pectoris, myocardial infarction, or diabetes mellitus, $n=263$. Subjects were between the ages of 30 and 79. This study was approved by the Institutional Review Board of Shiga University of Medical Science (Nos. 11–15, 1999).

Blood Pressure (BP) and Biochemical Examinations

sBP and diastolic blood pressure (dBP) were measured twice using a standard sphygmomanometer on the right arm while the subject was seated after having rested for at least 5 min. Korotokov's first and fourth points were regarded as the sBP and dBP, respectively, and the BP was measured by a well-trained nurse. The mean of the 2 measurements from each subject was used for the data analysis. In this study, participants were considered hypertensive if they had the following BP values: sBP≥140 mmHg or dBP≥90 mmHg. The non-fasting blood glucose level was measured by the hexokinase method. Participants were considered diabetic if they had a blood glucose level of 200 mg/dl or more, or if they were already being treated for diabetes. The body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m).

Assessment of Lifestyle Factors

The patient history regarding daily alcohol intake and number of cigarettes per day was assessed by face-to-face interview (*9*, *10*). The frequency of consumption during a typical week and the alcohol intake on each occasion were determined and used to calculate the alcohol intake per week, which was then divided by 7 to obtain the average intake per day. Subjects were asked to estimate their alcohol intake based on the "gou," a traditional Japanese drinking unit corresponding to 23 g of ethanol. Drinkers were defined as those consuming more than 0.3 gou a week. The participants who reported that they preferred salty foods in a simple questionnaire were defined as those "eating salty food."

Genetic Analysis

DNA was isolated from peripheral leukocytes and the AGT and ADD1 genotypes were determined as previously reported (*12*, *13*). Both genotypes, determined by the polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method for a total of 75 random samples consisting of 25 PCR products for each genotype, were confirmed by direct sequencing. Briefly, after fractionation of the PCR-RFLP products on 1% agarose gels (Nippon Gene, Tokyo, Japan), the desired DNA bands were excised, and the DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, USA), amplified with the above 5′ primer, and analyzed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Wellesley, USA).

Statistical Analysis

The Statistical Package for Social Science (SPSS ver. 11.0J; SPSS Japan, Tokyo, Japan) was used for the statistical analysis. Student's or Welch's t-test and the Wilcoxon rank sum test (for alcohol consumption) were used for comparisons of means between two categories. For comparisons of means among three or more categories, a one-way analysis of variance or the Kruskal-Wallis test (for alcohol consumption) was used according to the distributions. The χ^2 test was used to compare proportions. Age-adjusted prevalence was calculated directly. A multiple logistic regression analysis was

Table 1. Characteristics of Study Population by AGT M235T Polymorphism in Men and Women, Shigaraki Study in 1999

Risk characteristics	Men (638)				Women (1,009)			
	Met/Met	Met/Thr	Thr/Thr	*p*-value	Met/Met	Met/Thr	Thr/Thr	*p*-value
N (1,647)	29	168	441		40	303	666	
Age (years)	55.9±14.9	56.1±16.3	56.1±15.3	0.997	53.8±16.7	52.7±15.8	53.5±15.2	0.739
BMI (kg/m^2)	22.4±3.7	22.1±3.0	22.6±2.8	0.208	21.9±3.0	22.2±3.0	22.1±3.0	0.890
sBP (mmHg)	128.0±16.9	130.6±16.4	130.2±17.5	0.760	125.0±17.3	123.2±18.5	125.2±19.9	0.324
dBP (mmHg)	77.0±11.3	78.5±12.2	78.3±11.6	0.819	76.0±10.2	73.3±11.1	74.6±11.8	0.194
Alcohol consumption (gou/day)	1.38	0.80	0.80	0.251	0.06	0.07	0.07	0.145
Eating salty food (%)	31.0	25.6	19.5	0.119	15.0	6.9	7.5	0.191

N: number of subjects. Values are means±SD. AGT, angiotensinogen; BMI, body mass index; sBP, systolic blood pressure; dBP, diastolic blood pressure. Alcohol consumption: 1 gou=23 g of ethanol.

Table 2. Characteristics of Study Population by ADD1 Gly460Trp Polymorphism in Men and Women, Shigaraki Study in 1999

Risk characteristics	Men (638)				Women (1,009)			
	Gly/Gly	Gly/Trp	Trp/Trp	*p*-value	Gly/Gly	Gly/Trp	Trp/Trp	*p*-value
N (1,647)	123	305	210		201	497	311	
Age (years)	55.4±15.9	55.3±15.7	57.6±15.1	0.240	54.7±16.6	53.3±15.0	52.2±15.5	0.187
BMI (kg/m^2)	22.3±2.5	22.5±3.0	22.5±3.0	0.673	21.6±2.8	22.3±3.0	22.1±3.0	0.031
sBP (mmHg)	130.6±17.0	129.6±17.0	130.8±17.6	0.726	124.7±20.2	124.8±19.6	124.3±18.6	0.951
dBP (mmHg)	78.3±11.8	78.0±11.6	78.6±12.0	0.836	72.8±12.1	75.0±11.6	74.0±11.1	0.061
Alcohol consumption (gou/day)	0.77	0.87	0.79	0.390	0.06	0.08	0.07	0.352
Eating salty food (%)	24.4	22.3	19.0	0.482	8.0	8.7	5.8	0.322

N: number of subjects. Values are means±SD. BMI, body mass index; ADD1, adducin; sBP, systolic blood pressure; dBP, diastolic blood pressure. Alcohol consumption: 1 gou=23 g of ethanol.

used to clarify the contribution of each independent variable to hypertension. In this analysis, hypertension was regarded as a dependent variable, and each genotype and other factors were regarded as independent variables. The significance of the interaction of eating salty food with AGT and ADD1 genotypes was also tested using an interaction term in this model. All confidence intervals were estimated at the 95% level.

Results

Table 1 shows the characteristics of the study population according to the AGT M235T polymorphism. The frequencies of AGT genotypes Met/Met, Met/Thr, and Thr/Thr were 4.2%, 28.6%, and 67.2%, respectively. No significant differences were observed among the Met/Met, Met/Thr, and Thr/Thr groups with respect to age, BMI, sBP, dBP, alcohol consumption, and the habit of eating relatively more salty food, in comparison to the reported salt intake of other subjects. Table 2 shows the characteristics of the study population according to the ADD1 Gly460Trp polymorphism. The frequencies of ADD1 genotypes Gly/Gly, Gly/Trp, and Trp/Trp were 19.7%, 48.7%, and 31.6%, respectively. Results similar to those given above were obtained. In all, no significant differences were observed among the Gly/Gly, Gly/Trp, and Trp/Trp groups in terms of hypertension.

Table 3 shows the characteristics of the combined AGT and ADD1 polymorphism analysis, AGT M235T Thr/Thr and ADD1 Trp/Trp *vs.* other polymorphisms. There was a significant association between the combined genotypes AGT Thr/Thr and ADD1 Trp/Trp in men and hypertension ($p=0.035$). However, the statistical significance disappeared when we adjusted for age, although the magnitude of the percentage remained almost the same. After adjustments for age, BMI, alcohol consumption, eating salty food, family history of hypertension, and number of cigarettes per day were made, the multivariate prevalence odds ratio and 95% confidence interval (CI) of combined AGT and ADD1 polymorphisms for hypertension were, respectively, 1.33 and 0.88–2.02 for men, and 1.41 and 0.95–2.01 for women. The combined AGT and ADD1 polymorphisms were positively associated with hypertension in both men and women, with an odds ratio of almost the same magnitude; however, the association did not reach a level of statistical significance.

Table 4 shows the multivariate adjusted odds ratios of combined AGT and ADD1 polymorphisms for hypertension when the data for men and women were combined. Multiple

Table 3. Characteristics of Study Population by Combined AGT and ADD1 Polymorphisms in Men and Women, Shigaraki Study in 1999 (AGT M235T Thr/Thr and ADD1 Trp/Trp *vs.* Others)

Risk characteristics	Men (638)			Women (1,009)		
	Others	Thr/Thr and Trp/Trp	*p*-value	Others	Thr/Thr and Trp/Trp	*p*-value
N (1,647)	488	150		800	209	
Age (years)	55.3±15.8	58.6±14.6	0.026	53.4±15.5	52.7±15.4	0.549
BMI (kg/m^2)	22.4±2.9	22.8±3.0	0.189	22.1±3.0	22.1±2.9	0.971
sBP (mmHg)	129.6±16.9	132.0±18.0	0.140	124.3±19.3	125.8±19.7	0.335
dBP (mmHg)	77.9±11.7	79.6±11.9	0.125	74.2±11.6	74.7±11.5	0.598
Alcohol consumption (gou/day)	0.83	0.81	0.723	0.07	0.07	0.144
Family history of hypertension (%)	24.4	18.0	0.104	29.9	30.6	0.834
Eating salty food (%)	22.7	18.0	0.217	8.1	5.7	0.248
Hypertension (%)	28.9	38.0	0.035	23.4	27.3	0.241
Hypertension (%)†	28.9	35.9	0.127	23.4	28.1	0.125

Others: AGT M235T polymorphism, Met/Met and Met/Thr; and ADD1 Gly460Trp polymorphism, Gly/Gly and Gly/Trp. Other abbreviations are listed in Tables 1 and 2. †Age-adjusted prevalence was calculated by the direct method using the "others" group as the standard population.

Table 4. Multivariate Adjusted Relative Odds Ratios and 95% Confidence Intervals (CIs) of Combined Genetic AGT M235T Thr/Thr (TT) and ADD1 Trp/Trp (TT) Polymorphisms for Hypertension (*N*=1,647)

Risk characteristics	Odds ratio (95% CI)	*p*-values
AGT T/T and ADD1 T/T (both TT=1, others=0)	1.37 (1.03–1.82)	0.031
Age (years)	1.07 (1.06–1.08)	<0.001
BMI (kg/m^2)	1.18 (1.13–1.23)	<0.001
Alcohol consumption (gou/day)	1.39 (1.16–1.66)	<0.001
Eating salty food (yes=1, no=0)	1.25 (0.88–1.77)	0.218
Family history of hypertension	1.57 (1.18–2.07)	0.002
Smoking (number of cigarettes/day)	1.00 (0.99–1.01)	0.481
Sex (men=0, women=1)	1.09 (0.81–1.47)	0.481

Abbreviations are listed in Tables 1 and 2.

logistic regression analysis adjusting for age, BMI, alcohol consumption, eating salty food, family history of hypertension, number of cigarettes per day and sex showed that age, BMI, alcohol consumption, family history of hypertension, and combined AGT and ADD1 polymorphisms were associated with hypertension. However, there was no correlation between eating salty food and hypertension. In addition, there was no interaction between eating salty food and the AGT M235T Thr/Thr plus ADD1 Trp/Trp polymorphism (p=0.829).

Discussion

A number of genes have been tested for an association with hypertension, with controversial results. Salt sensitivity is possibly genetically determined. Salt-sensitive individuals tend to more frequently have a familial history of hypertension than do salt-resistant subjects, and there is a familial resemblance in the response of BP to sodium restriction (*14*, *15*). Such findings suggest the existence of genetic determinants that influence the sensitivity of BP to salt. Hunt and co-workers speculated that the AGT genotype affects BP in response to sodium and the development of hypertension. A greater reduction in BP following a reduction in sodium has been reported in subjects with the Thr/Thr genotype than in those with the Met/Met genotype (*16*). Similarly, Beeks and co-workers reported that the 460Trp variant of the ADD1 polymorphism is probably associated with a salt-sensitive form of hypertension (*17*). However, studies of African Americans, who are believed to have a higher prevalence of salt-sensitive hypertension, have not revealed any association between the ADD1 polymorphism and hypertension (*18*, *19*). These discrepancies may be difficult to reconcile. One possible explanation is that essential hypertension is a complex syndrome determined by both genetic and environmental factors. It is possible that the polymorphism of a single gene

exerts only a small effect on the development of hypertension, and this may be masked by differences in genetic phenotypes or environmental factors such as BMI, salt intake, and alcohol consumption (*9–11*). In the Ohasama study (*13*), the Gly460Trp polymorphism of ADD1 was associated with ambulatory BP and home BP, but not casual BP. Casual BP usually does not reflect basal BP, being influenced by physical or psychological stress and environmental factors. However, in the present study, we found an association between the AGT M235T Thr/Thr plus ADD1 Trp/Trp polymorphism and hypertension after adjustment for possible confounding lifestyle factors, which indicates the importance of clarifying the combined effects of certain candidate genes on hypertension. Here, we suggest that a combined genetic analysis for demonstrating the presence of both AGT and ADD1 polymorphisms is a good marker for hypertension, as defined by the casual BP. Therefore, we concluded that the accumulation of genetic risk factors increases the frequency of hypertension, irrespective of exposure to environmental risk factors for hypertension.

There were several limitations to the present study. First, we did not examine other candidate genes that might be associated with hypertension (*20, 21*). Second, the simple questionnaire regarding salt intake used in the present study did not reflect the actual salt intake of each participant. Instead, it might have been more suitable for the purposes of the present study to use a 24-h urinary sodium excretion test, or some other formula to estimate 24-h urinary sodium excretion based on spot urine samples (*22*).

In conclusion, as regards heredity, double homozygosity of 235Thr or 460Trp might be associated with essential hypertension. However, in the present study, no interaction between these genotypes and salt intake could be determined based on the results of a simple questionnaire. In addition, further investigation will need to be carried out using a large-scale sample.

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Original Article

Association of Dopamine β-Hydroxylase Polymorphism with Hypertension through Interaction with Fasting Plasma Glucose in Japanese

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Dopamine-β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine and is released from sympathetic neurons into the circulation. Several lines of evidence, including the finding of elevated plasma DBH activity in essential hypertension, suggest an important role of DBH in hypertension. Recently, a novel polymorphism (−1021C/T) in the 5′ flanking region of the DBH gene has been shown to account for 35–52% of the variation in plasma DBH activity. We therefore investigated the possible association between the DBH −1021C/T polymorphism and hypertension in a large Japanese population. Moreover, because the development of hypertension is considered to be due at least partly to gene-environmental interactions, we also investigated the possible interactions between the DBH −1021C/T polymorphism and environmental factors. Consequently, we found a significant interaction between the DBH −1021C/T polymorphism and fasting plasma glucose (FPG) in the association with hypertension. CC homozygotes showed a steeper increase in probability of hypertension with FPG than T allele carriers. We also found a marginally significant trend suggesting the presence of an interaction between the DBH −1021C/T polymorphism and FPG in the association with blood pressure. Consistent with the presence of the interaction, we found that a 19 bp sequence containing the DBH −1021C/T polymorphism includes two palindromic non-canonical E boxes separated by 5 bps, and closely resembles the glucose response element of the L-type pyruvate kinase gene. These findings could be helpful in conducting further molecular and biological studies on the relationship among glucose metabolism, the sympathetic nervous system, and hypertension. (*Hypertens Res* 2005; 28: 215–221)

Key Words: **dopamine-β-hydroxylase, essential hypertension, genetics, polymorphism, glucose**

Introduction

Hypertension is considered to be a complex trait to which genetic, environmental, and demographic factors contribute interactively (*1–5*). Dopamine-β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine and is released from sympathetic neurons into the circulation. Because the sympathetic nervous system is intimately involved in both the origin and the perpetuation of a hypertensive state (*6, 7*), DBH may play an important role in the pathogenesis of essential hypertension. Indeed, neonates with DBH deficiency show episodic hypotension (*8*). DBH activity, derived largely from sympathetic nerves, can be measured

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Table 1. Characteristics of Participants According to Hypertension Status

Variable	Normotensive (n=547)	Hypertensive (n=275)
Sex (male %)	78.8	89.1
Age (years)	52.7±8.6	57.3±8.5
Body mass index (kg/m^2)	22.6±2.8	23.8±2.9
SBP (mmHg)	112.6±10.7	143.2±17.4
DBP (mmHg)	72.0±9.1	89.1±9.9
Total cholesterol (mg/dl)	198.0±30.6	202.4±37.2
HDL cholesterol (mg/dl)	54.2±14.5	51.9±14.0
Triglyceride (mg/dl)	116.7±81.7	150.9±127.7
Fasting plasma glucose (mg/dl)	101.2±17.3	106.0±19.2

Data are mean±SD. Blood pressure readings before the start of antihypertensive medication were not available for 118 hypertensive subjects whose values were measured under treatment. SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein.

in human plasma (*9*, *10*), and elevated plasma DBH activity has also been shown in essential hypertension (*11*, *12*), although the conclusions have not been completely consistent (*13*). Moreover, DBH inhibitors have been shown to produce a dose-dependent decrease in mean arterial blood pressure (*14*, *15*).

The DBH gene, approximately 23 kb in length, is composed of 12 exons (*16*). Recently, a novel polymorphism (−1021C/T) in the 5′ flanking region of the DBH gene has been shown to account for 35–52% of the variation in plasma DBH activity in several ethnically different populations, including Japanese (*17*). The strong association of the DBH −1021C/T polymorphism with plasma DBH activity has also been replicated in a native Western European population (*18*). Thus, considering several lines of evidence for the relation between DBH and blood pressure, the DBH −1021C/T polymorphism appears to be an attractive candidate variable contributing to hypertension. Nevertheless, there have been few reports investigating the possible association between the DBH gene and hypertension. We therefore investigated the possible association between the DBH −1021C/T polymorphism and hypertension. Moreover, because the development of hypertension is considered to be due at least partly to gene-environmental interactions, we also investigated the possible interactions between the DBH −1021C/T polymorphism and environmental factors.

Methods

Subjects

According to the criteria described below, 275 hypertensive subjects and 547 normotensive subjects were selected from a population in the Hyogo region of Japan (Table 1) (*19*). All subjects were Japanese urban residents. They had participated in a medical check-up, and the mean values of variables in their personal health records were used in the analyses. All subjects gave their informed consent. The ethics committee of Ehime University approved the study.

Diagnostic Categories

Each subject was assigned to one of the blood pressure diagnostic categories defined by the following criteria. Hypertensive subjects had a previous diagnosis of hypertension and were being treated with antihypertensive medication, or their systolic/diastolic blood pressure (SBP/DBP) was ≥140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was <140/90 mmHg.

Subjects were considered to have impaired fasting glycemia (IFG) if their fasting plasma glucose (FPG) concentration was ≥110 mg/dl. Subjects were considered to have diabetes mellitus (DM) if their FPG was ≥126 mg/dl.

DNA Analysis

The TaqMan chemical method, which is an established and frequently used method (*20–23*), was used to detect the DBH −1021C/T polymorphism. The forward primer was 5′-GGATCAAGCAGAATGTCCTGAAG-3′, the reverse primer was 5′-GGCACCTCTCCCTCCTGTC-3′, the T-allele specific probe was 5′-Fam-CTCTCCCACAAGTAGA-MGB-3′, and the C-allele specific probe was 5′-Vic-CTC CCGCAAGTAGA-MGB-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated.

Statistical Methods

Statistical analysis was performed with SPSS statistical software. Comparisons of categorical variables were performed using the χ^2 test. Analysis of variance was used to assess differences in means and variances of continuous variables. Logarithmically transformed plasma triglyceride (TG) and FPG values were used in the analysis. Logistic regression models were used to assess whether the DBH −1021C/T polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the polymorphism and confounding factors. General linear regression models were used to assess whether the DBH −1021C/T polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and confounding factors. p values less than 0.05 were considered statistically significant.

Table 2. DBH Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Genotype frequency		*p* value	OR	95% CI
	Normotensive	Hypertensive			
DBH genotypes					
CC (%)	378 (69.1)	184 (66.9)			
CT (%)	153 (28.0)	86 (31.3)			
TT (%)	16 (2.9)	5 (1.8)	0.52*	0.90*	0.66–1.23*
DBH alleles					
C (%)	907 (83.1)	454 (82.5)			
T (%)	185 (16.9)	96 (17.5)	0.78	0.96	0.73–1.26

*p value, OR and 95% CI are for CC *vs.* CT+TT. DBH, dopamine-β-hydroxylase; OR, odds ratio; CI, confidence interval.

Table 3. Logistic Regression Model of FPG in the Association with Hypertension According to DBH Genotype

Genotype	Coefficient	Constant	*p* value for regression	OR	95% CI	*p* value for interaction
CC	3.12	−15.14	5.4×10^{-6}	22.59	5.90–86.55	
CT+TT	0.20	−1.53	0.82	1.22	0.22–6.78	0.0086

DBH, dopamine-β-hydroxylase; FPG, fasting plasma glucose; OR, odds ratio; CI, confidence interval.

Results

Association of DBH −1021C/T Polymorphism with Hypertension

A total of 822 Japanese individuals from the Hyogo region were categorized as hypertensive or normotensive and genotyped for the DBH −1021C/T polymorphism (Tables 1 and 2). The relative frequencies of the CC, CT and TT genotypes were 68%, 29% and 3%, respectively. The allele frequencies were 83% and 17% for the C and T alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium ($p>0.25$). Because of the relatively small number of subjects with the TT genotype, we analyzed differences between subjects with the CC genotype and those with the CT and TT genotypes. Statistical analysis failed to show a significant difference in the frequencies of the alleles ($p=0.52$) and genotypes ($p=0.78$ for CC *vs.* CT+TT) between the hypertensive and normotensive subjects (Table 2).

Interaction of DBH −1021C/T Polymorphism with FBS in the Association with Hypertension

We next analyzed possible interactions of the DBH −1021C/T polymorphism with confounding factors in the association with hypertension in logistic regression models, because the development of hypertension is attributable at least partly to gene-environmental interactions. The DBH −1021C/T polymorphism did not interact with sex, age, body mass index (BMI), plasma total cholesterol, high density lipoprotein (HDL)-cholesterol, or TG. In contrast, the DBH −1021C/T polymorphism significantly interacted with FPG ($p=0.0086$) (Table 3). The interaction was significant even after adjustment for sex and age ($p=0.014$), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG ($p=0.031$). Subjects with the CC genotype showed a steeper increase in probability of hypertension with FPG than those with the CT and TT genotypes (Fig. 1).

Because the distribution of logarithmically transformed FPG was still slightly skewed, we also examined this interaction using stratification of FPG by quartiles (first quartile <94 mg/dl, second quartile 94 to 99 mg/dl, third quartile 100 to 106 mg/dl, and fourth quartile >106 mg/dl). Consequently, the p value for the interaction was 0.014. The p value was 0.019 after adjustment for sex and age, and 0.037 after adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG. Moreover, stratified analyses showed that subjects with the CT and TT genotypes had a significantly higher probability of hypertension than those with the CC genotype in the first quartile (FPG <94 mg/dl) ($p=0.0056$; OR=2.58, 95% CI=1.32–5.05, where OR indicates odds ratio and 95% CI indicates 95% confidence interval).

Interaction of DBH −1021C/T Polymorphism with FBS in the Association with Blood Pressure

We next analyzed possible interactions of the DBH −1021C/T polymorphism with FPG in the association with blood pressure in general linear models. Analysis only of subjects not on current antihypertensive treatment showed that the DBH −1021C/T polymorphism significantly interacted with FPG ($p=0.045$) in the association with DBP (Table 4). The p value was 0.056 after adjustment for sex and age, and 0.055 after

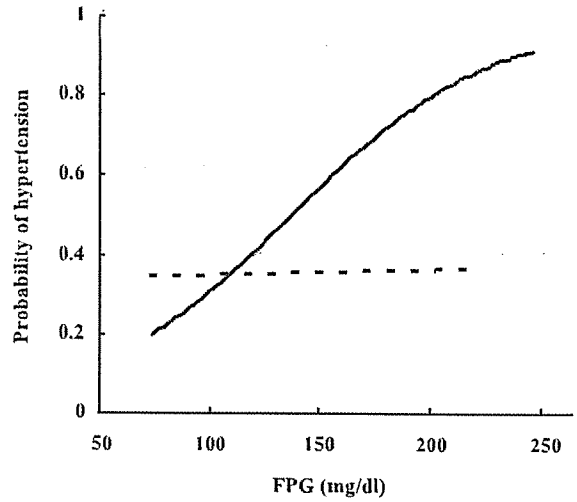

Fig. 1. *Genotype-specific regression slopes of hypertension on FPG. The simple line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and the probability of having hypertension in subjects with the CC genotype was represented by the equation:* $y = exp(0.02241x - 3.028)/\{1 + exp(0.02241x - 3.028)\}$. *The equation was:* $y = exp(0.00064x - 0.685)/\{1 + exp(0.00064x - 0.685)\}$; *in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes* ($p = 0.0086$).

adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG. Subjects with the CC genotype showed a steeper increase in blood pressure levels with FPG than those with the CT and TT genotypes (Fig. 2b). A similar trend of interaction was shown in the association with SBP ($p = 0.057$) (Table 4 and Fig. 2a). The p value was 0.092 after adjustment for sex and age, and 0.087 after adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG.

Analyses of the interaction using stratification of FPG by quartiles (first quartile <94 mg/dl, second quartile 94 to 98 mg/dl, third quartile 99 to 106 mg/dl, and fourth quartile >106 mg/dl) showed that the p value for the interaction was 0.089 for SBP and 0.025 for DBP. The p value was 0.091 for SBP and 0.033 for DBP after adjustment for sex and age. The p value was 0.10 for SBP and 0.035 for DBP after adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG.

Discussion

The present study provided evidence for the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension in a large Japanese population. There was also a marginally significant trend suggesting the presence of an interaction between the DBH −1021C/T polymorphism and FPG in the association with blood pressure. This lack of significance was possibly due to the unstable nature of blood pressure (*19*). In addition, the inclusion or exclusion of subjects who were receiving antihypertensive treatment influenced the distribution of blood pressure, and blood pressure readings before the start of antihypertensive medication were not available for 118 hypertensive subjects in our population.

In theory, the DBH −1021C/T polymorphism might be associated with hypertension, because this polymorphism is associated with plasma DBH activity (*17*, *18*) and plasma DBH activity is associated with hypertension (*11*, *12*). However, in practice, the present study failed to show a significant association between the DBH −1021C/T polymorphism and hypertension. This failure was possibly due to the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension. However, evidence for this possibility is insufficient, because data on plasma DBH activity were not available in our population. In addition, the previous reports showing that the DBH −1021C/T polymorphism is associated with plasma DBH activity did not analyze the interaction between the DBH −1021C/T polymorphism and FPG in the association with plasma DBH activity (*17*, *18*).

Supporting the interaction between the DBH gene and FPG, there is biological evidence showing that glucose and other sugars induce an increase of DBH (*24*). Indeed, rats with experimental diabetes have increased plasma DBH activity (*25*). Thus, the most important physiological influence on plasma DBH activity is considered to be the plasma glucose level (*26*). In addition, DBH-containing neurons in the hindbrain that innervate the hypothalamus have been implicated in the feeding response to glucose deprivation (*27*). In humans, the difference in sympathetic response to glucose ingestion related to family history of hypertension suggests the existence of genetic factors influencing the sympathetic response to glucose ingestion (*28*). The DBH gene may be one such genetic factor.

The precise mechanism of the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension remains elusive; a simple explanation may be that the CC genotype or a genotype in linkage disequilibrium with it might produce a controlled amount of DBH in association with the plasma glucose level, leading to increased blood pressure. In contrast, the CT and TT genotypes or genotypes in linkage disequilibrium with them might produce a constant amount of DBH irrespective of the plasma glucose level, leading to relatively stable blood pressure. This explanation may be in line with the observation in a previous study that all 19 chimpanzees were homozygous for the C allele (*29*).

Alternatively, depending on the genotype, glucose level could influence plasma insulin level, which in turn could influence blood pressure. However, the previous observation that insulin administration lowered plasma glucose level, but not plasma DBH activity, challenges this possibility (*24*). Moreover, in humans, activation of the sympathetic nervous

Table 4. General Linear Model for Regression of FPG in the Association with Blood Pressure According to DBH Genotype

BP	Genotype (*n*)	Coefficient	Constant	*p* value for regression	Determination coefficient	*p* value for interaction
SBP	CC (562)	12.1	23.5	0.00016	0.035	
	CT+TT (260)	2.9	106.7	0.75	0.00056	0.057
DBP	CC (562)	11.8	22.1	0.0034	0.021	
	CT+TT (260)	−3.1	91.0	0.65	0.0011	0.045

FPG, fasting plasma glucose; DBH, dopamine-β-hydroxylase; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

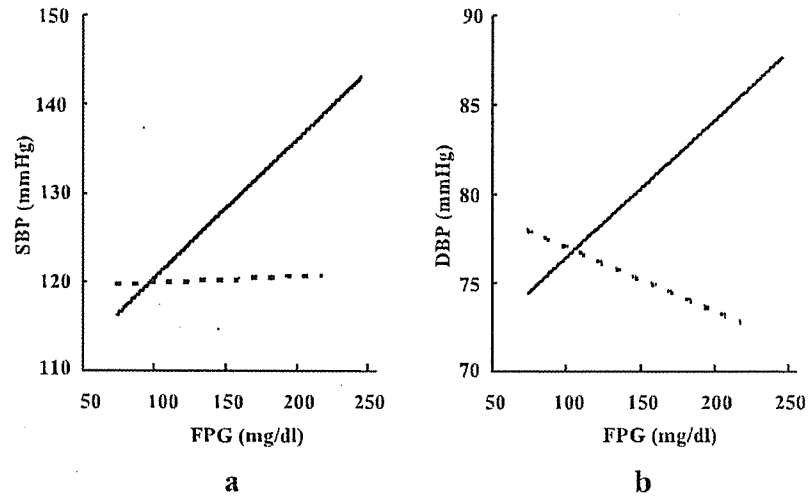

Fig. 2. *Genotypic variations in the relationship between FPG and blood pressure. a: The simple line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and SBP in subjects with the CC genotype was represented by the equation:* $y = 0.1558x + 104.71$. *The equation was:* $y = 0.0071x + 119.15$; *in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes* ($p = 0.057$). *b: The simple line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and DBP in subjects with the CC genotype was represented by the equation:* $y = 0.16x - 4.53$. *The equation was:* $y = 0.22x - 6.10$; *in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes* ($p = 0.045$).

system is related to plasma glucose level but not hyperinsulinemia or insulin hypersecretion in essential hypertension (*30*). However, because the etiology of hypertension, the effects of glucose, and the regulation of the sympathetic nervous system are all complicated, the above explanation remains completely speculative. Epidemiological studies in large populations with information on plasma DBH activity and plasma insulin level as well as biological studies could test this hypothesis.

With respect to the possible functionality of the DBH −1021C/T polymorphism, transient-transfection assays of the reporter gene construct in human neuroblastoma cell lines designed to assess whether this polymorphism directly alters transcriptional activation of the DBH gene have been negative to date (*31*, *32*). In this context, we found that a 19 bp sequence containing the DBH −1021C/T polymorphism (CCCTCAGTCTACTTGYGGG, where Y indicates the C/T polymorphism) includes two palindromic non-canonical E boxes separated by 5 bps, and closely resembles the glucose response element of the L-type pyruvate kinase gene (*33*). The DBH −1021C/T polymorphism resides in a critical 6-bp area. This suggests that the DBH −1021C/T polymorphism may alter the responsiveness to glucose, consistent with the interaction between the polymorphism and FPG, although direct molecular evidence is lacking.

In conclusion, the present study revealed a significant interaction between the DBH −1021C/T polymorphism and FPG in the pathogenesis of hypertension in a large Japanese population. This interaction was partly supported by other epidemiological and molecular biological evidence. Despite several limitations of this study, if our findings are confirmed, they could be helpful in conducting further molecular and biological studies on the relationship among glucose metabolism, the sympathetic nervous system, and hypertension.

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Resistin SNP-420 determines its monocyte mRNA and serum levels inducing type 2 diabetes

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Abstract

Resistin, secreted from adipocytes, causes insulin resistance in rodents. Its roles and main source in humans remain unknown. The G/G genotype of resistin single nucleotide polymorphism, SNP-420, induces type 2 diabetes mellitus (T2DM) by increasing promoter activity. We elucidated factors correlated with serum resistin and effects of SNP-420 on monocyte resistin mRNA. In 198 T2DM and 157 controls, fasting serum resistin was higher in T2DM. Multiple regression analysis revealed that SNP-420 genotype was the strongest determinant of serum resistin. In T2DM, 1-year duration of T2DM and 1% HbA1c was also correlated with 0.19 and 0.54 ng/ml serum resistin, respectively. Logistic regression analysis revealed that serum resistin was an independent factor for T2DM. In 23 healthy volunteers, monocyte resistin mRNA was positively correlated with its simultaneous serum levels and was higher in G/G genotype. Thus, SNP-420 determines monocyte mRNA and serum levels of resistin, which could induce T2DM.
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Keywords: Resistin; Insulin resistance; SNP; Polymorphism; Promoter; mRNA; Monocyte; Adipocyte; Type 2 diabetes; Serum level

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in insulin target tissues, namely, adipose tissue, skeletal muscle, and liver [1]. T2DM is thought to be polygenic disease, the major genetic factors of which remain to be identified [2]. Single nucleotide polymorphisms (SNPs) in intensively analyzed genes such as peroxisome proliferator-activated receptor γ (PPARγ), calpain-10, and adiponectin have been reported to be associated with T2DM [3–5]. Most recently, we found that the resistin gene promoter SNP-420 is associated with T2DM [6].

Resistin (resistance to insulin) is secreted from adipocytes and antagonizes insulin both in vitro and in vivo [7–14]. Serum levels of resistin are increased in obese diabetic mice and are reduced by PPARγ ligands, suggesting that levels of this molecule are correlated with whole body insulin resistance [7]. Transgenic mice that overexpress *resistin* in the liver have high serum resistin levels and are insulin-resistant [11]. *Resistin* (−/−) mice

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show lower fasting blood glucose levels, which are increased by resistin injection [13]. A reduction in resistin gene expression by antisense oligonucleotides improves and enhancement by adenovirus encoding resistin worsens insulin resistance [15,16]. These findings suggest that higher serum resistin levels cause insulin resistance and diabetes in rodents whereas some other studies did not agree with this role of resistin [17].

A link between *resistin* and human T2DM has not been clarified [18–26]. Most recently, we found that the G/G genotype of resistin promoter SNP-420 is associated with T2DM susceptibility [6]. A meta-analysis involving three previous reports confirms this association. Sp1 and Sp3 transcription factors specifically bind to the DNA element including −420G and enhance promoter activity. Serum resistin levels are highest in this genotype. Therefore, the specific recognition of −420G by Sp1/3 increases resistin gene promoter activity, which induces insulin resistance and human T2DM through enhanced serum resistin levels.

The issue of whether serum resistin levels are altered in human T2DM or obesity remains controversial [26–31]. Lee et al. [28] showed that serum resistin levels are not associated with T2DM or obesity. McTernan et al. [29] and Youn et al. [31] reported that serum resistin levels are elevated in T2DM, but are not associated with obesity. Fujinami et al. [30] and Azuma et al. [27] found that serum resistin levels are increased in T2DM, and in obesity, respectively. In addition to the SNP-420 genotype, other factors affecting serum resistin levels remain to be elucidated.

In humans, resistin is rarely expressed in adipose tissues and is highly expressed in monocytes or macrophages [32–34], in contrast to its dominant expression in adipose tissues in mice [7]. Since macrophages infiltrating into adipose tissues could account for insulin resistance in obese mice [35–37], the pathophysiological relevance of resistin in human monocytes or macrophages merits further investigation. Whereas Smith et al. [38] showed that resistin mRNA levels are higher in adipose tissues of obese human subjects with the SNP-420 G/G genotype, the association of its levels in monocytes with the genotype should be pursued.

In view of this, we examined which clinical parameters are associated with serum resistin levels in addition to the SNP-420 genotype. Effects of SNP-420 on resistin mRNA levels in monocytes and its relation to serum resistin levels were also assessed in healthy volunteers.

Materials and methods

Subjects. We randomly selected 198 T2DM and 157 control subjects whose fasting serum and DNA were available. All these subjects were unrelated and were native Japanese. In this study, 93 T2DM subjects were recruited from the previous study [6]. Diabetes mellitus was diagnosed based on the American Diabetes Association criteria, as reported in 1998 [39]. These 157 control subjects were chosen based on HbA1c levels of less than 5.6%, fasting plasma glucose levels of less than 110 mg/dl, no history of diabetes, and no evidence of diabetes within first degree relatives. The clinical characteristics of these subjects are given in Table 1.

To assess resistin mRNA in monocytes, another 23 healthy volunteers [11 males and 12 females, means ± SD (range), age; 24.7 ± 5.2 (21–46) years; BMI; 20.6 ± 1.5 (18.1–23.9)] were employed. They had normal glucose tolerance, as assessed by 75 g oral glucose tolerance test with no evidence of diabetes within the first degree relatives. Their routine blood tests were within normal ranges.

All subjects were informed of the purpose of the study and their consent was obtained. The study was approved by the Ethics Committee of the Ehime University Hospital and Ehime Prefectural Hospital.

SNP typing. PCR direct sequencing was performed as described previously [6,20,40]. To type SNP-420, sequences of minus strands were checked using the pr1R primer and the other strand was also sequenced, when required. Taqman analysis was also employed for typing SNP-420. The probes used were VIC 5′-CATGAAGACGG AGGCC-3′ for −420C and FAM 5′-ATGAAGAGGGAGGCC-3′ for −420G. Forward and reverse primers are 5′-CCACCTCCTGACCAG TCTCT-3′ and 5′-AGCCTTCCCACTTCCAACAG-3′, respectively.

Measurement of serum resistin levels. Serum resistin was measured using a human resistin ELISA kit (Linco Research), following the manufacturer's protocol [6]. We confirmed that linearity was maintained below 0.16 ng/ml, as described earlier [6].

Measurement of resistin mRNA levels in monocytes. Peripheral mononuclear cells were collected from whole blood samples using Lymphoprep Tube (Axis-Shield PoC AS, Oslo, Norway), and monocytes were isolated using an anti-CD14 antibody of the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) by following manufacturers' protocols. Total RNA was isolated from cell homogenates using an RNeasy Mini Kit (Qiagen Sciences, MD). Human resistin and internal control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantitated using the two-step TaqMan RT-PCR method using an ABI PRISM 7700 following the manufacturer's protocol (Perkin-Elmer Applied Biosystems, Foster City, CA). The primers and probes used were available as Assay on Demand (Perkin-Elmer Applied Biosystems). Five hundred nanograms of total RNA was incubated in 50 μl reaction mixture for the cDNA synthesis. Three microliters of the synthesized cDNA was then incubated in 25 μl of the RT-PCR mixture including 0.625 μl of either resistin or GAPDH probe. The comparative cycle of the threshold (C_T) method was used for quantitating this mRNA following the manufacturer's protocol (Perkin-Elmer). The resistin mRNA level was corrected by the GAPDH mRNA level. The corrected resistin mRNA level of undifferentiated THP-1 human monocyte cells was used as a control (defined as 1). Duplicate wells for each subject were

Table 1
Clinical characteristics of control and T2DM subjects

	Control	Type 2 diabetes
n (M/F)	157(77/80)	198(106/92)
Age (years)	62.8 ± 12.2	60.2 ± 10.8
Age of onset (years)	—	48.7 ± 11.7
Duration of diabetes (years)	—	11.2 ± 9.0
Height (cm)	155.8 ± 9.3	158.5 ± 8.6
BW (kg)	58.8 ± 12.0	60.2 ± 12.0
max BW (kg)	63.6 ± 12.1	70.1 ± 13.3
δBW(kg)	4.4 ± 4.5	9.5 ± 7.4
Age of max BW (years)	45.6 ± 17.9	44.6 ± 13.3
BMI (kg/m^2)	24.1 ± 3.4	23.9 ± 3.9
max BMI (kg/m^2)	25.9 ± 3.2	27.7 ± 4.2
δBMI (kg/m^2)	1.8 ± 1.8	3.7 ± 2.8
HbA1c (%)	5.1 ± 0.3	8.4 ± 1.8

Means ± SD are shown. BW, body weight; max BW, maximum body weight; δBW = max BW − BW; BMI, body mass index; δBMI = max BMI − BMI.