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Association of GNB3 gene with pulse pressure and clustering of risk factors for cardiovascular disease in Japanese

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Abstract

Heterotrimeric guanine nucleotide-binding proteins (G proteins) mediate many pathways including the β-adrenergic signaling pathway. The C825T polymorphism in the gene coding for the β3 subunit of G proteins (GNB3) has been shown to be associated with several phenotypes such as hypertension, obesity, and diabetes mellitus comprising the metabolic syndrome. The GNB3 C825T polymorphism may therefore be associated with many atherosclerosis-related phenotypes. On these grounds, we studied the C825T polymorphism in relation to atherosclerosis-related phenotypes in a large Japanese population. Analyses in general linear models showed that T carriers had a significantly wider pulse pressure ($P - 0.0089$) as well as a significantly higher systolic blood pressure ($P - 0.026$). In contrast, analyses in logistic regression models showed that the C825T polymorphism was not significantly associated with each of the four major classical risk factors for cardiovascular and cerebrovascular disease (obesity, hypertension, hypertriglyceridemia, and diabetes mellitus). However, a significantly higher percentage of subjects had none of the four disorders in CC homozygotes than in T carriers ($P - 0.026$). Thus, the C825T polymorphism was significantly associated with clustering of these four risk factors. Although the effect of the gene on each phenotype appears to be weak, considering the combined impact of the effects of the C825T polymorphism on risk factors, the GNB3 gene may be an important gene for human health.

Keywords: G protein; Hypertension; Atherosclerosis; Multiple risk factor clustering syndrome

Heterotrimeric guanine nucleotide-binding proteins (G proteins) couple seven transmembrane receptors to adenylyl cyclase, and mediate many pathways including the β-adrenergic signaling pathway. Each G protein is composed of three distinct subunits (α, β, and γ). Based on amino acid similarities of the α-subunits, G proteins are classified into four major classes (Gs, Gi/o, Gq/11, and G12/13) [1–3]. G proteins dissociate into the α-subunits and the β- and γ-subunits, which separately activate intracellular effector molecules. Recently, the β- and γ-subunits have been recognized as signal transduction molecules, and are now known to directly regulate as many different protein targets as the α-subunits [4].

More recently, a C825T polymorphism in the gene coding for the β3 subunit of G proteins (GNB3) was identified [5]. The 825T allele has been shown to be associated with hypertension [5–8] and obesity [9–11] in several populations, although the conclusions have been inconsistent. In the initial study, the 825T allele has been assumed to exert a dominant effect based on an observation that, stimulated by platelet-activating factor, levels of G protein activation of the TC genotype were similar to those of the TT genotype, but not to those of the CC genotype [5]. The 825T allele has also been shown to be associated with insulin resistance [12] and type 2 diabetes mellitus [13]. Thus, the 825T allele appears to be related to the metabolic syndrome, which is a complex condition composed of several disorders including hypertriglyceridemia, obesity, hypertension, and diabetes mellitus. The metabolic syndrome is a strong risk factor for cardiovascular and cerebrovascular disease. Indeed, as expected, the 825T allele has been shown to be associated with myocardial infarction [14] and clinical stroke [15]. However, association studies are

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often irreproducible. We therefore studied the association of the C825T polymorphism with several cardiovascular and cerebrovascular risk factors in a large Japanese population.

Methods

Subjects. We studied 806 Japanese subjects who participated in a medical check-up at a hospital. The values of variables in their personal health records were used in the analyses. All subjects provided their informed consent for participation in molecular-genetic studies, and the Ethics Committee of Ehime University approved the study.

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. Obesity was defined as body mass index ≥25 kg/m² (Japan Society for the Study of Obesity). Hypertriglyceridemia was defined as triglyceride ≥150 mg/dl [16]. Diabetic subjects were diagnosed according to the WHO98 definition of type 2 diabetes mellitus [17]. Subjects were considered to have diabetes mellitus if fasting plasma glucose concentration was ≥126 mg/dl.

DNA analysis. The TaqMan chemical method, which is an established and frequently used method [18–21], was used to detect the GNB3 C825T polymorphism. The forward primer was 5′-GGC AGA CCA GGA GCT GAT CT-3′, the reverse primer was 5′-TCG TCG TAG CCA GCG AAT AGT-3′, the T-allele-specific probe was 5′-Fam-ATC ACG TCT GTG GCC-MGB-3′, and the G-allele-specific probe was 5′-Vic-CAC GTC CGT GGC C-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 values were used in the analysis. Logistic regression models were used to assess whether the GNB3 C825T polymorphism made a statistically significant contribution to prediction of obesity, hypertension, hypertriglyceridemia, diabetes mellitus, and the number of disorders, with consideration of the effects of sex and age. General linear regression models were used to assess whether the C825T polymorphism made a statistically significant contribution to prediction of body mass index, blood pressure, triglyceride, total cholesterol, and fasting plasma glucose, with consideration of the effects of sex and age. P values less than 0.05 were considered statistically significant.

Results

Table 1 presents the actual values of the parameters associated with risk for cardiovascular and cerebrovascular disease as a function of the three genotypes. The relative frequencies of the TT, TC, and CC genotypes were 27%, 45%, and 29%, respectively. The allele frequencies were 49% and 51% for the T and C alleles, respectively. These results were consistent with the Hardy–Weinberg equilibrium, tested by the χ^2 test. Analysis of variance showed that the C825T polymorphism was significantly associated with SBP ($P - 0.026$) and PP ($P - 0.0089$), assuming a dominant effect of the T allele. General linear regression analysis showed that the association was significant even after adjustment for sex and age ($P - 0.050$ for SBP and $P - 0.018$ for PP).

Table 1
Clinical characteristics of subjects according to GNB3 genotype

Variable	Genotype			P value
	TT ($n = 215$)	TC ($n = 359$)	CC ($n = 232$)	
Sex (male %)	78.1	83.0	80.6	0.86
Age (years)	54.7 (8.7)	54.2 (8.7)	53.7 (9.1)	0.28
Body mass index (kg/m²)	22.9 (2.9)	23.2 (2.8)	22.7 (3.0)	0.13
Systolic blood pressure (mmHg)	126.0 (18.4)	124.8 (19.0)	122.0 (18.8)	0.026
Diastolic blood pressure (mmHg)	78.0 (11.7)	77.7 (12.0)	77.1 (13.1)	0.46
Pulse pressure (mmHg)	48.0 (13.3)	47.1 (13.0)	44.9 (10.9)	0.0089
Triglyceride (mg/dl)	131.1 (104.6)	126.3 (96.8)	119.0 (88.1)	0.12
Total cholesterol (mg/dl)	201.4 (32.1)	199.5 (33.1)	197.2 (32.9)	0.24
Fasting plasma glucose (mg/dl)	103.7 (18.9)	102.3 (17.6)	101.3 (15.0)	0.25

Data are means (SD). P values are for TT + TC vs. CC.

Table 2
Prevalence of disorders according to GNB3 genotype[a]

Disorders (%)[a]	Genotype			P value
	TT ($n = 215$)	TC ($n = 359$)	CC ($n = 232$)	
Obesity	25.6	29.5	23.7	0.21
Hypertension	35.3	33.4	30.2	0.28
Hypertriglyceridemia	46.0	39.0	34.5	0.060
Diabetes mellitus	6.5	5.6	4.3	0.36
Subjects with no disorders	30.7	32.6	40.1	0.026

P values are for TT + TC vs. CC.
[a] Disorders are obesity, hypertension, hypertriglyceridemia, and diabetes mellitus.

Table 2 shows the prevalence of four major disorders (obesity, hypertension, hypertriglyceridemia, and diabetes mellitus) correlated with the risk for cardiovascular and cerebrovascular disease according to the GNB3 genotype. Assuming a dominant effect of the T allele, logistic regression analyses showed that the C825T polymorphism was not significantly associated with any of the four disorders. However, a significantly higher percentage of subjects had none of the four disorders in CC homozygotes than in T carriers ($P - 0.026$) in a logistic regression model. Logistic regression analysis showed that the association was significant even after adjustment for sex and age ($P - 0.044$).

Discussion

The present study showed that the GNB3 C825T polymorphism was significantly associated with PP. T carriers had higher PP than CC homozygotes. This result is consistent with another result of this study that a T carrier state was significantly associated with SBP, whereas it was not significantly associated with DBP. Because the 825T allele has been shown to be associated with classical risk factors for atherosclerosis including hypertension, obesity, and diabetes mellitus [5–11,13], T carriers may tend to develop atherosclerosis. Atherosclerosis may lead to increased aortic stiffness, resulting in a wide PP as well as elevated SBP [22]. Conversely, a wide PP may be attributable to atherosclerosis. Indeed, elevated PP is increasingly being recognized as an independent risk factor for cardiovascular and cerebrovascular disease [22].

In the present study, we failed to show a significant association of the C825T polymorphism with each classical risk factor for cardiovascular and cerebrovascular disease. Consistent with this failure in our Japanese population, other studies have also failed to show a significant association of the C825T polymorphism with hypertension in Japanese populations [23,24]. The reason for this failure in Japanese is not clear. A possible reason may be the difference of environmental and genetic background between Japanese and other races. In this context, the C825T polymorphism has been shown to interact with the angiotensin I-converting enzyme (ACE) insertion/deletion (I/D) polymorphism in the association with myocardial infarction [14]. Thus, the lower frequency of DD homozygotes in Japanese than in other races could explain the failure. In addition, because, as expected, in each disorder, T carriers were consistently more frequent than CC homozygotes (Table 2), the failure could be partly attributable to lack of statistical power, suggesting that the effect of the C825T polymorphism on each classical risk factor may be weak. Consistent with this, a significantly higher percentage of subjects had none of the four disorders in CC homozygotes than in T carriers. This result is in line with the concept that the clinically manifest phenotype of each subject with genetic factors for the metabolic syndrome may depend on phenotype-specific genetic factors in each subject [25]. Similar analysis of the ACE I/D polymorphism has also shown a significant association of the I/D polymorphism with clustering of risk factors, despite a lack of association of the I/D polymorphism with each risk factor [26]. Indeed, more recently, the ACE gene has been increasingly recognized as a gene that is associated with many disorders [27,28]. Likewise, the GNB3 gene might have a similar impact on human health, because G proteins mediate many pathways and the GNB3 C825T polymorphism has been shown to be functional [5,29].

Although associations of the C825T polymorphism with classical risk factors for cardiovascular and cerebrovascular disease have been reported, the underlying mechanism remains to be explained. In this context, the T allele of the C825T polymorphism combines preferentially with the α-subunit of Gi proteins, and expression of the T allele is accompanied by enhanced sensitivity of Gi proteins to receptor activation [5,29]. Gi proteins are known to play an important role in adipogenesis. Increased expression of Gi α 2 in mouse embryo stem cells promoted their terminal differentiation to adipocytes [30]. Expression of constitutively active Gi α 2 in mouse embryonic fibroblasts stimulated adipogenesis [31]. Tumor necrosis factor α stimulated lipolysis in adipocytes by decreasing Gi protein concentration [32]. Indeed, the GNB3 825T allele was associated with impairment of the β-adrenergic control of lipolysis [33]. Thus, the association of the C825T polymorphism with obesity may be plausible. This association could result in association of the C825T polymorphism with hypertriglyceridemia, diabetes mellitus, and hypertension through insulin resistance. Indeed, adipose tissue expression of tumor necrosis factor-α induced obesity-linked insulin resistance [34].

In addition, because Gi proteins are coupled with many pathways, other mechanisms may also underlie the associations of the C825T polymorphism with hypertriglyceridemia, diabetes mellitus, and hypertension. Indeed, enhanced expression of Gi proteins has been shown to be associated with hypertension [35–38], and inactivation of enhanced expression of Gi proteins attenuated the development of hypertension [39]. With respect to hypertriglyceridemia, several nonselective β-blockers often cause hypertriglyceridemia [40,41]. In contrast, with respect to diabetes mellitus, biological evidence suggests that Gi proteins are associated with enhanced insulin action [42–44]. The apparently discrepant tendency of more frequent T carriers in diabetic subjects could have been by chance. However, the GNB3 825T allele has been shown to be associated with insulin resistance [12] and with type 2 diabetes mellitus

[13] in other association studies. Therefore, the apparent discrepancy between biological evidence and the results of association studies could imply that accumulated fat in T carriers might have stronger effects on insulin action than enhanced expression of Gi proteins in T carriers. Alternatively, gene–gene interactions might underlie the discrepancy. In this context, the GNB3 825T allele has been shown to interact with the insulin receptor substrate-1 972Arg variant in the association with type 2 diabetes mellitus [13]. Whatever the case, the combined effects of a higher percentage of T carriers in several major risk factors for atherosclerosis than CC homozygotes could explain the significantly wider PP in T carriers than in CC homozygotes in our population.

Although the effect of the gene on each phenotype may be weak, considering the combined impact of the effects of the gene, extensive investigation of the GNB3 gene in relation to various phenotypes may be required. In the present study, interaction analyses showed that the C825T polymorphism did not interact with any confounding factors shown in Table 1 (data not shown). However, the present study did not assess gene–gene interaction, which is a candidate factor modifying the evaluation of an association. In this context, interaction analyses of the GNB3 gene with other genes involved in G protein-mediated pathways may be helpful to improve the understanding of the relation between G protein-mediated pathways and cardiovascular disease.

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Original Article

Polymorphism of the Monocyte Chemoattractant Protein (MCP-1) Gene Is Associated with the Plasma Level of MCP-1 But Not with Carotid Intima-Media Thickness

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Monocyte chemoattractant protein-1 (MCP-1) plays an important role in atherosclerosis. Recently, single nucleotide polymorphisms (SNPs) in the MCP-1 regulatory region have been identified, and an *in vitro* study demonstrated that the SNP at position −2518 of the MCP-1 gene affected transcription of the gene. The purpose of this study was to clarify the association of the plasma level of MCP-1 and the SNP of the MCP-1 gene with carotid atherosclerosis in community-based subjects. The study subjects consisted of 325 community residents, aged 50 years or older (mean age, 70.5±9.4 years) and free from any cardiovascular complications. Carotid intima-media thickness (IMT) was measured in the right common carotid artery using ultrasonography. The plasma level of MCP-1 was measured by enzyme-linked immunosorbent assay (ELISA). The SNP of the MCP-1 gene was determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique. The plasma level of MCP-1 was significantly associated with IMT ($r=0.12$, $p<0.05$) and carotid arterial dimension ($r=0.13$, $p<0.05$). There was a significant difference in plasma MCP-1 level between the genotypes (AA, 166±36 ng/ml; GG+AG, 184±56 ng/ml; $p=0.028$). Analysis restricted to the subjects not receiving antihypertensive drugs or other medication further increased the statistical significance. However, carotid IMT and carotid arterial diameter were not significantly different among the MCP-1 genotypes. Stepwise regression analysis for plasma MCP-1 revealed that the MCP-1 genotype was an independent determinant of plasma MCP-1 level. These findings indicate that plasma MCP-1 is associated with carotid atherosclerosis. Although −2518 SNP is associated with the plasma level of MCP-1, it was not directly associated with carotid atherosclerosis. (*Hypertens Res* 2003; 26: 677–683)

Key Words: polymorphism, monocyte chemoattractant protein-1, carotid atherosclerosis

Introduction

There is accumulating evidence, both *in vivo* and *in vitro*, that monocyte chemoattractant protein-1 (MCP-1) plays an important role in atherosclerosis (*1*). MCP-1 shows potent chemotactic activity toward monocytes in response to inflammatory, inflammatory, and mechanical stimuli, such as balloon injury (*1–6*). Expression of MCP-1 has been demonstrated in atherosclerotic lesions in animal models and humans (*2–10*). The plasma level of MCP-1 has also been shown to increase in patients with myocardial infarction (*11, 12*), unstable angina (*13*), venous thrombosis (*14*), and Kawasaki disease (*15*). It has also been demonstrated that

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anti-MCP-1 neutralizing antibody prevents early inflammation and reduces subsequent coronary vascular medial thickening in N^{ω}-nitro-L-arginine methyl ester (L-NAME)-administered rats (*16*). These findings suggest that continuous activation of MCP-1 is involved in the progression of atherosclerosis. Nevertheless, there have been only a few studies evaluating the relationship between MCP-1 and carotid atherosclerosis in humans (*17*).

Recently, single nucleotide polymorphisms (SNPs) in the MCP-1 regulatory region, i.e., substitutions of −2518 G/A, have been identified and shown to affect transcription of the gene (*18*). It has also recently been reported that the GG genotype of the −2518 MCP-1 gene was associated with susceptibility to coronary arterial disease (*19*). Aguilar *et al.* (*20*) demonstrated an association between the presence of G at position −2518 in the MCP-1 promoter region and the presence of cutaneous vasculitis among patients with systemic lupus erythematosus. However, there has been no study evaluating the association between −2518 SNP and MCP-1 level, which may account for the process of arterial remodeling.

In the present study, the plasma level of MCP-1 and the −2518 SNP of the MCP-1 gene were determined in 325 community residents, and their relationship with the carotid intima-media thickness (IMT) thickening and carotid arterial dilation was investigated to address the following unresolved issues: 1) whether the plasma level of MCP-1 is associated with the degree of carotid atherosclerosis; 2) whether the SNP at −2518 is associated with the plasma level of MCP-1; and 3) whether the SNP at −2518 is associated with carotid atherosclerosis.

Methods

Subjects

The Shimanami Health Promoting Program (J-SHIPP) was started in 1999 in the Shimanami district, located in the southern part of Japan (*21*). J-SHIPP is a longitudinal study evaluating factors relating to cardiovascular disease, dementia, and death. The present study is a part of J-SHIPP performed in a single community that participated in previous J-SHIPP studies. All residents aged over 50 years were invited to participate in the program, which consisted of an interview, anthropometric measurement, blood sampling, and carotid ultrasonography. About 50% of residents aged over 50 participated in the program. Among them, subjects aged over 50 and free from any history or symptoms of cardiovascular disease such as stroke, transient ischemic attack (TIA), myocardial infarction, angina, congestive heart failure, and peripheral vascular disease were enrolled in the study. Subjects with inflammatory disease or infections were excluded from the study. Informed consent for the procedure was obtained from each subject. All procedures were approved by the ethical committee of the Ehime University School of Medicine. Three hundred and twenty-five subjects completed the whole procedures.

Evaluation of Carotid Artery

The right carotid artery was evaluated with an SSD-900 (Aloka Co., Ltd., Tokyo, Japan) using a 7.5-MHz probe. After having the subject rest for at least 10 min in the supine position with the neck in slight hyperextension, we evaluated an optimal visualization of the right common carotid artery (CCA), carotid bulb, and extracranial internal and external carotid arteries. From anterior, lateral, and posterior approaches, IMT of the far wall was measured in the right common carotid artery 1 cm proximal to the bulb and averaged to obtain the mean IMT (*22, 23*). Two-dimensionally guided M-mode tracings of the right CCA at 1 cm proximal to the bulb were recorded in real time. Peak-systolic internal diameters (Ds) were obtained by continuous tracing of the intimal-luminal interface of the near and far walls of the CCA in 3 cycles and averaged. The axial resolution of the M-mode system was 0.1 mm.

For the analysis of carotid atherosclerosis, carotid IMT thickening was defined as IMT>0.85 mm and carotid arterial dilatation was defined as Ds>7 mm based on the mean +0.5 SD values of studied subjects free from any known risk factors (*21*).

Evaluation of Risk Factors

Systolic and diastolic brachial blood pressure was measured twice at a 5-min interval in the supine position with an automatic oscillametric blood pressure recorder (HEM-705CP; OMRON Co., Ltd., Tokyo, Japan) during the carotid echo examination. The mean value of two measurements was obtained. The validity of the device and the reproducibility of its results have been established previously (*24*). Total cholesterol, high-density lipoprotein (HDL)-cholesterol, and glucose were determined by conventional methods.

Determination of Plasma Level of MCP-1 and SNP of the MCP-1 Gene

Blood was withdrawn into a tube containing EDTA. Plasma was quickly obtained by centrifugation, and kept at −80°C until assay. The plasma level of MCP-1 was measured in duplicate with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA). The interassay variability was 6.3%, and the intra-assay variability was 6.2%.

Genomic DNA was extracted from peripheral blood samples using an extraction kit (QIAGEN GmbH; Qiagen, Hilden, Germany) (*25*). SNP of the regulatory region of the MCP-1 gene, located at position −2518 (G or A), was determined according to the published method (*26*). In brief, a 930 bp DNA segment including the polymorphic site was amplified by polymerase chain reaction (PCR) using a set of oligonucleotide primers: 5′-CCGAGATGTTCCCAGCACA G-3′ and 5′-CTGCTTTGCTTGTGCCTCTT-3′. The PCR products were digested with one unit of PvuII and separated by 3% agarose gel electrophoresis. If the polymorphism was −2518A, a unique PvuII restriction site would be eliminated from this segment of the 5′-flanking region. The DNA segment from GG homozygous individuals was digested into 708 and 222 bp fragments.

Statistical Analysis

All values are expressed as the means±SD unless otherwise specified. Statistical comparisons among groups were performed by analysis of variance (ANOVA). Differences in the prevalence among groups and Hardy-Weinberg's equilibrium were analyzed by the χ^2 method. Stepwise regression analysis was performed to evaluate the association between plasma MCP-1 level, classical risk factors, and MCP-1 genotype. All analyses were performed using the software package JMP (SAS Institute, Cary, USA). Values of $p<0.05$ were considered to indicate statistical significance.

Table 1. Clinical Characteristics of Participants

n	325
Male (%)	33.5
Age (years old)	70.5±9.4
Body height (cm)	152.5±9.2
Body weight (kg)	53.9±10.1
BMI (kg/m^2)	23.1±3.2
SBP (mmHg)	138.9±24.1
DBP (mmHg)	75.5±10.9
Total cholesterol (mg/dl)	203±41
HDL-cholesterol (mg/dl)	54±15
Glucose (mg/dl)	111±33
Smoking (%)	11.1
Carotid artery	
IMT (mm)	0.79±0.13
Internal dimension (mm)	6.6±0.9

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; IMT, intima-media thickness.

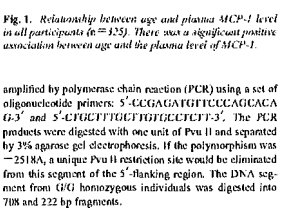

Fig. 1. *Relationship between age and plasma MCP-1 level in all participants (n=325). There was a significant positive association between age and the plasma level of MCP-1.*

Table 2. Simple Correlation Coefficients for Plasma Monocyte Chemoattractant Protein-1 (MCP-1)

Risk factors	*r*	*p*
Age	0.23	0.0001
Male (%)	0.01	0.84
BMI (kg/m^2)	0.05	0.34
SBP (mmHg)	0.11	0.051
DBP (mmHg)	0.01	0.90
Total cholesterol (mg/dl)	0.08	0.18
HDL-cholesterol (mg/dl)	0.01	0.98
Glucose	0.04	0.51
Smoking (%)	0.02	0.66
Medication	0.07	0.21

The abbreviations are the same as Table 1.

Results

Plasma MCP-1 and Carotid Atherosclerosis

The clinical characteristics of all participants are summarized in Table 1. The mean plasma level of MCP-1 was 181±54 ng/ml. The plasma MCP-1 level was significantly associated with age (Fig. 1). However, there were no significant associations between plasma MCP-1 and other classical risk factors, including sex, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, plasma glucose and smoking status, or between plasma MCP-1 and medication status (Table 2). The plasma level of MCP-1 was significantly associated with carotid IMT ($r=0.12$, $p<0.05$) and carotid arterial dimension ($r=0.13$, $p<0.05$). The relationship between carotid atherosclerosis and plasma MCP-1 is summarized in Fig. 2. Carotid IMT thickening defined as IMT>0.85 mm and carotid arterial dilatation defined as Ds>7 mm were associated with a significantly higher level of plasma MCP-1.

Effect of MCP-1 Gene Polymorphism on Plasma Concentration of MCP-1 and Carotid Atherosclerosis

The breakdown of the total 325 subjects by the −2518 SNP of the MCP-1 gene was as follows: 47 subjects had the AA genotype, 141 the GA genotype, and 137 the GG genotype. The genotype distribution of MCP-1 was in agreement with Hardy-Weinberg's equilibrium ($p=0.88$). The plasma levels of MCP-1 for each of the three genotypes are summarized in

Table 3. Plasma Monocyte Chemoattractant Protein-1 (MCP-1), Carotid Intima-Media Thickness (IMT), and Carotid Arterial Diameter (Ds) According to MCP-1 Genotype

	AA	GA	GG	GA+GG	AA vs. GA vs. GG		AA vs. GA+GG	
					F	*p*	*F*	*p*
Total population								
n	47	141	137	278				
MCP-1 (ng/ml)	166±36	184±59	184±54	184±56	2.2	0.11	4.4	0.036
IMT (mm)	0.78±0.13	0.80±0.13	0.80±0.14	0.80±0.13	0.2	0.79	0.4	0.50
Ds (mm)	6.7±0.9	6.7±0.9	6.5±0.8	6.6±0.9	3.8	0.024	0.9	0.34
Subjects without medication								
n	34	98	100	198				
MCP-1 (ng/ml)	163±34	192±59	182±53	187±56	3.5	0.031	5.6	0.019
IMT (mm)	0.77±0.13	0.79±0.13	0.78±0.13	0.79±0.13	0.4	0.65	0.6	0.44
Ds (mm)	6.5±0.8	6.6±0.9	6.4±0.9	6.5±0.9	1.9	0.16	0.01	0.98

Analysis of variance (ANOVA) with additive as well as dominant models of MCP-1 genotype was performed.

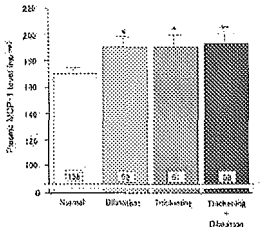

Fig. 2. *Plasma MCP-1 level in subjects divided into four groups according to the presence of carotid intima-media thickening and dilatation. Carotid arterial dilatation was defined as carotid arterial internal diameter >7.0 mm, and carotid thickening was defined as carotid intima-media thickness >0.85 mm. The plasma level of MCP-1 was significantly different among the four groups (F(3, 321)=4.10, p=0.005).*

Table 3. There was a significant difference in the plasma MCP-1 level between subjects with AA and those carrying the G allele (GG+GA). Subjects with the AA genotype had a lower plasma level of MCP-1 compared with G carriers (GG+GA). Analysis restricted to the subjects without medication further increased the statistical significance. However, carotid IMT as well as carotid arterial diameter were not significantly different among the MCP-1 genotypes (Table 3).

Multiple Regression Analysis of Plasma MCP-1

To further investigate whether the MCP-1 genotype independently affects plasma MCP-1 level, stepwise regression analysis of the plasma MCP-1 level was performed with the following parameters: age, sex, systolic blood pressure, total cholesterol, HDL-cholesterol, plasma glucose, smoking status, medication status, and MCP-1 genotype (in the dominant model). *The results revealed that the MCP-1 genotype*, in addition to age, was an independent determinant of the plasma MCP-1 level (Table 4).

Discussion

MCP-1 has been shown to be a key factor initiating the inflammatory process of atherogenesis and sustaining the proliferative response to vessel injury (*1, 2*). Elevated levels of MCP-1 have been reported in patients with myocardial infarction as well as after myocardial reperfusion (*7, 8, 27*). These findings are consistent with the features of an immediate-early gene (*1*). Transcription of MCP-1 occurs at the early stage after vascular injury (*28*). *Recently, Cipollone et al. (26)* demonstrated that the MCP-1 level after percutaneous transluminal coronary angioplasty (PTCA) could predict restenosis. They observed significantly higher MCP-1 levels sustained for 180 days after PTCA in subjects who developed restenosis compared with those who did not. A similar finding has also been reported in patients after stent implantation (*29*). Schmidt and Stern (*1*) proposed a chronic stage of the effect of MCP-1, in which prolonged MCP-1 production could have autocrine/paracrine effects on monocytes and smooth muscle cells to promote the development of atherosclerosis. On the other hand, plasma MCP-1 can also increase in response to the secondary leukocyte accumulation as well as in response to vascular alterations (*30*). Vascular stresses including high blood pressure have been shown to lead to the infiltration and activation of leukocytes in target tissues, where these cells contribute to the increment of plasma MCP-1 level (*31*).

Table 4. Stepwise Regression Analysis for Plasma Monocyte Chemoattractant Protein-1 (MCP-1)

Parameter	β	*p*
Age	0.23	<0.0001
MCP-1 genotype	0.11	0.043

Sex, BMI, systolic blood pressure, TMT, total cholesterol, HDL-cholesterol, glucose, and medication status were not entered in the equation.

In the present study, we observed a modest but significant positive association between plasma MCP-1 and carotid IMT in community residents free from any history or symptoms of cardiovascular disease. These findings indicate that circulating MCP-1 may play a role in the progression of chronic atherosclerotic lesions. We also observed that plasma MCP-1 was associated with carotid arterial dimension. These findings further support the hypothesis that MCP-1 may act in the chronic stage of arterial remodeling. However, the association between plasma MCP-1 level and carotid IMT observed in this study was weak compared with that in a previous report by Stork *et al. (17)*. One possible explanation for this discrepancy is the difference of study design. In short, *Stork and colleagues recruited study subjects who exhibited* at least one site of >1.0 mm IMT in the carotid artery system from their large cohort. Furthermore, they evaluated the mean maximum IMT in the carotid and femoral arteries, while our evaluation was carried out at 1 cm proximal to the carotid bulb. Because there are still no supportive epidemiological data available, this issue deserves further investigation. The influences of antioxidative factors and antiatherosclerotic reactance are another possible explanation. Further examination involving these factors may reveal a stronger association between plasma MCP-1 level and carotid atherosclerosis.

In the present study, the IMT of the far wall of the right common carotid was evaluated from anterior, lateral, and posterior approaches, and the mean of measurements of three points was calculated as the IMT. Although carotid arterial morphology evaluated with the present method has been shown to be associated with the status of several pathophysiological factors (*21, 32–38*), measurements of carotid IMT at more points could reflect carotid atherosclerosis more accurately. This finding leaves open the possibility that MCP-1 gene polymorphism could affect more subtle atherosclerotic changes.

Several studies have reported an age-dependent augmentation of plasma MCP-1 level (*39, 40*). We also observed a significant positive association between age and the plasma level of MCP-1. Although the exact mechanism of the age-dependent augmentation of plasma MCP-1 is not understood, Gerli *et al. (39)* postulated an age-dependent shift in the cytokine network. Age-dependent augmentation of the plasma level of MCP-1 may indicate occult atherosclerotic lesions (*40*). However, the result of our stepwise regression analysis indicated that aging itself determines the plasma level of MCP-1 independent of carotid IMT. Other known classical risk factors had no effect on plasma MCP-1.

SNP in the promoter region of the MCP-1 gene has been identified at position −2518 relative to the major transcriptional start site, and was shown to be related to the promoter activity (*26*). An *in vitro* study demonstrated that IL-1β-induced luciferase activity was significantly greater in cells transfected with constructs containing GA at the −2518 position than in those containing AA at this position. Furthermore, *it has also been observed that IL-1β-treated peripheral* blood mononuclear cells from G carriers (GA+GG), and especially from GG carriers, produced more MCP-1 than cells from individuals homozygous for AA. Our finding that subjects with the AA genotype had lower plasma MCP-1 compared with G carriers is consistent with these *in vitro* results. However, a co-dominant effect is certainly more in favor of a phenotype-genotype association. A previous report demonstrated the co-dominant inheritance in the IL-1β-induced MCP-1 production, but not in the basal production level, *in vitro (41)*. Since patients with known atherosclerotic disorders, who tend to produce high levels of plasma MCP-1 (*7, 8, 27*) and among whom there is a relatively high prevalence *of G carriers (19), were not admitted in the present study*, the plasma level of MCP-1 may mimic G-allele-dominant inheritance. To our knowledge, this is the first community-based report to examine the effect of the −2518 SNP on the plasma level of MCP-1.

We also investigated carotid morphology in the three genotypes of MCP-1. However, there was no association between MCP-1 genotype and either carotid atherosclerosis or carotid arterial dilatation. These findings do not exclude the possibility that the SNP is related to atherosclerotic disorders. With the knowledge that plasma MCP-1 is increased after myocardial events, and an increased level after PTCA is associated with restenosis, we cannot exclude the possibility that the plasma MCP-1 level responds to events such as myocardial infarction that could be affected by MCP-1 genotype. Recently, a case-control study reported that the frequency of the −2518 GG genotype was significantly higher in patients with coronary arterial diseases, including myocardial infarction (*19*). Although the genotype distribution was significantly deviated from Hardy-Weinberg's equilibrium, the findings indicate the possibility that the SNP of MCP-1 is associated with thromboembolic disorders. Furthermore, the positive association between plasma MCP-1 level and carotid IMT, examined using maximum IMT, was previously reported. Although only the mean IMT was evaluated in this study, the examination using maximum IMT, as well as plaque score, may clarify the involvement of the MCP-1 genotype on carotid atherosclerosis.

The prevalence of −2518 SNP in our study was different

from that previously reported in Asians (*26*). However, this previously reported prevalence was calculated using only 16 subjects. The allele frequency in our population was 36% for the A allele and 64% for the G allele. The previously reported G allele frequency in Caucasians (*n*=71) was 29%, suggesting a profound racial difference. A recent study from Hungary reported a G allele frequency of 23.9% in controls (*n*=320) and 29.9% in patients with coronary artery disease (*n*=318) (*19*). The higher prevalence of the G allele in the Japanese population and the relatively low prevalence of coronary arterial disease, together with the findings of the present study, indicate an ethnic difference in the role of the MCP-1 gene in the development of atherosclerosis.

There were several important limitations to this study. First, our subjects had no symptoms of cardiovascular disease. Thus we cannot exclude the possibility that the plasma *MCP-1 levels, as well as the MCP-1 polymorphism, would* show a stronger association with carotid atherosclerosis in subjects with known atherosclerotic disorders, particularly in light of a previous case-control study in which patients with the MCP-1 polymorphism were more susceptible to coronary artery disease (*19*). Second, the selection criteria of the study population were skewed to a relatively older population. We previously reported the age-related augmentation of genetic factors for the development of carotid atherosclerosis (*32*). In terms of the gene-age interaction, we considered that the present selection of subjects would provide specific predictive information for the development of carotid atherosclerosis. To ensure the present findings, however, further evaluation *with a wide range of subjects will be needed.*

In summary, the plasma level of MCP-1 showed a significant association with carotid IMT thickening and carotid arterial dilation. −2518 SNP in the promoter region of MCP-1 was significantly related to the plasma level of MCP-1 in this Japanese population. However, −2518 SNP did not directly correlate with carotid IMT or carotid diameter.

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Original Article

Association of Angiotensin II Type 2 Receptor Gene Variant with Hypertension

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The renin-angiotensin system plays an important role in blood pressure regulation by influencing salt-water homeostasis and vascular tone. Angiotensin II, the major biologically active component of this system, exerts its effect *via* two pharmacologically distinct subtypes of angiotensin II receptors, the angiotensin II type 1 receptor (AT$_1$-R) and the angiotensin II type 2 receptor (AT$_2$-R). Thus, the AT$_2$-R gene may be involved in hypertension. Accordingly, our objective was to examine whether polymorphisms of the AT$_2$-R gene are involved in hypertension. The entire AT$_2$-R gene including the promoter region was screened to find polymorphisms. As a result, two novel single nucleotide polymorphisms (SNPs), A1818T in intron 2 and G4303A in exon 3, as well as two known SNPs, A1675G in intron 1 and C4599A in exon 3, were identified. These four SNPs had similar allele frequencies, and the A1675G and C4599A polymorphisms were in almost complete linkage disequilibrium. Because the AT$_2$-R gene is located on the X chromosome, we analyzed the possible association between the C4599A polymorphism and hypertension in men and in women separately in two large Japanese populations. This analysis showed that the C4599A polymorphism was associated with hypertension in women (p=0.0058), but not in men. Moreover, this female-specific association was pronounced in premenopausal women. The female-specific association may be helpful in conducting further molecular and biological studies on the relationship among sex, the renin-angiotensin system, and hypertension. (*Hypertens Res* 2003; 26: 547–552)

Key Words: **angiotensin II receptor, hypertension, polymorphism, woman, estrogen**

Introduction

Hypertension is considered to be a complex trait to which genetic, environmental, and demographic factors contribute interactively (*1*). The renin-angiotensin system plays an important role in blood pressure regulation by influencing salt-water homeostasis and vascular tone (*2*). Angiotensin II, the major biologically active component of this system, exerts its effect *via* two pharmacologically distinct subtypes of angiotensin II receptors, the angiotensin II type 1 receptor (AT$_1$-R) and the angiotensin II type 2 receptor (AT$_2$-R). AT$_2$-R appears to act in opposition to and in balance with AT$_1$-R (*3–5*). Indeed, mice lacking the AT$_1$-R gene have been reported to have reduced blood pressure (*6, 7*), whereas mice lacking the AT$_2$-R gene show elevated blood pressure and an increased vasopressor response to injection of angiotensin II (*8, 9*). Thus, the AT$_2$-R gene may be an attractive candidate gene for hypertension.

The molecular structure of AT$_2$-R resembles that of the superfamily of G protein-coupled receptors, which contain seven transmembrane regions (*10, 11*). The AT$_2$-R gene is located on the X-chromosome and spans about 5 kb (*12*). The AT$_2$-R gene has three exons, with the entire coding region on the third exon (*13*). Regulatory elements are located in the first intron in addition to the promoter region (*14*). The complete nucleotide sequence of the human AT$_2$-R gene including the promoter region has been elucidated (*13*) and is publicly available *via* the World Wide Web in the NCBI sequence database (http://www.ncbi.nlm.nih.gov/) (*12*). On these grounds, we screened the entire AT$_2$-R gene including the promoter region to find polymorphisms, and examined the possible association between a polymorphism and hypertension.

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Table 1. Characteristics of Male Participants According to Hypertensive Status

Variable	Population 1		Population 2		Population 1 and 2	
	Normotensive (n=1,478)	Hypertensive (n=660)	Normotensive (n=432)	Hypertensive (n=239)	Normotensive (n=1,910)	Hypertensive (n=899)
Age (years)	51.0 (8.8)	55.1 (6.4)	53.0 (8.6)	56.8 (8.3)	51.4 (8.8)	55.6 (7.0)
Body mass index (kg/m^2)	22.9 (2.7)	24.4 (3.0)	22.7 (2.8)	23.9 (2.6)	22.8 (2.7)	24.2 (2.9)
SBP (mmHg)	124.3 (9.4)	150.9 (10.0)	116.0 (11.3)	143.8 (16.6)	122.4 (10.4)	149.0 (12.5)
DBP (mmHg)	72.5 (6.0)	88.0 (6.1)	73.1 (8.7)	89.9 (9.7)	72.7 (6.7)	88.5 (7.3)
Total cholesterol (mg/dl)	195.9 (34.7)	200.7 (30.7)	195.2 (28.6)	201.0 (35.5)	195.7 (31.6)	200.8 (32.0)
HDL-cholesterol (mg/dl)	59.5 (12.7)	60.0 (13.4)	51.4 (12.8)	54.3 (13.6)	57.6 (13.21)	57.7 (14.0)
Triglyceride (mg/dl)	135.9 (77.3)	160.2 (86.0)	127.2 (85.2)	158.0 (134.2)	133.9 (79.3)	159.6 (101.1)

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein. Data are mean (SD).

Table 2. Characteristics of Female Participants According to Hypertensive Status

Variable	Population 1		Population 2		Population 1 and 2	
	Normotensive (n=231)	Hypertensive (n=77)	Normotensive (n=121)	Hypertensive (n=31)	Normotensive (n=352)	Hypertensive (n=108)
Age (years)	48.2 (9.6)	54.2 (5.4)	51.8 (8.8)	60.1 (8.8)	49.4 (9.5)	55.9 (7.0)
Body mass index (kg/m^2)	22.3 (3.2)	25.1 (3.6)	21.6 (2.4)	23.2 (5.0)	22.1 (2.9)	24.6 (4.1)
SBP (mmHg)	118.9 (11.0)	149.4 (9.3)	111.9 (12.3)	143.5 (16.3)	116.5 (11.9)	148.1 (11.3)
DBP (mmHg)	67.7 (6.5)	83.6 (6.3)	68.3 (9.1)	84.5 (10.6)	67.9 (7.5)	83.9 (7.7)
Total cholesterol (mg/dl)	199.6 (29.0)	220.7 (31.7)	207.6 (36.5)	215.1 (46.1)	202.5 (32.1)	219.1 (36.3)
HDL-cholesterol (mg/dl)	66.5 (15.6)	63.2 (14.2)	64.7 (15.2)	60.0 (15.6)	65.8 (15.5)	62.3 (14.6)
Triglyceride (mg/dl)	90.7 (38.4)	132.1 (73.9)	78.8 (51.8)	99.4 (50.4)	86.2 (44.1)	122.6 (69.3)

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein. Data are mean (SD).

Methods

Subjects

The clinical characteristics of the subjects included in the study are shown in Tables 1 and 2. Population 1 (n=2,446) originated from the Ehime region of Japan, and population 2 (n=823) was from the Hyogo region of Japan. All subjects were Japanese urban residents. Subjects in population 1 participated in medical check-ups 1 to 11 times (average 6.2 times per person), and the mean values of variables in the personal health records were used in analyses. Subjects in population 2 also underwent a medical check-up, and the values of variables in the personal health records were used in analyses. All subjects gave their informed consent, and the study was approved by the ethics committee of Ehime University.

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 medications, 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. Menopausal status was defined based on the average age of menopause in Japan, 51 years old (Japan Society of Obstetrics and Gynecology, Tokyo, Japan).

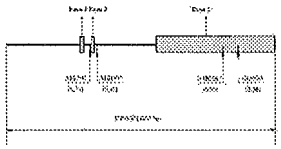

*Fig. 1. Schematic representation of the human AT$_2$-R gene and position of identified SNPs. The minor allele frequency for each SNP is shown in parentheses. * Newly identified SNPs*

Screening for Polymorphisms in the Human AT$_2$-R Gene Including the Promoter Region

The full genomic sequence of the human AT$_2$-R gene including the promoter region (GenBank accession no. U20860; Fig. 1) was divided into 13 overlapping fragments for polymorphism detection by sequencing. All fragments were around 500 bp. To identify polymorphisms and to obtain an estimate of allele frequencies, we examined genomic DNA from 30 healthy unrelated Japanese men. The DNA was amplified and the polymerase chain reaction (PCR) products were sequenced in both directions using a BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, USA). Primers used for sequencing were the same as those used for the initial PCR.

AT$_2$-R Gene Polymorphism Genotyping

The SNaPshot (PE Applied Biosystems) ddNTP primer extension method and capillary electrophoresis were used to detect the AT$_2$-R A1675G polymorphism. The forward primer was 5′-CTTCTGTTTTCCTGTGTTTT-3′, the reverse primer was 5′-TGACTTACTGGTTGTTGA-3′, the A-allele extension primer was 5′-ACTGTATTTTGCAAAACTCCT-3′, and the G-allele extension primer was 5′-GAAACAGCAGCTAAATAATT-3′. The TaqMan (PE Applied Biosystems) chemical method was used to detect the AT$_2$-R C4599A polymorphism. The forward primer was 5′-GGATTATTCAGGCTTTAGGCATATTG-3′, the reverse primer was 5′-TCTATATTCGTAGCCCTATTCAATATGTAGTT-3′, the C-allele specific probe was 5′-Fam-TCTTTAAAAACGCTATAAAT-MGB-3′, and the A-allele specific probe was 5′-Vic-TCTTTAAAAAGCTATAAATTMGB-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. We validated the TaqMan chemical method with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in 300 selected subjects (*15*).

Statistical Methods

Measures of linkage disequilibrium (Lewontin's D' and correlation coefficient Δ) were calculated according to Devlin and Risch (*16*). Analysis of variance was used to assess differences in means and variances of continuous variables. Logistic regression models were used to assess whether the AT$_2$-R C4599A polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of the effects of confounding factors. Sex, age, body mass index, plasma total cholesterol, high-density lipoprotein-cholesterol, and triglyceride levels were considered to be confounding factors (Tables 1 and 2). Logarithmically transformed plasma triglyceride values were used in the analyses. General linear regression models were used to assess whether the C4599A polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of the effects of confounding factors. P values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software.

Results

Identification of Polymorphisms

The full genomic sequence of the human AT$_2$-R gene including the promoter region was screened to find polymorphisms by sequencing 30 randomly selected Japanese men. This screening identified two novel single nucleotide polymorphisms (SNPs), A1818T in intron 2 and G4303A in exon 3, as well as two known SNPs, A1675G in intron 1 (*17*) and C4599A in exon 3 (*18*) (Fig. 1). G4303A and C4599A were in the 3′-untranslated region. Minor allele frequencies for these four polymorphisms were 0.33 for A1675G, 0.32 for A1818T, 0.31 for G4303A, and 0.36 for C4599A. Assessment of linkage disequilibrium between the A1675G and C4599A polymorphisms by several measures as well as the χ^2 test showed that they were in almost complete linkage disequilibrium (χ^2 test p<0.0001, Lewontin's D'=0.93, and correlation coefficient Δ=0.89).

Association of AT$_2$-R C4599A Polymorphism with Hypertension

Because these four SNPs had similar allele frequencies, the A1675G and C4599A polymorphisms were in almost complete linkage disequilibrium, and AT$_2$-R is located on the X-chromosome, we analyzed the possible association between the C4599A polymorphism and hypertension in men and in women separately in our two populations (Table 3). The genotype distribution of the C4599A polymorphism did not significantly deviate from the expected genotype frequencies deduced from Hardy-Weinberg equilibrium.

Logistic regression analysis showed a marginally significant difference in the frequencies of the genotypes

Table 3. AT$_2$-R C4599A Genotype and Allele Frequencies in Normotensive and Hypertensive Subjects According to Sex

Genotype and allele	Population 1				Population 2				Population 1 and 2			
	Normotensive	Hypertensive	p value	OR	95% CI	Normotensive	Hypertensive	p value	OR	95% CI	Normotensive	Hypertensive	p value	OR	95% CI
Male															
A-	531 (36%)	235 (36%)	0.89	1.01	0.84–1.23	155 (36%)	88 (37%)	0.81	0.88	0.65–1.20	686 (36%)	323 (36%)	1.00	1.00	0.84–1.18
C-	947 (64%)	425 (64%)				277 (64%)	151 (63%)				1,224 (64%)	576 (64%)			
Female															
AA	30 (13%)	17 (22%)	0.091			11 (9%)	7 (23%)	0.044			41 (12%)	24 (22%)	0.021		
AC	108 (47%)	37 (48%)				58 (48%)	17 (54%)				166 (47%)	54 (50%)			
CC	93 (40%)	23 (30%)				52 (43%)	7 (23%)				145 (41%)	30 (28%)			
AA	30 (13%)	17 (22%)	0.055	0.53	0.27–1.02	11 (9%)	7 (23%)	0.038	0.34	0.11–0.96	41 (12%)	24 (22%)	0.0058	0.46	0.26–0.81
AC+CC	201 (87%)	60 (78%)				110 (91%)	24 (77%)				311 (88%)	84 (78%)			
A allele	168 (36%)	71 (46%)	0.037	0.67	0.46–0.97	72 (30%)	21 (34%)	0.53	0.83	0.50–1.48	240 (34%)	92 (43%)	0.023	0.70	0.51–0.95
C allele	294 (64%)	83 (54%)				169 (70%)	41 (66%)				464 (66%)	124 (57%)			

AT$_2$-R, angiotensin II type 2 receptor; OR, odds ratio; CI, confidence interval.

(p=0.055 for AA *vs.* AC+CC; OR=0.53; 95% CI=0.27–1.02, where OR indicates odds ratio and 95% CI indicates 95% confidence interval) between the hypertensive and normotensive women in population 1 (Table 3). In contrast, the C4599A polymorphism was not associated with hypertension in men (p=0.89; OR=1.01; 95% CI=0.84–1.23). In population 2, the C4599A polymorphism was significantly associated with hypertension in women (p=0.038; OR=0.34; 95% CI=0.11–0.96), but not in men (p=0.81; OR=0.88; 95% CI=0.65–1.20). Analysis combining populations 1 and 2 yielded a p value of 0.0058 for the association between the C4599A polymorphism and hypertension in women (OR=0.46; 95% CI=0.26–0.81). This female-specific association remained significant even after adjustment for age (p=0.0085) and for all confounding factors (p=0.0063).

Given the female-specific association, we next analyzed the association according to menopausal status. This analysis combining populations 1 and 2 showed a lower OR of 0.29 for the association between the C4599A polymorphism and hypertension in premenopausal women (p=0.027; 95% CI=0.10–0.86) (Table 4). In contrast, in postmenopausal women, a higher odds ratio of 0.56 was shown for the association between the C4599A polymorphism and hypertension (p=0.089; 95% CI=0.29–1.10).

More quantitatively, we further analyzed the association between the C4599A polymorphism and blood pressure in women. This analysis failed to show any significant association (Table 5). However, there was a non-significant tendency for women with the AA genotype to show higher blood pressure than those with the AC and CC genotypes in both populations.

Analysis of the association between the C4599A polymorphism and blood pressure according to menopausal status also failed to show any significant association (Table 6). However, the probability values tended to be lower, and the difference in blood pressure tended to be larger, in premenopausal women than in postmenopausal women (Table 6).

Discussion

The present study identified two novel SNPs as well as two known SNPs in the AT$_2$-R gene. The four SNPs had similar allelic frequencies, and the A1675G and C4599A polymorphisms were in almost complete linkage disequilibrium. These results suggest that the AT$_2$-R region has a low mutation rate and a low recombination rate, implying that this region is extremely stable compared even with relatively stable regions, such as the angiotensin converting enzyme (ACE) gene region (*19*) and the angiotensinogen gene region (*20, 21*). In contrast to these regions, the calpain-10 region appears to be unstable, because the region contains many SNPs with different allele frequencies and with relatively high density (*22*). However, the difference in the stability of the regions might be partly attributable to racial differences, be-

Table 4. Association of AT₂-R C4599A Genotype with Hypertension According to Menopausal Status

Menopausal status	Genotype frequency		p value	OR	95% CI
	Normotensive (n=352)	Hypertensive (n=108)			
Premenopausal women					
AA	18 (10%)	5 (28%)			
AC+CC	159 (90%)	13 (72%)	0.027	0.29	0.10–0.86
Postmenopausal women					
AA	23 (13%)	19 (21%)			
AC+CC	152 (87%)	71 (79%)	0.089	0.56	0.29–1.10

The abbreviations are the same as Table 3.

Table 5. Blood Pressure According to AT₂-R C4599A Polymorphism

Blood pressure	AA	AC+CC	p value
Population 1			
n	47	261	
SBP (mmHg)	128.8 (17.9)	126.1 (16.7)	0.32
DBP (mmHg)	73.0 (9.3)	71.4 (9.4)	0.29
Population 2			
n	18	134	
SBP (mmHg)	123.5 (23.3)	118.0 (17.7)	0.24
DBP (mmHg)	72.6 (14.9)	71.5 (11.0)	0.71
Population 1 and 2			
n	65	395	
SBP (mmHg)	127.3 (19.5)	123.4 (17.5)	0.10
DBP (mmHg)	72.9 (11.0)	71.4 (10.0)	0.29

Data are mean (SD). AT₂-R, angiotensin II type 2 receptor; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 6. Blood Pressure According to Menopausal Status and AT₂-R C4599A Polymorphism

Menopausal status	AA	AC+CC	p value
Premenopausal women			
n	23	172	
SBP (mmHg)	121.5 (18.9)	117.6 (14.8)	0.24
DBP (mmHg)	69.9 (10.0)	68.2 (8.8)	0.40
Postmenopausal women			
n	42	223	
SBP (mmHg)	130.5 (19.3)	127.8 (18.5)	0.39
DBP (mmHg)	74.5 (11.4)	73.9 (10.3)	0.74

Data are mean (SD). The abbreviations are the same as Table 5.

cause the ACE and calpain-10 regions have only been studied in Caucasians thus far. Moreover, because our screening to find polymorphisms was carried out in only 30 randomly selected Japanese men, polymorphisms with low allele frequencies could have been missed.

In addition, the present study showed a female-specific association between the AT₂-R C4599A polymorphism and hypertension, particularly in premenopausal women. Women with the AA genotype were significantly more likely to develop hypertension than those with the AC and CC genotypes. Consistent with this association, women with the AA genotype in the present study also tended to have higher blood pressure than those with the AC or CC genotypes, although this tendency was not significant. This finding may be attributable to the unstable nature of blood pressure and to the presence of treated hypertensive subjects (23). Some previous studies have also shown a similar tendency or association, lending further support to the idea of an association between the *AT₂-R C4599A polymorphism and hypertension.* One study reported that women with the AA genotype were more likely to have hypertension than those with the AC or CC genotypes, though again, this tendency did not reach the level of statistical significance (15). Another study has shown that the C allele of the AT₂-R C4599A polymorphism is significantly associated with a low probability of hypertrophic cardiomyopathy in women, but not in men (24). Nevertheless, given the fact that association studies are often irreproducible (25), and because the previous studies and the present study were all conducted in relatively small populations, replication studies in large populations will be indispensable for establishing the association (26, 27).

The female-specific association between the AT₂-R *C4599A polymorphism and hypertension cannot be readily* explained. However, because recent studies have shown that estrogen upregulates AT₂-R (28, 29), a possible genotypic difference in reactivity to estrogen may explain the female-specific association. From this point of view, a simple explanation may be that women with the AC and CC genotypes or genotypes in linkage disequilibrium with them might react normally to estrogen, whereas those with the AA genotype or a genotype in linkage disequilibrium with it might have a lesser reaction to estrogen. If this is the case, in women with the AC and CC genotypes, estrogen may upregulate AT₂-R, resulting in reduced blood pressure. In contrast, in women with the AA genotype, estrogen may fail to upregulate AT₂-*R and may, in turn,* fail to reduce blood pressure, resulting in their relatively high probability of developing hypertension. Such an explanation would also be consistent with our observation that the association was pronounced in premenopausal

women, because estrogen levels fall after menopause.

The present study has several limitations. First, although several factors are known to influence blood pressure, including glucose metabolism, cigarette smoking, and alcohol consumption, quantitative parameters of these factors were not available in our population. Second, the present study did not assess A1675G, A1818T, or G4303A. Third, the present study did not assess gene–gene interactions, which may have modified the evaluation of an association. In this context, analyses of the interaction between the AT₂-R gene and other genes involved in the renin-angiotensin system may help to improve our understanding of the relation between the renin-angiotensin system and hypertension. Finally, because the present study included few women, the results of this study need to be assessed in large populations.

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Original Article

Genome-Wide Linkage Disequilibrium Mapping of Hypertension in Japan

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Hypertension is a common, complex phenotype resulting from the interaction between genetic and environmental factors. To select candidate regions potentially responsible for hypertension, we are conducting a genome-wide linkage disequilibrium (LD) mapping of hypertension using dinucleotide repeat markers in 146 hypertensive and 136 normotensive subjects. Although the LD mapping is still underway, 19 alleles of 15 markers have already shown a nominally significant association (p<0.05), with odds ratios ranging from 0.08 to 5.12, suggesting the presence of many hypertension-related loci with weak effects in the human genome. These markers should be further assessed, adjusting for confounding factors and considering gene–gene and gene–environmental interactions in additional samples. In this report, we discuss our ongoing LD mapping project and describe the 15 markers thus far discovered. Among the 15 markers, D10S537 had a highly significant association with hypertension (p=5.3×10⁻⁴; OR=3.80; 95% CI=1.98–7.27; where OR *indicates the odds ratio and 95% CI indicates the 95% confidence interval). Further analysis in a large* Japanese population showed that D10S537 was significantly associated with hypertension (p=0.044; OR=1.27; 95% CI=1.01–1.59). D10S537 was more significantly associated with hypertension in subjects with normotriglyceridemia in our population (p=0.007; OR=1.47; 95% CI=1.11–1.95).

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Key Words: hypertension, linkage disequilibrium mapping, microsatellite

Introduction

Hypertension is a common, complex disorder that results from interactions between genetic and environmental factors *and has been intensively studied to identify susceptibility* loci. One widely used strategy for identifying candidate polymorphisms for hypertension is the candidate gene approach. And in fact, polymorphisms in many candidate genes have been tested for their association with hypertension. However, association studies have reached divergent conclusions (1), and even the conclusions of meta-analyses have been inconsistent (2–5). Moreover, the candidate gene approach in large Japanese populations has also failed to show conclusive results (6–17). Thus, there is currently no *genotypic polymorphism that has been proven to be associat*ed with hypertension in humans.

Another strategy to search for candidate polymorphisms for hypertension is linkage mapping in humans, and large-scale linkage mappings of hypertension have led to the identification of several candidate regions (18–29). However, no region showed highly significant linkage according to a proposed criterion (30), and the candidate regions have been inconsistent, suggesting that the development of hypertension may not be attributable to a few strong genetic factors. Instead, many weak-to-moderate genetic factors may contribute to the development of hypertension, although the number of such genetic factors and their relative risks remain unknown. Indeed, the authors of a large-scale linkage study concluded that the lack of a highly significant maximum logarithm of odds score values in their study demonstrated just how difficult genome-wide searches for genes influencing complex traits can be (9). This difficulty may be attributable to several characteristics of linkage analysis. First, if we assume that many weak-to-moderate genetic factors may con*tribute to the development of hypertension, then the relative*ly weak statistical power of linkage analysis could fail to identify them. Second, the results of linkage analysis could be influenced by wide regions of chromosomes over the range of linkage disequilibrium (LD), and such wide regions may contain more than several genetic factors influencing the development of hypertension. For example, at least three functional polymorphisms can exist in relation to hypertension even within a single gene, as has been reported for the β-2 adrenergic receptor gene (31, 32). The interpretation of the results may therefore be complicated, and the statistical power of linkage analysis to find a single genetic factor may be even weaker. Finally, linkage analysis has proven difficult *to conduct when considering a number of gene–environmen*tal and gene–gene interactions, despite the fact that the development of hypertension is considered to be at least partly attributable such interactions (33).

Owing to the complex nature and late onset of common disorders, LD mapping of genes in population samples has been suggested as an alternative to conventional linkage mapping. Indeed, large-scale LD mappings of Alzheimer's disease have identified several candidate loci (34, 35). However, to date, no large-scale LD mapping of hypertension has been conducted. We therefore planned to conduct an efficient large-scale LD mapping of hypertension using dinucleotide repeat markers. And although this mapping is still underway, 15 markers have already been *found* to be nominally associated with hypertension. In this report, we discuss our ongoing LD mapping project and describe the 15 markers thus far *discovered.*

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Table 1. Characteristics of Participants in Linkage Disequilibrium Mapping

	First group		Second group		Third group	
	HT1	NT1	HT2	NT2	HT3	NT3
Number of subjects	48	48	48	48	50	40
Age (years)	53.1 (6.3)	55.5 (2.5)	54.1 (5.1)	54.8 (3.0)	54.1 (4.0)	55.8 (7.5)
Number on treatment	17	0	17	0	25	0
SBP (mmHg)	162.5 (12.1)	111.1 (6.4)	156.4 (9.0)	114.3 (5.5)	160.4 (9.1)	111.3 (5.5)
DBP (mmHg)	85.7 (8.4)	77.7 (9.1)	81.0 (11.3)	73.8 (10.4)	80.7 (10.7)	73.0 (8.8)

Data are mean (SD). Each number following HT or NT means group number. HT, hypertensive subject; NT, normotensive subject; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Methods

Subjects

According to the criteria described below, 146 hypertensive and 136 normotensive subjects were selected from a population composed of 2,426 subjects who worked in a company in the Ehime region of Japan. All subjects were Japanese. They participated in medical check-ups 1 to 11 times (average, 6.2 times per person), and the mean values of variables in their personal health records were used in analyses. These *146 hypertensive and 136 normotensive subjects were fur*ther assigned to one of three groups (Table 1) according to the available amount of DNA. For each of the six subgroups thus created, a DNA pool was established by taking an equal amount of genomic DNA from each member. All subjects gave their informed consent and the study was approved by the ethics committee of Ehime University.

Diagnostic Categories

In our view, a case-control study should be performed in order to sensitively screen candidate polymorphisms for hypertension. In contrast, a population study should be performed in order to assess the extent to which candidate polymorphisms affect the development of hypertension, with the goal of establishing a genetic diagnosis. Therefore, in the genome-wide LD mapping of hypertension, we set very strict criteria for hypertensive subjects but relaxed criteria for statistical significance, whereas in our population study, we set relaxed criteria for hypertensive subjects with stringent criteria for significance. The criteria for hypertensive subjects in the genome-wide LD mapping of hypertension were defined as follows (Table 1): 1) male; 2) age ≤60 years; and 3) systolic blood pressure (SBP) ≥160 mmHg without hypertensive medication or SBP ≥150 mmHg with antihypertensive medication. The criteria for normotensive subjects in the genome-wide LD mapping of hypertension

Table 2. Characteristics of Participants in Population Study According to Hypertensive Status

Variable	Normotensive (n=1,687)	Hypertensive (n=739)
Sex (male %)	86.4	89.3
Age (years)	51.6 (7.7)	55.2 (6.1)
Body mass index (kg/m^2)	22.8 (2.7)	24.4 (3.0)
SBP (mmHg)	123.8 (9.7)	150.9 (9.9)
DBP (mmHg)	72.1 (6.3)	87.7 (6.3)
Total cholesterol (mg/dl)	196.7 (31.6)	202.9 (31.6)
HDL cholesterol (mg/dl)	60.4 (13.3)	60.4 (13.6)
Triglyceride (mg/dl)	130.9 (75.8)	156.2 (85.1)
Smoking (heavy smoker %)	28.5	24.6
Alcohol (moderate to heavy drinker %)	29.2	38.2

HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are mean (SD). Blood pressure readings prior to the start of antihypertensive treatment were not available for 141 hypertensive subjects whose values were measured under treatment.

were defined as follows: 1) male; 2) age between 49 and 60 years; 3) SBP ≤115 mmHg; and 4) no history of angina pectoris or myocardiac infarction. In the population study, the criteria for hypertensive and normotensive subjects were defined as follows (Table 2). Hypertensive subjects had previously been diagnosed with hypertension and were being treated with antihypertensive medication, or their SBP/diastolic blood pressure (DBP) was ≥140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was <140/90 mmHg. Hypertriglyceridemia was defined as triglyceride ≥150 mg/dl (Japan Atherosclerosis Society, Tokyo, Japan).

DNA Analysis

DNA was extracted from whole blood with a QIAamp Blood Kit (Qiagen K.K., Tokyo, Japan). Multiplex fluorescent-based genotyping was performed using the ABI Prism Linkage Mapping Set, HD-5 (PE Biosystems, Foster City, USA). Polymerase chain reaction (PCR) analysis was used to genotype dinucleotide repeat markers. Sizes of the PCR products were determined with an ABI 3100 genetic analyzer (PE Biosystems). Peak heights derived from electropherograms of pooled DNA amplifications were converted to 96 allele-frequency counts. The average spacing between markers was 5 centimorgans (cM).

Statistical Methods

All statistical analyses were performed on a personal computer using SPSS software (Version 10.0J for Windows; SPSS Inc., Chicago, USA). Categorical variables were compared using the χ^2 statistic or Fisher's exact test, as appropriate. Logistic regression models were used to assess whether the polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the polymorphism and confounding factors. Sex, age, body mass index, plasma total cholesterol, high density lipoprotein-cholesterol, triglyceride levels, smoking status, and alcohol consumption were considered to be confounding factors (Table 2). Logarithmically transformed plasma triglyceride values were used in the analyses. General linear regression models were used to assess whether the polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and confounding factors. A probability (p) value of less than 0.05 was considered statistically significant.

Results

Genome-Wide Linkage Disequilibrium Mapping of Hypertension

To conduct a large-scale LD mapping of hypertension efficiently, we used a multi-layered design and pooled DNA screening. First, 453 of 811 dinucleotide repeat markers were genotyped using the pooled DNA of the first subgroup and analyzed for an allelic association with hypertension, whereas the other 358 markers remain to be genotyped. As a result, 205 of 453 markers showed a nominal association. Subsequently, 203 of the 205 markers were genotyped and analyzed using the pooled DNA of the second group, with the other two markers remaining to be genotyped. As a result, 151 of 203 markers showed a nominal allelic association in the group made up of the first and second groups combined. After this rough screening of markers associated with hypertension using pooled DNA, 77 of the 151 markers were individually genotyped and analyzed in the group made up of the first and second groups combined. The other 74 markers remain to be genotyped. As a result, 28 of the 77 markers were confirmed to show a nominal allelic association in the group made up of the first and second groups combined. Further, 23 of the 28 markers were genotyped in the third group and analyzed in the group made up of the first, second, and third groups, whereas the other five markers remain to be genotyped. As a result, 15 of the 23 markers showed a nominal allelic association in the group made up of the first, second, and third groups (Table 3). Thus, although the LD screening of hypertension is still underway, 15 markers showed a nominal allelic association in the group made up of the first, second, and third groups combined, as well as in the group made up of the first and second groups combined.

Subsequent comparisons of individual allele frequencies at these loci between the hypertensive and normotensive subjects revealed only one allele at each locus showing significance at 11 loci and two alleles at each locus showing significance at the other four loci, resulting in detection of a total of 19 alleles showing significance (Table 3). This suggests the presence of 19 hypertension-related loci in close proximity to the 15 loci. The levels of statistical significance of the 19 alleles ranged from 0.038 to $10^{-4}\times5.3$. Of the 19 alleles, 10 alleles appeared to have a risk-increasing effect on the development of hypertension, while nine alleles appeared to have a protective effect against the development of hypertension. The odds ratios (ORs) of the 19 alleles ranged from 0.08 to 5.12. It should be noted that in this LD screening of hypertension, we could detect an OR of 2.6 with 80% power at a 5% type I error probability.

Table 3. Summary of Alleles Associated with Hypertension in Combined Group of First, Second, and Third Groups

Maker	Locus	No. of alleles	Allele*	Allele frequency HT	Allele frequency NT	OR	95% CI	p value
D1S2667	1p36.23	10	3	0.369	0.259	1.67	1.16–2.40	0.006
D1S2667	1p36.23	10	8	0.075	0.143	0.48	0.28–0.84	0.010
D1S2785	1q43	10	8	0.195	0.117	1.82	1.14–2.92	0.013
D2S2163	2p16.3	10	3	0.415	0.309	1.59	1.14–2.28	0.011
D3S1581	3p21.31	14	11	0.004	0.045	0.08	0.02–0.38	0.002
D4S2912	4p15.1	11	5	0.040	0.008	5.12	1.29–20.33	0.020
D4S2912	4p15.1	11	8	0.435	0.224	0.54	0.34–0.86	0.009
D5S630	5p15.2	24	3	0.187	0.120	1.68	1.03–2.74	0.036
D7S493	7p15.3	19	14	0.026	0.066	0.37	0.16–0.88	0.024
D7S493	7p15.3	19	15	0.004	0.031	0.11	0.64–0.02	0.014
D7S515	7q22.1	12	3	0.031	0.000			0.006
D7S515	7q22.1	12	4	0.069	0.132	0.49	0.27–0.89	0.019
D9S288	9p24.2	13	3	0.049	0.014	3.57	1.07–11.88	0.038
D10S537	10q22.2	10	8	0.138	0.040	3.80	1.98–7.27	5.3×10^{-5}
D10S217	10q26.3	12	7	0.100	0.190	0.48	0.29–0.79	0.004
D13S1320	13q21.1	8	3	0.019	0.068	0.27	0.10–0.71	0.008
D14S1051	14q32.33	7	1	0.939	0.888	1.94	1.04–3.63	0.037
D16S520	16q24.3	10	4	0.179	0.103	1.90	1.12–3.25	0.018
D16S3075	16p13.3	9	3	0.062	0.121	0.48	0.26–0.88	0.018

*Marker allele showing association in single-allele test. HT, hypertensive subject; NT, normotensive subject; OR, odds ratio; CI, confidence interval.

Table 4. D10S537 Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Genotype frequency: Normotensive (n=1,687)	Genotype frequency: Hypertensive (n=739)	p value	OR	95% CI
D10S537 genotypes					
Allele 8 carrier	251 (14.9%)	134 (18.1%)			
Allele 8 non-carrier	1,436 (85.1%)	605 (81.9%)	0.044	1.27	1.01–1.59
D10S537 alleles					
Allele 8	260 (7.7%)	138 (9.3%)			
Other alleles	3,114 (92.3%)	1,340 (90.7%)	0.057	1.23	0.99–1.52

OR, odds ratio; CI, confidence interval.

Association of D10S537 with Hypertension in the General Population

D10S537 showed a highly significant association with hypertension among the 15 markers thus far selected through the ongoing genome-wide LD mapping (Table 3). We therefore analyzed the association between D10S537 and hypertension in the whole population from which the 146 hypertensive and 136 normotensive subjects were selected. This analysis showed that D10S537 was significantly associated with hypertension even in the whole population (p=0.044; OR=1.27; 95% CI=1.01–1.59; where 95% CI indicates 95% confidence interval) (Table 4). Adjustment for all confounding factors showed a non-significant but similar trend (p=0.069; OR=1.26; 95% CI=0.98–1.62). This associa-

Table 5. Association of D10S537 Genotype with Hypertension According to TG Status

Genotype	Genotype frequency: Normotensive (n=1,687)	Genotype frequency: Hypertensive (n=739)	p value	OR	95% CI
Normotriglyceridemia (n)					
Allele 8 carrier (272)	184 (14.9%)	88 (20.5%)			
Allele 8 non-carrier (1,392)	1,051 (85.1%)	341 (79.5%)	0.007	1.47	1.11–1.95
Hypertriglyceridemia (n)					
Allele 8 carrier (113)	67 (14.8%)	46 (14.8%)			
Allele 8 non-carrier (649)	385 (85.2%)	264 (85.2%)	0.995	0.99	0.70–1.50

TG, triglyceride; OR, odds ratio; CI, confidence interval.

Table 6. Association of D10S537 Genotype with Blood Pressure According to TG Status

Genotype	SBP (mmHg)	DBP (mmHg)
Normotriglyceridemia (n)		
Allele 8 carrier (272)	132.3 (16.1)	76.7 (9.7)
Allele 8 non-carrier (1,392)	129.4 (15.3)	75.2 (9.1)
p value	0.005	0.016
Hypertriglyceridemia (n)		
Allele 8 carrier (113)	136.6 (15.2)	79.8 (9.3)
Allele 8 non-carrier (649)	136.8 (15.8)	79.9 (9.4)
p value	0.941	0.896
Total (n)		
Allele 8 carrier (385)	133.6 (16.0)	77.6 (9.7)
Allele 8 non-carrier (2,041)	131.8 (15.8)	76.7 (9.5)
p value	0.038	0.093

TG, triglyceride. Data are mean (SD). SBP, systolic blood pressure; DBP, diastolic blood pressure.

tion was pronounced in subjects with normotriglyceridemia (p=0.007; OR=1.47; 95% CI=1.11–1.95) (Table 5). The association in subjects with normotriglyceridemia was significant even after adjustment for all confounding factors (p=0.049; OR=1.36; 95% CI=1.00–1.84). In contrast, D10S537 was not associated with hypertension in subjects with hypertriglyceridemia (p=0.995; OR=0.99; 95% CI=0.70–1.50).

More quantitatively, we further analyzed the association between D10S537 and blood pressure (Table 6). This analysis showed a significant association between D10S537 and SBP (p=0.038). Adjustment for all confounding factors showed a non-significant but similar trend (p=0.101). The analysis also showed a non-significant trend supporting the presence of an association between D10S537 and DBP (p=0.093). After adjustment for all confounding factors, the p value was 0.260. Analysis of the association between D10S537 and SBP according to stratified triglyceride levels showed a significant association between D10S537 and blood pressure (p=0.005 for SBP and p=0.016 for DBP). Adjustment for all confounding factors showed a non-significant but similar trend (p=0.058 for SBP and p=0.169 for DBP). In contrast, D10S537 was not associated with blood pressure in subjects with hypertriglyceridemia (p=0.941 for SBP and p=0.896 for DBP).

Discussion

We are presently conducting a large-scale LD mapping of hypertension using dinucleotide repeats and a multi-layered design. Two similar large-scale LD mappings of Alzheimer's disease have been conducted using tri- and tetranucleotide repeat markers (*34, 35*), and most of the markers were shown to overlap with each other. However, most of the markers that showed a significant association were different between the two mappings. This may have been attributable to the insufficient sample sizes in these mappings, and/or to their relaxed criteria for significance. Nevertheless, one of the markers associated with Alzheimer's disease in the first large-scale LD mapping of Alzheimer's disease (*34*), D10S1423, was repeatedly shown to be associated with Alzheimer's disease in ethnically different independent populations (*36*). Moreover, the region including this marker was coincidentally shown to be associated with Alzheimer's disease in the second large-scale LD mapping of Alzheimer's disease (*37*), although the region has never been detected in linkage mappings. In addition, in the case of diabetes mellitus, the insulin gene has been detected in association studies, but not in linkage mappings (*38, 37, 38*). Thus, linkage mappings may not be appropriate to find candidate regions influencing the development of common disorders with weak effects. In contrast, large-scale LD mappings may be potentially useful to select such candidate regions. However, large-scale LD mappings have several drawbacks, including the need to analyze a number of polymorphic markers in numerous subjects to increase their reliability.

In addition, large-scale LD mappings of hypertension are influenced by LD existing throughout a population. In this context, although the size of LD blocks is dependent on the loci, and the maximum size of LD blocks in Japanese appears to be larger than 2.3 cM (*39*), the mean size of LD blocks in Japanese remains to be estimated. However, given the finding that the mean size of LD blocks in northern Europeans is unlikely to be larger than 100 kb (*40*), our large-scale LD mapping using markers with an average spacing of 5 cM is far from able to detect all of the hypertension-related loci, and thus further mappings using more densely spaced markers are warranted.

Each of the two large-scale LD mappings of Alzheimer's disease employed approximately 50 cases and 50 controls in their initial mappings (*34, 35*). The numbers of loci detected in their mappings were 6 and 22, respectively (*34, 35, 41, 42*). In apparent agreement with these numbers, our mapping of hypertension detected 15 loci that were nominally associated with hypertension (Table 3). However, our mapping is still underway, and if our current rate of loci detection were to continue, the resulting number of hypertension-associated loci in the human genome would likely be too high. This may suggest that our mapping contained some false positive results. On the other hand, our high rate of loci detection might be partly attributable to the complex nature of hypertension. For example, the functions of many organs have an effect on blood pressure including the heart, vasculature, kidneys, adrenal gland, thyroid, sympathetic nerves, and brain. Moreover, many non-genetic factors, including age, body weight, stress, smoking, alcohol consumption, and diet, influence blood pressure. The effects of such non-genetic factors on blood pressure are also thought to be modified by genetic factors.

As a result of our ongoing large-scale LD mapping, D10S537 was selected for its highly significant association with hypertension in selected hypertensive and normotensive subjects (Table 3). However, our large-scale LD mapping is only a screening based on a relaxed criterion for significance to lessen false negatives, and the results of large-scale LD mapping may include false positives. For this reason, the results should be further examined in general populations. In this context, D10S537 was associated with hypertension also in our population (Table 4), supporting the efficacy of the screening to some extent. However, the association in the population was relatively weak, despite the fact that the 146 hypertensive and 136 normotensive subjects being used in the screening were included in the population. This stresses the need for studies in additional general populations, although the weak association in the population may have been due to the difference between the severe criteria for hypertensive subjects in the genome-wide LD mapping of hypertension and the relaxed criteria for hypertensive subjects in the population study. Moreover, because obvious candidate genes for hypertension have not yet been identified in the close proximity to D10S537, the biological plausibility of the association between D10S537 and hypertension, particularly in subjects with normotriglyceridemia (Table 5), remains to be investigated; such an association could simply represent a false positive error. Nevertheless, gene hunters might consider an examination of the surrounding markers. However, it will be worth having rigorous evidence in hand before undertaking positional cloning to avoid the unpleasant prospect of chasing a phantom locus (*38*). We therefore plan to examine D10S537 in additional populations with sufficient information on environmental factors.

The association between D10S537 and hypertension in subjects with normotriglyceridemia will require further consideration. In general, hypertriglyceridemia was associated with hypertension, although the mechanism remains obscure. Therefore, if a gene in close proximity to D10S537 is on a pathway from hypertriglyceridemia to hypertension, a polymorphism in the gene could alter the effect of hypertriglyceridemia on hypertension. If this is the case, allele 8 carriers might activate the pathway constantly, leading to a relatively constant level of blood pressure. In contrast, allele 8 non-carriers might activate the pathway depending on triglyceride levels, leading to a changing level of blood pressure depending on triglyceride levels.

Our large-scale LD mapping has additional limitations. First, because the markers used in the mapping are sparsely spaced, we likely missed many hypertension-related loci. More densely spaced markers should therefore be used in future studies. Moreover, LD is not complete even within an LD block. Microsatellite markers in LD with a functional polymorphism responsible for hypertension should therefore be less sensitive than the functional polymorphism itself in detecting an association with hypertension. Second, the sample size may have been insufficient to detect hypertension-related loci with minor effects on blood pressure or to show statistically conclusive evidence using a more strict p value corrected by the number of hypotheses tested. The detected markers should therefore be further examined in additional samples. Adjustment for confounding factors and analysis in the light of gene–gene and gene–environmental interactions could also be helpful to assess these results.

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Original Article

Association of the *GNAS1* Gene Variant with Hypertension Is Dependent on Alcohol Consumption

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The β-adrenoceptor (β-AR)–stimulatory guanine nucleotide-binding (Gs) protein system has been shown to play important roles in the cardiovascular system. The gene encoding the α-subunit of Gs proteins (*GNAS1*) is a candidate genetic determinant for hypertension. Because alcohol consumption is known to affect blood pressure partly through the β-AR–Gs protein system, we examined the possible interaction between *GNAS1* T393C polymorphism and drinking status in the association with hypertension in the present study. As a result, a non-significant but reasonable trend supporting the presence of an interaction was shown (p=0.076). In line with this trend, the T393C polymorphism significantly interacted with drinking status in the association with systolic blood pressure (p=0.028). Moreover, supporting the presence of an interaction, T allele carriers consistently had a higher probability of hypertension, higher systolic blood pressure, and higher diastolic blood pressure than CC homozygotes in non-drinkers and light drinkers. In contrast, CC homozygotes consistently had a higher probability of hypertension, higher systolic blood pressure, and higher diastolic blood pressure than T allele carriers in moderate to heavy drinkers. The present study also showed a significant interaction between the T393C polymorphism and drinking status in the association with pulse pressure (p=0.028), reflected by a significant association between the T393C polymorphism and pulse pressure in moderate to heavy drinkers (p=0.026). These findings may be helpful in conducting further molecular and biological studies on the relationship among the effects of alcohol, the β-AR–Gs protein system, and hypertension. (*Hypertens Res* 2003; 26: 439–444)

***Key Words*: guanine nucleotide-binding proteins, β-adrenoceptor, hypertension, polymorphism, alcohol**

Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) couple seven transmembrane receptors to adenylyl cyclase. Each G protein is composed of three distinct subunits (α, β, and γ). Based on amino acid similarities of the α-subunits, G proteins are classified into four major classes (Gs, Gi/o, Gq/11, and G12/13) (*1–3*). Ubiquitously expressed Gs proteins mediate signal transduction across cell membranes. Stimulation of the Gs subfamily activates adenylyl cyclase, resulting in accumulation of the second messenger, cAMP (*1–3*).

The β-adrenoceptor (β-AR)–Gs protein system has been shown to play important roles in the cardiovascular system. To date, three distinct β-AR subtypes have been identified (β1-AR, β2-AR, and β3-AR) (*4–6*). Signals of all three β-AR subtypes are transmitted by coupling to Gs proteins. However, in the cardiovascular system, the α-subunit of Gs proteins couples to β1-AR and β2-AR (*7*). The gene encoding the α-subunit of Gs proteins (*GNAS1*), comprising 13 exons, maps to 20q13.2–q13.3 (*8*).

Recently, based on several lines of biological evidence suggesting an association of the α-subunits of G proteins with hypertension (*9–11*), an initial study examined the association between a common silent polymorphism (T393C) in *GNAS1* and hypertension (*12*). This study showed that the T393C polymorphism was significantly associated with hypertension. Subsequently, we also studied this association in a large Japanese population (*13*), resulting in replication of the results of the initial study. Additionally, in the same population, we showed that the T393C polymorphism significantly interacted with cigarette smoking in the pathogenesis of hypertension (*13*). Because alcohol consumption, like cigarette smoking, is known to affect blood pressure at least partly through the β-AR–Gs protein system (*14–16*), we speculated that the T393C polymorphism could also interact with alcohol consumption in the pathogenesis of hypertension. Because information on alcohol consumption was not available at the time of this earlier study, we were unable to examine the possible interaction between the T393C polymorphism and drinking status. However, because, more recently, such information was available in subjects included in the population, we were able to examine the possible interaction in the present study.

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Table 1. Characteristics of Participants According to Hypertensive Status

Variable	*Normotensive (n=1,609)*	*Hypertensive (n=699)*	*p value*
Sex (male %)	86.1	89.6	NS
Age (years)	51.2 (8.1)	55.1 (6.2)	<0.001
Body mass index (kg/m²)	22.8 (2.7)	24.4 (3.0)	<0.001
Systolic blood pressure (mmHg)	123.7 (9.7)	150.9 (10.1)	<0.001
Diastolic blood pressure (mmHg)	72.0 (6.2)	87.6 (6.3)	<0.001
Pulse pressure (mmHg)	51.7 (5.1)	63.2 (6.7)	<0.001
Total cholesterol (mg/dl)	196.5 (31.2)	203.4 (32.0)	<0.001
HDL cholesterol (mg/dl)	60.4 (13.4)	60.5 (13.7)	NS
Triglyceride (mg/dl)	130.0 (74.0)	157.2 (84.8)	<0.001
Smoking (heavy smoker %)	28.7	24.7	NS
Alcohol (moderate to heavy drinker %)	29.1	37.9	<0.001

Data are mean (SD). HDL, high density lipoprotein. NS, not significant. Blood pressure readings prior to the start of antihypertensive treatment were not available for 141 hypertensive subjects whose values were measured under treatment.

Methods

Subjects

According to the criteria described below, 699 hypertensive subjects and 1,609 normotensive subjects were selected from among the employees of a company in the Ehime region of Japan (Table 1) (*13*). All subjects were Japanese. They had participated in medical check-ups 1 to 11 times (mean 6.2 times per person), and the mean values of variables in their personal health records were used in the analyses. All subjects provided their informed consent to participate, and 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 medications, 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. Heavy smokers were defined as subjects smoking 20 or more cigarettes per day. Drinkers (moderate to heavy) were defined as subjects drinking 25 g of ethanol or more per day.

DNA Analysis

The polymerase chain reaction (PCR) was used to detect the *GNAS1* T393C polymorphism (*12*). The sense oligonucleotide primer was 5′-CTCCTAACTGACATGGTGCAA-3′ and the antisense primer was 5′-TAAGGCCACACAAGTCGGGGT-3′. The PCR mixture contained 10 ng genomic DNA, 10 pmol of each primer, 250 μmol/l dNTP, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.4, and 1 U Taq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) in a final volume of 25 μl. After initial denaturation at 94°C for 5 min, the DNA was amplified by 35 PCR cycles: of denaturation at 94°C *for 45 s, annealing at 55°C for 45 s, and extension at*

Table 2. *GNAS1* Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype	Genotype frequency		p value	OR	95% CI
	Normotensive (n=1,609)	Hypertensive (n=699)			
GNAS1 genotypes					
TT	500 (31%)	238 (34%)			
TC	776 (48%)	342 (49%)			
CC	333 (21%)	119 (17%)	0.046*	1.27*	1.01–1.60*
GNAS1 alleles					
T	1,776 (55%)	818 (59%)			
C	1,442 (45%)	580 (41%)	0.036	1.15	1.01–1.30

*p values, OR and 95% CI are for TT+TC *vs.* CC. *GNAS1*, α-subunit of stimulatory guanine nucleotide-binding protein gene; OR, odds ratio; CI, confidence interval.

Table 3. Association of *GNAS1* Genotype with Hypertension According to Drinking Status

Genotype	Genotype frequency		p value	OR	95% CI
	Normotensive (n=1,609)	Hypertensive (n=699)			
Non-drinkers and light drinkers					
TT+TC	897 (79%)	367 (85%)			
CC	244 (21%)	67 (15%)	0.0084	1.49	1.11–2.00
Moderate to heavy drinkers					
TT+CC	379 (81%)	213 (80%)			
CC	89 (19%)	52 (20%)	0.84	0.96	0.66–1.41

The abbreviations are the same as Table 2.

72°C for 2 min, followed by final extension at 72°C for 7 min. The amplified PCR products were digested with 3 U of the restriction enzyme, *Fok*I. The digested samples were separated by electrophoresis through an agarose gel and visualized under ultraviolet light after ethidium bromide staining. A thymine at nucleotide position 393 was shown by a fragment of 345 base pairs (bp), whereas a cytosine at nucleotide position 393 was shown by two fragments of 263 bp and 82 bp. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated (*13*).

Statistical Methods

Analysis of variance was used to assess differences in means and variances of continuous variables. Logistic regression models were used to assess whether the *GNAS1* T393C polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the T393C polymorphism and drinking status. Sex, age, body mass index, plasma total cholesterol, high density lipoprotein cholesterol, triglyceride levels, smoking status, and alcohol consumption were considered to be confounding factors (Table 1). Logarithmically transformed plasma triglyceride values were used in the analyses. General linear regression models were used to assess whether the T393C polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and drinking status. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software.

Results

Association of *GNAS1* T393C Polymorphism with Hypertension

A total of 2,308 Japanese individuals from the Ehime region were categorized as hypertensive or normotensive and genotyped for the T393C polymorphism (Table 2). The frequencies in both hypertensive and normotensive subjects were in Hardy-Weinberg equilibrium. Logistic regression analysis showed a significant difference in the frequencies of the alleles (p=0.036) and genotypes (p=0.046 for TT+TC *vs.* CC) between the hypertensive and normotensive subjects, as shown also in our previous study (*13*) (Table 2).

Interaction of *GNAS1* T393C Polymorphism with Alcohol Consumption in the Association with Hypertension

In the present study, we analyzed the possible interaction of the *GNAS1* T393C polymorphism with drinking status in the association with hypertension in a logistic regression model.

Table 4. Association of *GNAS1* Genotype with Blood Pressure According to Drinking Status

Genotype	Systolic blood pressure	Diastolic blood pressure	Pulse pressure
Non-drinkers and light drinkers			
TT+TC (1,264)	130.4 (16.2)	75.7 (9.7)	54.7 (7.7)
CC (311)	129.1 (15.2)	74.7 (9.3)	54.4 (7.5)
p value	0.18	0.092	0.51
Moderate to heavy drinkers			
TT+TC (592)	135.3 (14.5)	79.2 (8.5)	56.0 (7.6)
CC (141)	137.8 (16.3)	80.2 (9.0)	57.6 (8.2)
p value	0.074	0.23	0.026
Total			
TT+TC (1,856)	132.0 (15.8)	76.8 (9.5)	55.1 (7.7)
CC (452)	131.8 (16.0)	76.4 (9.5)	55.4 (7.9)
p value	0.82	0.39	0.50

Data are mean (SD). The abbreviations are the same as Table 2.

This analysis showed a non-significant trend supporting the presence of an interaction (p=0.076). Given the suggestive trend, we next analyzed the association between the T393C polymorphism and hypertension according to stratified alcohol consumption (Table 3). This analysis revealed that the T393C polymorphism was associated with hypertension in non-drinkers and light drinkers (p=0.0084, OR=1.49, 95% CI=1.11–2.00, where OR indicates odds ratio and 95% CI indicates 95% confidence interval). This association remained significant even after adjustment for all confounding factors (p=0.046, OR=1.36, 95% CI=1.01–1.90). In contrast, the T393C polymorphism was not associated with hypertension in moderate to heavy drinkers (p=0.84, OR=1.04, 95% CI=0.71–1.52) (Table 3).

More quantitatively, we further analyzed the interaction between the T393C polymorphism and drinking status in the association with blood pressure in general linear regression models. This analysis showed significant interactions between the T393C polymorphism and drinking status in the association with SBP (p=0.028) and with pulse pressure (p=0.026). The analysis also showed a non-significant trend supporting the presence of an interaction between the T393C polymorphism and drinking status in the association with DBP (p=0.059).

Given these interactions and trends, we next analyzed the association between the T393C polymorphism and blood pressure according to stratified alcohol consumption in general linear regression models (Table 4). This analysis showed a significant association between the T393C polymorphism and pulse pressure in moderate to heavy drinkers. However, except for this association, the analysis failed to show any significant association between the T393C polymorphism and blood pressure in stratified alcohol consumption groups.

Discussion

We previously showed a significant interaction between the *GNAS1* T393C polymorphism and cigarette smoking status in the association with hypertension in a Japanese population (*13*). Prompted by the presence of this interaction, in the present study, we assessed the interaction between the *GNAS1* T393C polymorphism and alcohol consumption in the association with hypertension in the same population. As a result, a non-significant trend supporting the presence of an interaction was shown. In line with this trend, our results also showed a significant interaction between the T393C polymorphism and drinking status in the association with SBP, and a non-significant trend supporting the presence of an interaction between the T393C polymorphism and drinking status in the association with DBP. These results suggest that the apparent effect of the T393C polymorphism differed depending on alcohol consumption. Association analyses of the T393C polymorphism with blood pressure in stratified alcohol consumption groups also supported this difference. Although a significant association was shown only in non-drinkers and light drinkers, subjects with the TT and TC genotypes consistently had a higher probability of hypertension, higher SBP, and higher DBP than subjects with the CC genotype in this group (Tables 3 and 4). In contrast, subjects with the CC genotype consistently had a higher probability of hypertension, higher SBP, and higher DBP than subjects with the TT and TC genotypes in moderate to heavy drinkers (Tables 3 and 4).

The present study also showed a significant interaction between the *GNAS1* T393C polymorphism and alcohol consumption in the association with pulse pressure, reflected by a significant association between the T393C polymorphism and pulse pressure in moderate to heavy drinkers. A wide pulse pressure resulting largely from excessive large artery stiffness is associated with systolic hypertension (*17*). Consistent with this established association, the present study showed a non-significant but strong trend supporting an association between the T393C polymorphism and SBP in moderate to heavy drinkers (Table 4).

Taking these results together, the present study suggested an interaction between the *GNAS1* T393C polymorphism and alcohol consumption in the association with hypertension and with pulse pressure. Because alcohol is known to affect blood pressure through the β-AR–Gs protein system (*14–16*), an interaction between the T393C polymorphism and alcohol consumption in the association with hypertension seems reasonable. This interaction could be reflected by the interaction between the T393C polymorphism and alcohol consumption in the association with pulse pressure, because hypertension promotes atherosclerosis (*18, 19*), which results in large-vessel stiffening and increased wave reflection, and thereby amplifies pulse pressure (*20*). However, the precise mechanism of these interactions remains elusive. Previous studies have provided evidence that the T allele of the T393C polymorphism is associated with poor responsiveness to β-blockade (*12*) and that the T393C polymorphism interacts with cigarette smoking status in the pathogenesis of hypertension (*13*). Based on this evidence, we previously speculated that the TT and TC genotypes or genotypes in linkage disequilibrium with them might produce a constant amount of the α-subunit of Gs proteins independent of activation of the sympathetic nervous system (*13*). In contrast, the CC genotype or genotype in linkage disequilibrium with it might produce a controlled amount of α-subunit of Gs proteins. Indeed, subjects with the CC genotype tended to be more strongly affected by alcohol consumption than subjects with the TT and TC genotypes in the association with hypertension (Tables 3 and 4). Thus, the above explanation appears also to be applicable to the interaction between the T393C polymorphism and alcohol consumption in the association with hypertension. Alternatively, depending on the genotypes, alcohol could influence glucose metabolism, which in turn could influence blood pressure (*21*). Indeed, *GNAS1* gene knockout mice have been shown to exhibit a significant increase in insulin sensitivity (*22*). Data on the parameters of glucose metabolism, however, were not available in our population, preventing assessment of the association between the T393C polymorphism and glucose metabolism. Another possible explanation for the interaction between the T393C polymorphism and alcohol consumption in the association with hypertension and with pulse pressure might be that this interaction might reflect the interaction between the T393C polymorphism and cigarette smoking status. Indeed, in our population, alcohol consumption was associated with cigarette smoking status (data not shown). Moreover, the T393C polymorphism interacted significantly with cigarette smoking status in the association with hypertension (p=0.00050) and with pulse pressure (p=0.00067). However, considering that both cigarette smoking and alcohol consumption could affect blood pressure through the β-AR–Gs protein system, the final explanation may be less plausible than the former two.

The present study has additional limitations. Information on the history of alcohol consumption and the actual amount of alcohol drunk by subjects was not available in our population, preventing quantitative assessment of alcohol consumption. In this regard, analysis of the aldehyde dehydrogenase 2 gene may be helpful to some extent (*23*). Moreover, the present study did not assess gene-gene interaction, which is a candidate factor for modifying the evaluation of an association. In this context, interaction analyses of the *GNAS1* gene with other genes involved in the β-AR–Gs protein system may be helpful to improve understanding of the relation between the β-AR–Gs protein system and hypertension.

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Association of Endothelin-1 Gene Variant With Hypertension

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Abstract—Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide produced by endothelial and smooth muscle cells. Many lines of biological evidence suggest that the ET-1 gene is a candidate gene for hypertension. Moreover, recent association studies suggested that a G/T polymorphism with an amino acid substitution (Lys/Asn) at codon 198 in exon 5 of the ET-1 gene interacts with body mass index (BMI) in association with blood pressure. They suggested that T carriers are more sensitive to weight gain than GG homozygotes in association with blood pressure. However, association studies are often irreproducible, and the first study often suggests a stronger genetic effect than is found by subsequent studies. We therefore assessed the interaction in 2 large Japanese populations. The present study showed a nonsignificant but similar trend to the results of previous reports. Moreover, in line with previous reports, this study revealed a significant interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with hypertension in our populations ($P=0.027$). The interaction was significant, even after adjustment for gender and age ($P=0.045$) and for all confounding factors ($P=0.044$). T carriers were more sensitive to weight gain than GG homozygotes in association with hypertension. Considering the combined impact of obesity and hypertension on the development of cardiovascular and cerebrovascular disorders, T allele carriers might represent elective targets for therapy to lower their body weight. (*Hypertension*. 2003;41:163-167.)

Key Words: endothelin ■ hypertension, essential ■ genetics ■ polymorphism ■ body mass index

Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide produced by vascular endothelial cells.[1] Some patients with moderate-to-severe essential hypertension, similar to some experimental rat models with severe blood pressure elevation, exhibit enhanced endothelial expression of the ET-1 gene.[2] Plasma ET-1 concentration is elevated in hypertensive patients.[3–6] An endothelin-receptor antagonist significantly lowered blood pressure in patients with essential hypertension.[7] Given these lines of biological evidence, the ET-1 gene is a candidate responsible for hypertension.

Hypertension is a common, complex phenotype and has been intensively studied to identify susceptibility loci in humans. Nonetheless, there is no known genotypic polymorphism consistently associated with hypertension in humans, thus far. Moreover, albeit that the development of hypertension is considered to be due at least partly to gene-gene and gene-environmental interactions, fewer interaction analyses have been conducted than simple association analyses. In this regard, the ET-1 gene is an attractive candidate because, in addition to its biological function, this gene has been shown to interact with body mass index (BMI) in association with blood pressure in 3 large populations.[8,9] However, association studies are often irreproducible, and the first study often suggests a stronger genetic effect than is found by subsequent studies.[10] We therefore assessed the interaction in 2 large Japanese populations. The present study showed a significant interaction between the ET-1 K198N (G/T) polymorphism and BMI in the association with hypertension.

Methods

Subjects

The clinical characteristics of the subjects included in the study are shown in Table 1. Population 1 (n=2460) originated from the Ehime region of Japan, and population 2 (n=806) from the Hyogo region of Japan. The initial rate of participation in the present study was 99% and 67% for populations 1 and 2, respectively. All subjects were Japanese urban residents. Subjects in population 1 participated in medical checkups 1 to 11 times (average 5.2 times per person), and the mean values of variables in the personal health records were used in analyses. Subjects in population 2 also underwent a medical checkup, and the values of variables in the personal health records were used in analyses. All subjects gave informed consent, and the study was approved by the ethics committee of Ehime University.

Diagnostic Categories

Each subject was assigned to one of the blood pressure diagnostic categories defined by the following criteria. Hypertensive subjects were those who had a previous diagnosis of hypertension and were being treated with antihypertensive medications (7.6%) or whose systolic/diastolic blood pressure was ≥140/90 mm Hg. Normotensive subjects were those who had never been treated with medication for hypertension and whose systolic/diastolic blood pressure was <140/90 mm Hg. Blood pressure was measured with a mercury sphygmomanometer fewer than 3 times per year in a sitting position in clinics. Obesity was defined as BMI ≥25 kg/m² (Japan Society for the Study of Obesity). Hypertriglyceremia was defined as triglyceride (TG) ≥150 mg/dL (Japan Atherosclerosis Society).

DNA Analysis

The TaqMan chemical method was used to detect the ET-1 K198N (G/T) polymorphism. The forward primer was 5′-GGT CGG AGA CCA TGA GAA ACA-3′, the reverse primer was 5′-TGT GGG TCA CAT AAC GCT CTC T-3′, the T allele specific probe was 5′-Fam-AAG GCA ATC CCT C-Tamura-3′, and the G allele specific probe was 5′-Tet-AAG GCA AGC CCT C-Tamura-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. We validated the TaqMan chemical method with more standard polymerase chain reaction (PCR)/single-strand conformation polymorphism and PCR/sequencing methods in selected 57 subjects.

Statistical Methods

Analysis of variance was used to assess differences in means and variances of continuous variables. Comparisons of categorical variables were performed using the χ^2 test. General linear models were used to assess whether the ET-1 K198N (G/T) polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and BMI in regression models. Logistic regression models were used to assess whether the ET-1 K198N (G/T) polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the polymorphism and BMI in regression models. Logarithmically transformed plasma TG values were used in the analyses. Probability values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software (SPSS Inc).

Results

Frequencies of Alleles and Genotypes

Table 1 presents the clinical characteristics of the participants as a function of the 3 genotypes. The relative frequencies of the GG, GT, and TT genotypes were 53%, 38%, and 8%, respectively. The allele frequencies were 72% and 28% for the G and T alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium. The frequencies of the genotypes and the alleles in Japanese were similar to, but significantly different from, those in Caucasians.

Interaction of ET-1 G/T Polymorphism With BMI in the Association With Blood Pressure and Hypertension Status

Because the Etude Cas-Temoin de l'Infarctus Myocarde (ECTIM) study[9] has shown a strong interaction of the ET-1 K198N (G/T) polymorphism with BMI in association with blood pressure, we analyzed the interaction in our 2 populations. This analysis showed that the interaction between the polymorphism and BMI was not significant (Table 2, Figure 1). In relation to the interaction, the ECTIM study also showed that both systolic and diastolic blood pressure in T allele carriers were significantly higher than those in GG homozygotes in obese subjects but nonsignificantly lower in lean subjects. Similar analyses in our populations showed that both systolic and diastolic blood pressures in T allele carriers were nonsignificantly higher than those in GG homozygotes in obese subjects and nonsignificantly lower in lean subjects (Table 3). These results were similar when subjects on current antihypertensive treatment were excluded.

However, blood pressure readings before the start of antihypertensive treatment were not available for 248 hypertensive subjects, and the inclusion or exclusion of subjects with antihypertensive treatment could influence the distribution of blood pressure. In addition, blood pressure is unstable even in the resting condition. Therefore, considering that logistic regression analyses may be more suitable than linear regression analyses and t statistics, we analyzed the possible interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with hypertension status in our populations. These analyses showed a significant interaction in population 1 ($P=0.035$; OR=1.070, 95% CI=1.005 to 1.134, where OR indicates odds ratio and 95% CI indicates 95% confidence interval) (Figure 2a). A similar but nonsignificant interaction was shown in population 2 ($P=0.55$; OR=1.038, 95% CI=0.918 to 1.174) (Figure 2b). Analysis combining populations 1 and 2 yielded a probability value of 0.027 for the interaction between the polymorphism and BMI in association with hypertension (Figure 2c). The interaction was significant even after adjustment for gender and age ($P=0.045$) and for all confounding factors ($P=0.044$).

Discussion

Association studies are often irreproducible.[10] Replication studies in large populations are indispensable to establishing an association.[11] The ECTIM study showed a strong interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with both systolic and diastolic resting blood pressure levels.[9] That is, the study has shown that T carriers are more sensitive to weight gain than GG homozygotes in association with blood pressure. Moreover, the study has shown that, in obese subjects, both systolic and diastolic resting blood pressure levels are significantly higher in T carriers than GG homozygotes.

However, other studies have shown similar but not the same results. The Glasgow Heart Scan study showed a similar strong interaction between the polymorphism and BMI in association with the maximum blood pressure achieved during a treadmill exercise test, but not with the resting blood pressure.[8]

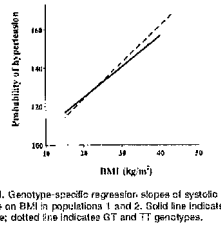

Figure 1. Genotype-specific regression slopes of systolic blood pressure on BMI in populations 1 and 2. Solid line indicates GG genotype; dotted line indicates GT and TT genotypes.

The Ohasama study did not assess the interaction, but has shown a significant association, that is, in obese subjects, casual diastolic blood pressure level is significantly higher in T carriers than in GG homozygotes.[9] The study has also shown a similar trend in the relation of the polymorphism with casual systolic blood pressure in obese subjects.

Finally, the present study showed a similar trend that T carriers are more sensitive to weight gain than GG homozygotes in association with blood pressure. This study also showed a similar trend that, in obese subjects, both systolic and diastolic resting blood pressure were higher in T carriers than in GG homozygotes. Thus, the trends were all similar to those in previous reports. Moreover, in line with previous reports, the present study revealed a significant interaction between the ET-1 K198N (G/T) polymorphism and BMI in the association with hypertension in population 1, and a similar trend was shown in population 2.

Taken together, all of the studies have shown a similar trend, that is, that T allele carriers are more sensitive to weight gain than GG homozygotes in association with blood pressure and with hypertension (chance occurrence <0.063), providing evidence in favor of interaction between the polymorphism and BMI in association with hypertension. Consequently, the ET-1 gene may be a promising candidate responsible for hypertension. However, the first study often suggests a stronger genetic effect than is found by subsequent studies, as in the case of the ET-1 polymorphism.[10] Therefore, further studies in large populations and a systematic meta-analytical approach are needed to accurately assess the genetic effect of the ET-1 gene. Moreover, haplotype studies, as well as examinations of variants in linkage disequilibrium with the polymorphism, are also needed.

To establish an association, it is also important that reported associations make biological sense and that associated alleles affect the gene product in a physiologically meaningful way.[11] In this context, plasma ET-1 levels were shown to be significantly higher in obese normotensives than in lean normotensives, suggesting an influence of obesity on plasma ET-1 level.[12,13] Indeed, weight loss significantly decreased the plasma ET-1 level in both obese normotensives and obese hypertensives.[12–14] Thus, the interaction between the ET-1 polymorphism and BMI may make biological sense. However, thus far, there is no evidence showing that the ET-1 K198N (G/T) polymorphism affects the gene product in a physiologically meaningful way, although the polymorphism changes the corresponding amino acid (Lys/Asn). Therefore, it is required to investigate a possible biological change of the gene product by the K198N (G/T) polymorphism or another variant in linkage disequilibrium with it.

In conclusion, the present study revealed a significant interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with hypertension in large Japanese populations. This result is in line with biological evidence on ET-1 and with the results of 3 previous association studies. Considering the combined impact of obesity and hypertension on the development of cardiovascular and cerebrovascular disease, T allele carriers might represent elective targets for therapy to lower their body weight.

Perspectives

Thus far, the interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with blood pressure and hypertension was assessed in 5 large populations including ours. Although these populations were studied in different design and differed in characteristics including BMI and race, all of the results showed a similar trend, suggesting the presence of the interaction. Consequently, together with several lines of biological evidence, the ET-1 gene may be a promising candidate gene for hypertension. Meanwhile, this study may have a broad implication, the importance of categorical analyses of blood pressure. The present study showed a significant interaction between the ET-1 K198N (G/T) polymorphism and BMI in association with hypertension, but a nonsignificant trend in association with blood pressure. This is possibly due to the unstable nature of blood pressure and to the presence of treated hypertensive subjects. Thus, in some populations, blood pressure may have much information content but less correct information than categorical hypertension status.

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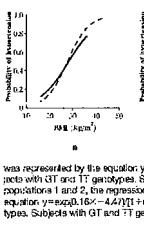

Figure 2. Genotypic variations in relationship between BMI and hypertension. a, In population 1, the regression between BMI and the probability of having hypertension in subjects with GG genotype was represented by the equation $y=\exp(0.16\times-4.53)/[1+\exp(0.16\times-4.53)]$. The equation was $y=\exp(0.22\times-5.10)/[1+\exp(0.22\times-5.10)]$ in subjects with GT and TT genotypes. Subjects with GT and TT genotypes show a steeper slope than those with GG genotype ($P=0.035$). b, In population 2, the regression between BMI and the probability of having hypertension in subjects with GG genotype was represented by the equation $y=\exp(0.21\times-5.73)/[1+\exp(0.21\times-5.73)]$ in subjects with GT and TT genotypes. Subjects with GT and TT genotypes show a steeper slope than those with GG genotype ($P=0.55$). c, Combining populations 1 and 2, the regression between BMI and the probability of having hypertension in subjects with GG genotype was represented by the equation $y=\exp(0.16\times-4.47)/[1+\exp(0.16\times-4.47)]$. The equation was $y=\exp(0.22\times-5.97)/[1+\exp(0.22\times-5.97)]$ in subjects with GT and TT genotypes. Subjects with GT and TT genotypes show a steeper slope than those with GG genotype ($P=0.027$).

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TABLE 1. Clinical Characteristics of Participants According to ET1 G/T Polymorphism

	Population 1				Population 2				Populations 1 and 2			
Characteristics	GG (n=1283)	GT (n=977)	TT (n=200)	P	GG (n=421)	GT (n=327)	TT (n=53)	P	GG (n=1704)	GT (n=1229)	TT (n=269)	P
Men, %	37.1	38.9	33.5	0.50	41.9	38.9	54.1	0.42	65.9	64.5	63.6	0.32
Age, y	52.1 (0.2)	51.9 (0.3)	51.3 (0.6)	0.37	54.8 (0.5)	53.5 (0.5)	53.5 (1.0)	0.030	52.7 (0.2)	52.3 (0.2)	51.8 (0.5)	0.099
BMI, kg/m²	23.3 (0.1)	23.2 (0.1)	23.2 (0.2)	0.28	23.6 (0.1)	22.9 (0.2)	22.7 (0.3)	0.54	23.2 (0.1)	23.1 (0.1)	23.1 (0.2)	0.27
SBP, mm Hg	121.4 (0.4)	122.3 (0.5)	120.3 (1.0)	0.55	125.1 (0.9)	124.5 (1.1)	121.3 (2.2)	0.53	122.8 (0.4)	123.0 (0.5)	122.4 (0.9)	0.87
DBP, mm Hg	76.5 (0.3)	76.8 (0.3)	75.0 (0.6)	0.91	77.8 (0.6)	77.5 (0.7)	77.9 (1.5)	0.99	76.8 (0.3)	77.1 (0.3)	75.7 (0.6)	0.53
T-Cho, mg/dL	198.5 (0.9)	198.1 (1.0)	197.5 (2.2)	0.68	200.5 (1.6)	198.5 (1.9)	198.5 (4.1)	0.39	199.0 (0.9)	198.2 (0.9)	197.7 (1.9)	0.43
HDL-Cho, mg/dL	60.3 (0.4)	60.6 (0.4)	61.2 (0.9)	0.41	54.4 (0.7)	53.1 (0.8)	55.6 (2.0)	0.09	58.8 (0.3)	58.7 (0.4)	59.9 (0.9)	0.78
TG, mg/dL	139.5 (2.4)	137.9 (2.4)	128.6 (4.6)	0.27	130.5 (4.9)	131.2 (6.0)	115.6 (10.7)	0.76	136.3 (2.2)	136.4 (2.3)	125.5 (4.3)	0.52

Data are means (SE). *P* value is for GG vs GT+TT. Blood pressure readings before the start of antihypertensive medication were not available for 248 hypertensive subjects whose values were measured under treatment. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; T-Cho, total cholesterol; HDL-Cho, HDL cholesterol; and TG, triglyceride.

TABLE 2. General Linear Model for Regression of BMI in Association With Blood Pressure According to Genotype

Blood Pressure	Subjects	Genotype (n)	Coefficient	Constant	P (Regression)	Determination Coefficient	P (Interaction)
Systolic blood pressure	Population 1	GG (1283)	1.58	94.5	<0.0001	0.091	
		GT+TT (1183)	1.86	88.8	<0.0001	0.121	0.17
	Population 2	GG (421)	1.52	90.1	<0.0001	0.046	
		GT+TT (385)	1.93	80.1	<0.0001	0.076	0.40
	Populations 1 and 2	GG (1704)	1.61	92.5	<0.0001	0.077	
		GT+TT (1568)	1.92	85.5	<0.0001	0.108	0.10
	ECTIM study*	GG (450)	0.75	111.9	0.002	0.022	
		GT+TT (506)	1.99	80.9	<0.0001	0.133	<0.001
Diastolic blood pressure	Population 1	GG (1283)	1.05	51.9	<0.0001	0.110	
		GT+TT (1183)	1.15	49.9	<0.0001	0.126	0.44
	Population 2	GG (421)	1.16	51.1	<0.0001	0.057	
		GT+TT (385)	1.16	50.8	<0.0001	0.056	0.95
	Populations 1 and 2	GG (1704)	1.07	52.0	<0.0001	0.093	
		GT+TT (1568)	1.14	50.4	<0.0001	0.103	0.59

*See Tiret et al.[9]

TABLE 3. Blood Pressure According to ET1 G/T Polymorphism and Obesity Status

	Lean Subjects				Obese Subjects			
Blood Pressure	GG	GT	TT	P	GG	GT	TT	P
Population 1, n	937	746	157		346	231	43	
SBP, mm Hg	120.2 (0.5)	120.1 (0.6)	122.2 (1.2)	0.58	137.3 (1.8)	139.2 (2.0)	135.1 (2.0)	0.22
DBP, mm Hg	75.0 (0.3)	75.5 (0.3)	73.7 (0.7)	0.61	80.5 (0.5)	81.0 (0.6)	79.2 (1.2)	0.76
Population 2, n	328	256	54		93	66	9	
SBP, mm Hg	123.1 (1.0)	122.6 (1.1)	118.5 (2.2)	0.42	131.9 (2.1)	133.2 (2.3)	137.2 (5.8)	0.55
DBP, mm Hg	76.5 (0.6)	76.3 (0.7)	76.5 (1.7)	0.85	82.5 (1.4)	83.8 (1.5)	85.9 (3.1)	0.43
Populations 1 and 2, n	1265	1002	211		439	297	56	
SBP, mm Hg	127.6 (0.5)	128.2 (0.5)	125.0 (1.1)	0.97	138.1 (0.7)	137.9 (0.9)	136.3 (1.9)	0.18
DBP, mm Hg	75.4 (0.3)	75.7 (0.3)	74.4 (0.7)	0.77	80.9 (0.5)	81.5 (0.6)	80.2 (1.2)	0.48

Data are mean (SE). *P* value is for GG vs GT+TT.

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ELECTRONIC LETTER

Patients with the R133C mutation: is their phenotype different from patients with Rett syndrome with other mutations?

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Rett syndrome is an X linked dominant neurodevelopmental disorder with an incidence of 1:10 000 females in Australia.[1] It is characterised by apparently normal development between 6 and 18 months, followed by a period of regression with loss of purposeful hand use, deceleration of head growth, and onset of repetitive, stereotypic hand movements.[2] Affected people also manifest gait ataxia and apraxia, autistic features, epileptic seizures, respiratory dysfunction, autonomic dysfunction, and decreased somatic growth.[2,3] In recent years it has become apparent that the phenotypic range of this disorder is much wider than previously thought. Some patients may have a milder phenotype and retain the ability to walk or speak and others have an earlier onset and more severe features. People who have some but not all of the necessary criteria have been categorised as atypical[4] or as one of six variant forms.[5]

Rett syndrome has now been shown to be associated with mutations in the methyl-CpG-binding protein 2 (MeCP2).[6] For many genetic disorders, the next stage in research after the identification of the gene involves describing the relation between genotype and phenotype, and the phenotypic diversity produced by different mutations in the same gene. Some research has found that people with missense *MECP2* mutations may have a milder phenotype than those with truncating mutations.[7,8] Weaving *et al*[9] found that age at onset of hand stereotypies was later and speech and height (but not head growth) were slightly more normal in those with missense mutations whereas Nielsen *et al*[10] found no difference in severity between these mutation types. In the study of Amir *et al*[11] breathing abnormalities were found to be more common with truncating mutations and scoliosis more common with missense mutations. Hoffbuhr *et al*[12] concluded that patients with missense mutations in the methyl binding domain (MBD) and mutations truncating the entire transcription repression domain (TRD) were more severely affected than those with missense and nonsense mutations in the TRD and C terminal segment. Similarly, in another study, a milder phenotype was associated with late compared with early truncating mutations.[13] In a recent publication, Huppke *et al*[14] considered those mutations in the nuclear localisation signal (NLS) as a separate category from the other truncating mutations. Overall, they found that cases with mutations leading to a partial or complete truncation of the NLS were more severe than those with mutations downstream of the TRD. Although some phenotypic associations with different classes of mutations within *MECP2* are emerging, the reports are hampered by relatively small sample size. In one recent review, it was actually suggested that a simple relation between clinical severity and type of mutation may not even exist.[15]

The location of *MECP2* on the X chromosome makes it very likely that altered patterns of X inactivation also contribute to the phenotypic variation as seen in heterozygous females from the same family.[16] The closeness of the correlation between skewed X inactivation patterns and the phenotype seen remains uncertain. In the two most comprehensive reported studies, Hoffbuhr *et al*[12] found skewing (defined as >85% of one X allele active) in 6/39 (15%) cases whereas Weaving *et al*[9] with a more liberal definition (>75% of one X allele active) found it in 31/72 (43%).

Key points

- Rett syndrome is now known to be caused by mutations in the *MECP2* gene.
- Over 200 pathogenic mutations have been identified with eight common ones accounting for two thirds of cases.
- Grouping of mutations is not providing consistent genotype-phenotype relations across studies and we suggest there is a need to examine individual mutations.
- We ask the question for clinicians: "Is the phenotype of a girl with an R133C mutation likely to differ from what one would normally expect in Rett syndrome?"
- We found that the phenotype of a patient with an R133C mutation is milder overall with better ambulation and hand use and a greater likelihood of being able to use speech.
- It is important for clinicians to have this information when such patients are diagnosed.

Functional analysis of specific mutations associated with Rett syndrome is now also being undertaken. Similar to some other missense mutations in the MBD, the R133C mutation has been shown to abrogate methylation specific binding to the DNA template.[7] However, in a later study Kudo *et al*[8] found the R133C mutant to be functionally almost equivalent to the wild type protein; if this were the case, it could be hypothesised that people with the R133C mutation may have a milder phenotype.

The purpose of this study was to examine whether the clinical phenotype of patients with the R133C mutation is different from the clinical phenotype in those with other pathogenic mutations. Phenotype has been defined by scales that have already been used to describe the clinical variation in the Australian cohort.[17] Although included in the group of eight

Abbreviations: DHPLC, denaturing high performance liquid chromatography; EDTA, ethylene diamine tetraacetic acid; FQ2000, Australian follow up 2000 study instrument; MBD, methyl binding domain; MeCP2, methyl-CpG-binding protein 2; NLS, nuclear localisation signal; PCR, polymerase chain reaction; RettBASE, IRSA *MECP2* gene variation database; TRD, transcription repression domain; WeeFIM, functional independence measure for children.

Table 1 Study demographics

	All R133C cases	Australia	Japan	United Kingdom	Other mutations
No	24	11	4	9	98
Age (years)					
Mean	14.8	16.6	10.4	17.0	13.9
SD	7.0	8.2	2.2	6.3	6.2
Min	4.5	6.9	9.5	5.5	5.0
Max	31.1	31.1	12.2	29.0	28.6
p value, t test*	0.2448	0.2377	0.2298	0.1575	
X inactivation					
Random	13	6	2	5	41
Skewed	2	2	0	0	29
Not tested	9	3	2	4	28
p value, t test†	0.0225	0.2869			
Classification‡					
Mild (22%)		3	3		19
Classical (55%)		7	0		55
Early onset (12%)		1	1		24

*Comparing mean to other mutation group mean. †Comparing mean of log(P/(1−P)), where P is the percentage of the less commonly expressed allele. ‡[illegible] from the United Kingdom not classified.

common mutations which account for almost two thirds of pathogenic *MECP2* mutations,[2] the R133C mutation currently represents only 4.1% of all *MECP2* variations reported in the IRSA *MECP2* gene variation database (RettBASE).[18] To enhance the power of the analysis the R133C case group of 11 Australians was expanded by the addition of four Japanese and nine United Kingdom patients with this mutation.

MATERIALS AND METHODS

The Australian Rett Syndrome Database is an ongoing national registry of patients diagnosed with Rett syndrome born in 1976 and subsequently.[19] At the end of 2001, 227 verified cases had been reported to the registry. Fourteen (6%) of these had died. Mutation screening had been carried out on 175 (77%) of these patients as well as on 19 Australian adult patients with a clinical diagnosis of Rett syndrome. A pathogenic mutation was identified in 121/175 (69%) of registry patients and 15/19 (79%) adult patients. Australian patients with an R133C mutation represented 9/121 (7%) registry cases and 2/15 (13%) adult patients with a pathogenic mutation. X inactivation studies have now been completed on 145 registry patients, including 8/9 with R133C mutations. X inactivation data were also available for 2/4 Japanese R133C patients and 5/9 United Kingdom R133C patients (table 1).

The Australian follow up 2000 study instrument (FQ2000) consists of 15 sections, one of which is a questionnaire version of the functional independence measure for children (the WeeFIM).[20] It also contains items which allowed us to provide Rett syndrome severity scores for three different scales which we have termed the Kerr, Percy, and Pineda scales.[17] The Kerr scale was originally published to provide guidelines for describing the clinical features of patients with *MECP2* mutations.[21] Parents of seven of the nine registry patients with the R133C mutation completed the FQ2000 from the follow up study, as did the parent of one adult patient with the R133C mutation. For the other three Australian patients with the R133C mutation, we used data from the original family and clinicians' questionnaires and a telephone interview with one parent to obtain the necessary data items. Parents of all 98 people on the database with a positive mutation in the comparison group completed the FQ2000.

For the Japanese patients, a brief protocol was developed to capture the same information as provided by the FQ2000 questionnaire. The WeeFIM section was translated into Japanese and administered by telephone in one case and by interview with parents in three cases. For the patients in the United Kingdom, clinical data in the format published in Cheadle *et al*[7] were sought using a clinical data sheet that recorded key diagnostic points.[22]

Chi-squared tests were performed on categorical variables and *t* tests were used to compare means of the continuous variables with the SAS package.[23] Growth charts for Japanese females were used to assess Z scores and centile ranges for the four Japanese patients. Centre for Disease Control growth charts[24] were used for the rest of the cohort.

Mutation analysis

DNA was extracted from blood samples collected in ethylene diamine tetra-acetic acid (EDTA) anticoagulant. Using a variety of template primers,[7] the *MECP2* coding region was amplified by polymerase chain reaction (PCR) and then screened for mutations by direct sequencing (bidirectional).[7] Screening was also performed in some cases by denaturing high performance liquid chromatography (DHPLC) followed up by sequence analysis of fragments shown to have an altered elution profile by DHPLC.[7]

Inactivation of the X chromosome

Inactivation of the X chromosome was measured by examining the methylation of each allele of the androgen receptor locus, after the method of Pegoraro *et al*.[25] Microsatellites were analysed using Genescan (Automated DNA Analysis Facility, University of New South Wales, Sydney, Australia). For scoring the X inactivation pattern, we calculated the percentage of the smaller allele present. This was converted to a logistic transformation[26] and values for R133C and comparison cases were compared with *t* tests.[26] Skewing was defined as greater than 75% of one X allele active.

RESULTS

For this study, the case population consisted of 11 Australian, four Japanese, and nine United Kingdom patients with Rett syndrome, who have been identified as having an R133C mutation. Four clinical scales, the WeeFIM, Percy, Kerr, and Pineda scales, have been used to compare this case group with the group within the Australian follow up 2000 study of 98 patients considered to have a pathogenic mutation other than R133C.

Table 1 shows the age distribution, country of ascertainment, and clinical classifications for case and comparison groups. There were six major groups of genotype based on type and domain which covered 91% of the 98 comparison case

Table 2 Individual clinical data items (categorical variables): R133C cases and comparison group

	R133C cases (24)			Other mutations (98)	
	p Value	Percentage in category	Missing	Percentage in category	Missing
Feeding	<0.0001		4		2
Uses spoon		25		0	
Finger feeds		50		22	
No attempt		25		78	
Speech	<0.0001		1		5
More than single words		4		0	
Single words		48		2	
More than no vocalisation but no single words		17		65	
Lost speech		22		33	
Never acquired		4		0	
Gross motor function	<0.0001		0		5
Walks normally		54		12	
Walking impaired		38		27	
Previously or has never walked		8		61	
Age at losing social interaction	0.0011		10		6
<18 months		86		39	
6–18 months		14		61	
Sleep disturbance	0.0015		9		5
No disturbance reported		40		8	
Disturbance either past or present		27		22	
Disturbance both past and present		33		70	
Hand use	0.0017		1		5
Acquired and retained		29		7	
Lost purposefulness 2–5 years or acquired manipulation		29		21	
Lost purposefulness <2 years or acquired grasping		23		18	
Acquired and lost		10		53	
Never acquired		0		5	
Respiratory	0.0075		2		6
No dysfunction		45		18	
Hyperventilation and/or apnoea		55		82	
Disturbed awake breathing rhythm	0.0102		5		6
Never		53		20	
Rare to daily		21		39	
Daily to constantly		26		41	
Frequency of hand stereotypies	0.0292		5		5
Never		5		1	
Rare to dominating		21		5	
Dominating to constantly		74		94	
Voluntary hand use	0.0307		4		1
Normal, feeding independently		20		7	
Some hand use, feeding with help		55		38	
None		25		55	
Scoliosis	0.0339		71		5
No scoliosis		65		39	
Scoliosis		35		36	
Scoliosis corrected		0		25	
Ambulation	0.0353		91		2
First walked <18 months and still walking		33		35	
First walked <18 months then lost ability, or first walked 18–30 months and still walking		40		16	
First walked 18–30 months then lost ability		20		5	
First walked >30 months and still walking		7		4	
Never acquired		0		40	
Epilepsy	0.0656		5		12
Never		21		27	
Previous seizures, or present and controlled with medication		74		50	
Uncontrolled		0		22	
Early epilepsy <3 months		5		1	
Present weight	0.0677		24		8
Z score ≥ −1		59		37	
−2 < Z score < −1		6		20	
Z score ≤ −2		35		43	
Early developmental progress	0.0998		1		5
Normal progress		61		39	
Suboptimal progress		39		54	
No or virtually no progress		0		8	
Present HC	0.1567		5		6
Above 10th centile		51		35	
3rd–10th centile		26		21	
Below 3rd centile		23		43	
Present height	0.1980		88		0
Z score ≥ −1		35		31	
−2 < Z score < −1		21		13	
Z score ≤ −2		44		56	

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Table 2 continued

	R133C cases (24)			Other mutations (98)	
	p value	Percentage in category	Missing	Percentage in category	Missing
Age at sitting alone	0.7770		1		7
Acquired <8 months		77		69	
Acquired 8–26 months		23		29	
Never acquired		0		2	

*Includes eight patients from the United Kingdom, one from Japan, and one from Australia; †excludes all nine patients from the United Kingdom; ‡excludes six patients from the United Kingdom; §excludes seven patients from the United Kingdom; ¶includes all nine patients from the United Kingdom and two from Australia.

mutations. These were missense MBD (24.5%), nonsense TRD-NLS (21.4%) frameshifts in the C terminal (13.3%), missense TRD (12.2%), and nonsense mutations positioned between the MBD and the TRD (9.2%). The most frequent mutations found in the 98 comparison cases were T158M (n = 14), R168X (n = 11), R270X (n = 11), R294X (n = 9), R255X (n = 8), and R306C (n = 7).

The proportion of cases in each category for the R133C case group (n = 24) for selected categorical items used in the adapted Kerr, Percy, and Pineda scales are contrasted with the comparison group (n = 98) in table 2. Results for continuous variables and the four severity scales are shown in table 3.

Patients were significantly less likely to be in the most severe category for most of the individual clinical data items:

- Hand use (voluntary and finger feeding)
- Speech
- Gross motor function and ambulation
- Age at losing social interaction
- Sleep disturbance
- Respiratory dysfunction
- Awake breathing rhythm
- Age at onset and frequency of hand stereotypies
- Scoliosis.

Also, all four severity scales were significantly different between the patients with the R133C mutation and the comparison group.

X inactivation status was available for 15 R133C cases and 70 of the comparison group. In the Australian cases and comparison group, skewing was found in 31/78 (40%) cases. Skewing was less common in the R133C cases than the comparison group (p = 0.0217) with the mean allele percentages for the less commonly expressed allele being 35.8% for cases and 27.1% for the comparison group (logistic transformations −0.27 and −0.50 respectively). Kerr, Percy, and Pineda scales were uniformly but not significantly higher (24.5, 24.0, and 14.2) in the two cases with skewed than in the eight with random X inactivation (15.9, 17.6, and 11.1; p = 0.09, 0.37, 0.41). Similarly, the mean WeeFIM score was lower (23.0) in the two with skewed, compared with 53.4 in the seven with random X inactivation (p = 0.23).

DISCUSSION

This is the first report among cases of Rett syndrome that shows and measures the extent of the milder phenotype associated with the R133C mutation by contrast with cases that have mutations elsewhere in *MECP2*. These data show that patients harbouring the R133C mutation have better function overall. These girls and young women are more likely to have learned to walk and then remain ambulatory, to have better speech (single words or better), and to be able to use a spoon or finger feed. Among patients with the R133C mutation, hand stereotypies were less dominating and age of onset was later. Scoliosis, breathing, and sleep disturbances were less common.

Of interest is the fact that we have found no evidence to suggest that the mild phenotype in R133C cases is related to non-random X inactivation patterns. X inactivation was in fact more likely to be random in this group than in the comparison cases. This contrasts to the findings of Hoffbuhr *et al*[4] who found a linear relation between X inactivation ratios and clinical severity score for a small group of cases with missense MBD and nonsense mutations in the interdomain region. However, their complete study group of 73 cases with mutations only contained two cases with the R133C mutation and it is not known whether these were included in the group of 35 patients who had informative X inactivation results.

Although the quantitative data from the individual items and the composite scoring systems showed significantly milder disease overall, some R133C cases can be identified with phenotypes and severity scales in the range compatible with classical Rett syndrome. However, although equivalent

Table 3 Individual clinical data items (continuous variables) and severity scales: Australian R133C cases and comparison group

	R133C cases (17)				Other mutations (98)		
	Mean	SD	p value	Missing	Mean	SD	Missing
Age at onset of hand stereotypies (months)	41.1	32.2	0.0149	2	28.3	14.6	22
Age at onset of epilepsy (years)	7.0	6.0	0.0825	1	4.7	2.2	34
Latest weight Z score	−1.99	2.6	0.4820	0	−1.7	3.4	8
Latest height Z score	−2.22	1.2	0.5979	1	−2.3	1.7	9
Age at sitting alone (months)	7.7	1.8	0.6528	3	8.5	9.9	8
Age at onset of regression (months)	22.1	25.3	0.9733	2	22.3	22.7	5
Pineda scale (max = 31)	13.1	2.7	0.0030	0	16.2	4.3	0
WeeFIM score (max = 126)	37.9	17.9	0.0265	2	38.4	11.5	8
Kerr scale (max = 37)	19.8	4.6	0.0251	0	22.0	4.2	0
Percy scale (max = 42)	20.7	6.4	0.0455	0	30.0	6.3	0

Table 4 References to the MECP2 R133C mutation in Rett syndrome in published reports

Study	No of patients	Country	Case ID	Phenotype data provided
Amano et al[33]	2	Japan	Sag012	Genotype data only provided
Uchino et al[31]			Sag024	Uchino adds "few words" only
Amir et al[6]	2	United States of America		Analysed as missense truncating
Amir et al[34]				
Amir et al[11]				
Buyse et al[35]				
Sung et al[36]				
Wan et al[16]				
Auranen et al[37]	3	Finland		Overall milder clinical phenotype; all learnt to walk and developed normally <10 months
			[illegible]	Born 1992: hands: uses purposefully
				No epilepsy; no scoliosis
			[illegible]	Born 1979: walking until 9–10 years
			[illegible]	Born 1973: [illegible]
Chae et al[38]	1	Korea		Compared missense and nonsense severity scores
			17	Age: 7 years 5 months
				Hands: grasps briefly
				Speech: a few words
				Walking: walks alone
				Epilepsy: no abnormal respiration
Cheadle et al[7]	4 including 2 sisters	United Kingdom	8	Hands: uses spoon/finger feeds/holds a cup or bottle
				Speech: few words, possibly with meaning
				Walking: independently
			13	Hands: grasps or holds briefly
				Speech: few words, possibly with meaning
				Walking: independently
			31	Hands and speech: not applicable
				Walking: independently
				Neurological
			84	Hands: uses spoon/finger feeds/holds a cup or bottle
				Speech: understands and says some words
				Walking: independently
Erlandson et al[39]	1	Sweden		No phenotype data, different case than that reported by Xiang et al[40]
Hoffbuhr et al[12]	1	United States of America	R133a	Hands: limited preserved hand use
				Speech: preserved
				Walking: ambulatory
				Decelerated head circumference: [illegible]
Huppke et al[13]	1	Germany	26	No loss of ability to sit, walk, or speak
				Head circumference >3rd centile
Laccone et al[41]	6			No phenotype data
Trappe et al[42]	3			No phenotype data, includes 2 patients, 1 maternal origin
Inui et al[30]	2	Japan	3	14 years, no gait apraxia
			5	8 years, no head growth deceleration, no severely impaired language, no severe psychomotor retardation
Milunsky et al[43]	2	United States of America		Some cases without microcephaly
Nielsen et al[10]	1	Denmark	6	Born 1979
				Hand: can hold glass; uses spoon or fork
				Gait: walks independently
				Eye gaze: [illegible], uses eye pointing
Nielsen et al[44]	1	Denmark	2	Born 1970; preserved speech variant
				Hands: uses hands for eating and drinking
				Speech: lost speech at 5 years, regained at 6 years at [illegible] level [illegible]
				Walking: relatively effortlessly
				Head circumference normal, no scoliosis or kyphosis
Vacca et al[45,46]	3	Italy/United Kingdom	N4	No phenotype data provided
			N19 N22	All United Kingdom cases
Xiang et al[40]	1	Sweden	41	No phenotype data provided
Yamada et al[47]	2	Japan	R02	Diagnosed at 3.1 years
				Speech: says a few words, possibly with meaning
				Walking: since 17 months
				Epilepsy: no head growth deceleration
			R17	Diagnosed at 17 years
				Speech: none
				Walking: no walking
				Epilepsy
Yamashita et al[29]†	2	Japan	1	8 years, onset hand stereotypies at 6 years
				Hands: can use spoon

Cont

Table 4 continued

Study	No of patients	Country	Case ID	Phenotype data provided
Kondo and Yamagata[48]‡	15			Speech: short sentences
				Walking: at 3 years could run and take stairs; normal growth
			3	9 years, preserved speech variant, onset hand stereotypies at 5 years
				Hands: hand function relatively preserved
				Speech: speaks 3–4 words
				Walking: unsupported
				onset of epilepsy at 7 years, no microcephaly
Zappella et al[32]	2	Italy	368.OR	10 years, no scoliosis, first words >24 months
			397.R	30 years, no scoliosis, first words >24 months, >2 word sentences

*Three cases are included in the United Kingdom cases for the current study. †Japanese cases included in the current study.

information on their representativeness is not available for the cases from either the Japanese or the United Kingdom, we know that the cases from Australia have been sourced from a national population database and are thus representative of patients with the R133C mutation in this country. The fact that such a sound epidemiological framework has underpinned this particular study provides us with the confidence to generalise the findings. Within R133C cases we do not know what factors predict a milder or more severe phenotype but we have found no evidence to suggest that skewed X inactivation is protective (but rather noted a tendency to the reverse). As noted by Hoffbuhr *et al*[4] relative to their six cases which showed preferential use of one X chromosome, patients with skewing seem to be either more mildly or more severely affected than other patients with the same type of mutation. Like Hoffbuhr *et al*,[4] in this study we were unable to determine whether it was the normal or mutated X chromosome that was preferentially active in those patients with skewing. If it were the mutated chromosome this could account for the direction of our findings.

Until now, reports of patients with the R133C mutation have involved only a description of affected patients who also have given an impression of milder disease (table 4). The R133C mutation has previously been documented in girls with preserved speech[29 30] and in a family with two mildly affected sisters and a clinically unaffected mother.[7] Based on a case sample with a total of only 10 patients with preserved speech (two of whom had an R133C mutation) and with no comparison group, Zappella *et al*[32] have commented that the presence of this mutation is one factor likely to contribute to a milder phenotype. Ability to confirm this association statistically in our study depended upon the existence of the Rett syndrome epidemiological database in which most patients have now been genetically screened. However, because Rett syndrome is a rare disorder and because fewer than 1/20 of these patients may have the R133C mutation, it takes this optimal level of ascertainment of juvenile Rett syndrome in the Australian total population of almost 19.5 million people to generate nine such affected childhood cases. As well as adequate sample size, which is clearly facilitated by national population based data collections as well as international collaborations, the two other important ingredients necessary for success are comprehensive mutation analysis and systematic collection of relevant clinical data.

The systems for classification of phenotypes which have been developed so far have partly resulted from individual researchers taking advantage of the information they had already collected on various clinical items and using these to develop a score.[1 8 24] The scores for two (Percy and Kerr) of the scales used to describe the phenotype in our Australian population database[9] increased with age. This did not occur with the Pineda scale (derived from the study of Monros *et al*[8]) which was based more on developmental data than on current clinical features. Because the milder phenotype associated with the R133C mutation is so marked, confounding by age was not likely to be a problem in this study. However, this may be an issue in future studies.

Although around 200 different pathogenic mutations have now been identified in the *MECP2* gene,[4] it is thought that eight common mutations represent about two thirds of the total number of pathogenic mutations.[20] Among these eight recurrent mutations, R133C is one of three missense mutations in the MBD along with R106W and T158M, the commonest of all mutations. Even in the more complex classification systems,[4] these two (R133C and T158M) would be grouped together as missense MBD mutations but in reality may have very different phenotypes. Despite including what we have now shown to be a mild mutation, missense MBD mutations were estimated to be the second most clinically severe group in the analysis of Hoffbuhr *et al*.[4] Therefore, it is now apparent that these common mutations must be assessed individually rather than being treated as part of a larger group. The R133C mutation may well be at one end of the clinical range of Rett syndrome phenotypes associated with *MECP2* mutations. As this is clinically useful information it is apparent that each of the other recurrent mutations also requires more detailed evaluation.

In recommending that future genotype-phenotype analyses should focus on individual mutations rather than groupings, it is important to consider the reasons for undertaking such studies of genotype-phenotype. The primary reason is to gather information which will allow clinicians to assess the likely clinical course for children in whom the diagnosis of Rett syndrome has been confirmed by molecular testing. In the past, the need for adherence to clinical criteria to make the diagnosis has constrained clinicians in their diagnostic practice and perhaps resulted in missing those atypical patients, who have symptoms which are either milder or more severe than classical Rett syndrome. Our experience with the R133C mutation further suggests that the phenotypic boundaries of Rett syndrome extend well beyond the original Trevathan criteria.[3] It is important not only that a diagnosis of Rett syndrome is confirmed in this group of children but also that clinicians are aware of their generally milder phenotype when counselling families about future prognosis.

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A novel giant gene CSMD3 encoding a protein with CUB and sushi multiple domains: a candidate gene for benign adult familial myoclonic epilepsy on human chromosome 8q23.3–q24.1

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Abstract

We identified a novel giant gene encoding a transmembrane protein with CUB and sushi multiple domains on the human chromosome 8q23.3 q24.1 in which benign adult familial myoclonic epilepsy type 1 (BAFME1/FAME, OMIM:601068) has been mapped. This giant gene consists of 73 exons and spans over 1.2 Mb on the genomic DNA region. It showed significant homology to two genes, CSMD1 gene on 8p23 and CSMD2 gene on 1p34, at reduced amino acid sequence level and hence we designated as CSMD3. The CSMD3 gene was expressed mainly in adult and fetal brains. We performed mutation analysis on the CSMD3 gene for seven patients with BAFME1/FAME, but no mutation was found in the coding sequence of the CSMD3 gene. Comparative genomic analysis revealed a conserved family of CSMD genes in the mouse and fugu genomes. Possible functions of the CSMD gene family are discussed.

Keywords: BAFME1; FAME; CUB; Sushi; CSMD; Epilepsy; Brain; Comparative genomics; Chromosome 8

We have constructed a BAC contig of 30 Mb corresponding to the human chromosome 8q22–q24.1, and performed a genomic DNA sequencing followed by extensive computer-aided gene annotation in combination with manual and experimental examination. We identified 120 genes, among which we focused on a giant gene (named CSMD3) of 1.2 Mb consisting of 73 exons. We determined the complete structure of CSMD3 gene and found that it encodes a transmembrane protein of CUB and sushi multiple domains and it is expressed mainly in fetal and adult brains, suggesting a good candidate for the pathogenic gene for the benign adult familial myoclonic epilepsy type 1 (BAFME1/FAME, OMIM:601068) which has been mapped to this chromosomal region.

The BAFME1/FAME is characterized by adult-onset tremulous finger movement, myoclonus, epileptic seizures, and a non-progressive course [1]. The age of onset of the disorder is between 18 and 50 years old. The BAFME1/FAME showed a clear autosomal dominant pattern of inheritance. Linkage analysis of Japanese patients indicated that the responsible gene for the BAFME1/FAME is located to chromosome 8q23.3–q24.1 [2,3]. Mikami et al. [3] showed a maximum two-point LOD score of 4.31 for a marker D8S555 with a recombination fraction of zero and a maximum multi-point LOD score of 5.42 for the interval between D8S555 and D8S1779 for a large Japanese pedigree. In this family, no recombination was observed with three markers, D8S1830, D8S555, and D8S1779, which are located within an 8-cM interval between D8S1784 and D8S1694, indicating that this 8-cM region contains a causative gene for BAFME1. For the linkage analysis of FAME families by Plaster et al. [2], we reviewed their data according to precise order of microsatellite markers based on the finished human genome sequence. We then found that the FAME locus is mapped on a region proximal to D8S514, which is overlapped with BAFME1 candidate region. According to these information, we focused on the 8-cM interval between D8S1784 and D8S1694 as target region of BAFME1/FAME. The mapped locus is distinct from three other epilepsy-related loci on chromosome 8q (KCNQ3, ECA1, and FEB1) [4–6].

Here, we report a novel giant gene CSMD3 of 1.2 Mb encoding a transmembrane protein and describe initial characterization of the gene in terms of its genomic structure, transcript variants, tissue expression profiles, evolutionary relationship with the CSMD family genes in a phylogenetic tree, and mutation analysis as a candidate gene for a type of epilepsy BAFME1/FAME.

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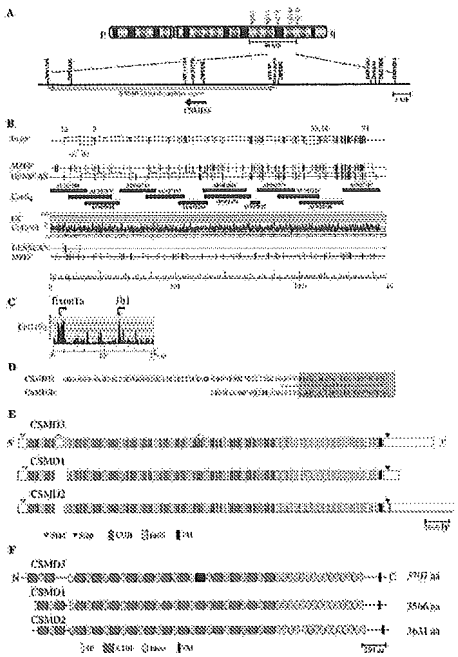

Fig. 1. A novel human gene CSMD3: chromosomal location, gene structure, CpG islands, and protein domains. (A) Diagram of chromosome 8, STS markers linked to BAFME1/FAME and location of CSMD3 gene. White arrow indicates BAFME1/FAME candidate region. Black arrow indicates 5′ to 3′ direction of the gene. (B) The exon–intron structure of the CSMD3 gene, positions of exons on the upper DNA strand predicted by GENSCAN and MZEF, BAC contig and accession numbers of BAC clone sequence data, GC/CpG contents, positions of predicted exons on the lower DNA strand. (C) Location of CpG islands and 5′ ends of two first exons. (D) N-terminal amino acid sequences of protein translated from two transcripts (CSMD3a and CSMD3b). A part of CUB domain is shown by dark gray. (E) Exon structures of CSMD transcripts. The positions of start and stop codons are indicated by open and solid arrowheads, respectively. Exons encoding CUB, sushi, and transmembrane domains (TM) are depicted by dark gray, light gray and filled boxes, respectively. The connected lines above the transcript indicate alternative splicing of exons 7, 33, and 34. CSMD3 represents the transcript started at exon 1a. (F) Predicted structure of the CSMD proteins. Rectangles, squares, ovals, and vertical hatches represent putative signal peptides, CUB domains, sushi domains, and putative transmembrane domains, respectively. The blackened CUB domain is subject to skipping by alternative splicing. CSMD3 represents the protein translated from CSMD3a.

Materials and methods

BAFME1/FAME patients. The DNA samples were obtained from healthy individuals and patients with BAFME1/FAME after receiving informed consent for this study [2,3].

Construction of BAC contigs and sequencing. BAC contig of 30 Mb covering 8q22.1–q24.1 was constructed by digital hybridization method using Keio BAC library [7,8]. These BAC clones were sequenced by shotgun sequencing method. We generated contiguous genomic sequence by combining our data with those generated by other sequencing groups for chromosome 8. For this process, we utilized the BLAST program to identify overlapping BAC clones and confirmed their relationship by dot matrix analysis.

Gene prediction. Gene prediction was performed by using two different programs GENSCAN and MZEF. Predicted exons were analyzed with SMART to identify CUB and sushi domains. All analyses were performed with default parameters.

Table 1
List of partial cDNA fragments for reconstruction of full length CSMD3 cDNA

Fragment	Size (bp)	Exons	Position in coding region[a]	Position in cDNA	Comments
B73	221	1a	5′-158:63	1:221	5′ RACE
B72	279	1a–2	13:291	172:450	
B74	168	1b1–1b2	5′-171[b]:5′-4[b]	1[b]:168[b]	5′ RACE
B71	357	1b2–3	5′-40[b]:432	132[b]:506	
B70	488	3–4	210:697	369:856	
B68	277	4–5	561:837	720:996	
B67	267	5–6	754:1020	913:1179	
B66	880	6–12	933:1812	1092:1971	
B61	138	11–12	1675:1812	1834:1971	
B60	1067	12–18	1811:2877	1970:3036	
B54	397	18–20	2856:3252	3015:3411	
B52	357	20–22	3204:3560	3363:3719	
B51	563	21–24	3463:4025	3622:4184	
B48	237	24–25	3940:4176	4099:4335	
B47	416	25–27	4089:4504	4248:4663	
B45	649	27–30	4396:5044	4555:5203	
B44	797	28–32	4573:5368	4731:5527	
B40	648	32–36	5279:5926	5438:6085	
B36	558	36–41	5825:6382	5984:6541	
B31	476	41–43	6327:6802	6486:6961	
B29	430	43–45	6714:7143	6873:7302	
B27	237	45–47	7066:7292	7225:7451	
B26	242	46–48	7204:7445	7363:7604	
B24	372	48–50	7408:7771	7559:7930	
B22	697	50–53	7711:8407	7870:8566	
B21	661	51–54	7951:8611	8110:8770	
B18	402	54–56	8494:8895	8653:9054	
B16	867	56–61	8855:9721	9014:9880	
B12	449	60–62	9588:10036	9747:10195	
B11	237	61–63	9837:10073	9996:10232	
B10	432	62–65	9956:10387	10115:10546	
B7	1627	65–71	10230:3′-732	10389:12015	
B2	1174	71	3′-435:3′-1608	11718:12891	
B1	401	71	3′-1466:3′-1865	12749:13148	3′ RACE
B0	257	71	3′-1581:3′-1865	12864:13148	3′ RACE

[a] "5′" indicates base position upstream from the initiation codon. " 3′" indicates base position downstream from the last base of stop codon.
[b] Base position based on the numbering of exon 1a transcript (However, −40 and 1 in those columns are counted based on the numbering of exon 1b transcript).

Table 2
Exon/intron structure of human CSMD3 gene

Exon number	Exon size (bp)	cDNA (nt)	Genome position NCBI build 33 chr8 (bp)
1a	336	1–336	114113034–114113699
1b1	81	1–81	114058175–114058995
1b2	148	82–229	114057907–114057760
2	223	337–559	113995815–113995593
3	113	560–672	113959726–113959614
4	195	673–867	113854938–113854744
5	208	868–1075	113779985–113779778
6	113	1076–1188	113700201–113700089
7	312	1189–1500	113657170–113656859
8	78	1501–1578	113635783–113635706
9	88	1579–1666	113628899–113628812
10	125	1667–1791	113602773–113602649
11	122	1792–1913	113540288–113540167
12	104	1914–2017	113510811–113510708
13	113	2018–2130	113481296–113481184
14	183	2131–2313	113371072–113370890
15	327	2314–2640	113366754–113366428
16	195	2641–2835	113363658–113363464
17	139	2836–2974	113347437–113347299
18	188	2975–3162	113337363–113337176
19	189	3163–3351	113331371–113331183
20	117	3352–3468	113326247–113326131
21	216	3469–3684	113319933–113319718
22	189	3685–3873	113318027–113317839
23	170	3874–4043	113268257–113268088
24	157	4044–4200	113254679–113254523
25	192	4201–4392	113237976–113237785
26	127	4393–4519	113233742–113233616
27	203	4520–4722	113231895–113231693
28	192	4723–4914	113198247–113198056
29	139	4915–5053	113187851–113187713
30	188	5054–5241	113184999–113184812
31	195	5242–5436	113173705–113173511
32	117	5437–5553	113153729–113153613
33	114	5554–5667	113090054–113089941
34	96	5668–5763	113089453–113089340
35	204	5764–5967	113087749–113087546
36	125	5968–6092	113071810–113071686
37	97	6093–6189	113064685–113064589
38	105	6190–6294	113061478–113061374
39	119	6295–6413	113033556–113033438
40	70	6414–6483	113032266–113032197
41	117	6484–6600	113027235–113027119
42	210	6601–6810	113022708–113022499
43	189	6811–6999	113018753–113018565
44	178	7000–7177	113017851–113017674
45	146	7178–7323	113016496–113016351
46	81	7324–7404	113001003–113000923
47	114	7405–7518	112999972–112999859
48	189	7519–7707	112998639–112998451
49	147	7708–7854	112995074–112994928
50	189	7855–8043	112992188–112992000
51	186	8044–8229	112987214–112987029
52	195	8230–8424	112985937–112985743
53	174	8425–8598	112983988–112983815
54	174	8599–8772	112977038–112976855
55	174	8773–8946	112973733–112973559
56	186	8947–9132	112972717–112972532
57	174	9133–9306	112970540–112970387
58	183	9307–9489	112968268–112968086
59	177	9490–9666	112962372–112962196
60	180	9667–9846	112946612–112946433
61	174	9847–10,020	112944834–112944661

Table 2 (continued)

Exon number	Exon size (bp)	cDNA (nt)	Genome position NCBI build 33 chr8 (bp)
62	174	10,021–10,194	112936449–112936276
63	74	10,195–10,268	112935348–112935275
64	112	10,269–10,380	112928153–112928042
65	180	10,381–10,560	112925595–112925416
66	66	10,561–10,626	112922807–112922742
67	159	10,627–10,785	112918370–112918212
68	113	10,786–10,898	112915499–112915387
69	88	10,899–10,986	112912654–112912567
70	136	10,987–11,122	112909913–112909778
71	1999	11,123–13,119	112905952–112903954

Amplification and sequencing of cDNA between predicted exons. Primers with forward and reverse directions were designed according to the sequence of each predicted exon, which putatively encoded CUB and sushi domains. We attempted to amplify partial cDNAs among juxtaposed exons with those primers in proper combination using cDNAs of adult brain, fetal brain, and testis in Human Multiple Tissue cDNA panels (MTC panels; Clontech) as template. We then examined possible connection among these putative genes by PCR. The final sets of PCR primers to recover the entire cDNA of CSMD3 gene are listed in Table 4. Amplified cDNAs were sequenced with appropriate primers and BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA) using ABI Prism 3700 DNA analyzer. Quality of the sequence was proven with Phred/Phrap/Consed program. Homology plot analysis was performed using DNASIS-Mac software version 3.5 (Hitachi Software Engineering, Japan).

RACE PCR. The 5′ and 3′ sequences of the CSMD3 gene were determined by RACE using Marathon Ready cDNA kits of human fetal brain or adult testis (Clontech) according to the manufacturer's instructions. For exon 1a, first 5′-RACE amplification was performed between adapter primer 1 (AP1: CCATCCTAATACGACTCACTATAGGGC) and 5′ anti-sense primer (CTTGCCAGGCTCCCAGGGTTTGGATTCCTT) using human fetal brain RACE template, and then second amplification was performed between adapter primer 2 (AP2: ACTCACTATAGGGCTCGAGCGGC) and second 5′ anti-sense primer (GCAGTGGAGTTGTTGCTGTTGTTGGTG). For exon 1b, first 5′-RACE was performed between AP1 and first 5′anti-sense primer (TGCTATTATTACCCATGTGCAG) using human testis RACE template, and then second amplification was performed between AP2 and second 5′ anti-sense primer (ACCGGGGTTGTGCTGAAGAGGTTGTTTA: this primer was designed according to a draft version of AC055788. Later, this original sequence was updated to be ACCGGGTTGTGCTGAAGAGGTTGTTTA in the finished version of AC055788. However, the original primer worked to obtain proper RACE product). 3′-RACE amplifications were performed between AP1 and 3′ specific first sense primer (CTGCTTGGCTCTATCTCTACAACATTTA), and then performed between AP2 and 3′ specific second sense primer (TTTTAAATTTTATGCCATCAGTTATT). The PCR products were purified and sequenced.

Table 3
SNP[a] candidates in CSMD3 gene region

Amino acid change	Exon/intron	Position of nucleotides	Allele frequency	NCBI SNP cluster ID
None	1a	-78 G/A	3/11	None
None	1b2	IVS1b2−22 T/C	6/8	None
None	1b2	IVS1b2−216 A/G	1/13	rs4876512
I219M	4	657 A/G[a]	30/20	rs2219898
None	4	IVS4-58 A/C	1/13	rs2030506
None	4	IVS4-5 T/A	13/1	None
None	11	IVS11-21 C/T	10/4	None
None	14	IVS14−56 A/G	1/13	None
None	15	IVS15−68 C/T	6/8	None
None	18	IVS18-5 T/G	4/10	None
None	19	IVS19−91 C/A	4/10	None
None	23	IVS23−88 C/T	7/7	None
None	23	IVS23−99 G/A	7/7	None
None	27	IVS27-99 A/T	3/11	None
None	28	IVS28−30 C/T	12/2	rs2853244
None	29	IVS29-3 C/T	2/12	None
None	31	IVS31-78 T/G	12/2	None
Y2068Y	39	6204 T/C[a]	33/17	None
None	39	IVS39-38 A/G	7/7	None
None	45	IVS45-208 A/G	9/5	None
None	53	IVS53-8 T/C	7/7	rs4876462
None	56	IVS56-109 A/T	7/7	None
None	57	IVS57−46 T/C	7/7	rs1861755
None	61	IVS61-115 A/G	7/7	rs1861753
None	61	IVS61-53 T/C	2/12	None
N3621H	70	10861 A/C[a]	5/9	rs1592624
None	70	IVS70−21 C/T	6/8	None

[a] Numbers from the first base of initiation codon of exon 1a transcript.

Mutation search. PCR primers of 50–250 bp flanking exon–intron boundaries were designed manually or by the program Primer3. Genomic DNA (10 ng) from patients or control individuals was used for amplification of each exon. For exons of more than 400 bp, two or more pairs of primers were designed to keep size of PCR products less than 500 bp. All primer sequences are listed in Table 5. The PCR products were sequenced by ABI Prism 3700 DNA sequencer. These sequences were assembled and searched for SNPs by Consed with polyPhred [9].

Multiple-tissue cDNA panel and Northern blot analysis. Expression analysis of CSMD3 gene by PCR was performed using MTC panels (Clontech; I, II, fetal and immune system panels) consisting of cDNAs from 27 human adult and fetal tissues with primer pairs of A:(CGC AAAGGGGAAAGCCGAGCAAAGGA/CTTGTCACTGGAGGT GGCAGATGGAATC), B:(TTGCGACTCATAAACAACCTCTTC AG/CTTGTCACTGGAGGTGGCAGATGGAATC), and C:(AGCT CGCCTAATGTTACGCATA/AAAGCTGCTTGGCCATTGTTAT) as shown in Fig. 1. Twenty picograms of cDNAs was used as templates.

Northern blot hybridization was performed with Human MTN Blots 1-3 (Clontech) loaded with 2 μg of poly(A)$^{+}$ RNA from 27 human adult and fetal tissues. Twenty nanograms of partial cDNA fragment amplified by PCR with a pair of primers (AGGACTTAA TGGCACTATAGAA/GAACAGGAAAATCCCACGAAGC) was labeled with [α-^{32}P]dCTP by random primer extension method. Hybridization was carried out according to the manufacturer's protocol. Autoradiography was carried out using IP plate (Fujifilm, Japan) for 24 h and then hybridization signals were detected by FLA-3000G system (Fujifilm, Japan).

Sequence analysis. Homology search of nucleotide and amino acid sequences in the database was performed through the BLAST server at NCBI using BLASTN, BLASTP, and BLASTX. The fugu genomic DNA sequences corresponding to CSMD gene family (Scaffold_3752; CSMD1, Scaffold_380; CSMD3a, Scaffold_55; CSMD3b, and Scaffold_73; CSMD2) were downloaded from Ensembl genome server and analyzed by DOTTER program to compare with cDNA sequences of human CSMD. Identified exons were joined to construct putative cDNA sequences and used for further analysis.

Evolutionary analysis. The amino acid sequences deduced from the coding sequences of *CSMD* family genes of human, mouse, and *fugu* were aligned using the Clustal X (ver 1.82). An evolutionary neighbor joining tree was constructed by Clustal X. The phylogenetic tree was drawn by TreeView (ver 1.5). Graphic representation of the aligned amino acid sequence was obtained by BOXSHADE 3.21.

GenBank accession numbers. Accession numbers of BAC clones covering the entire CSMD3 gene are as follows: AC055788, AC102890, AP005015, AP004713, AC027189, AC021264, AP002846, AP003470,

Table 4
Primers used for amplification of CSMD3 cDNAs

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)
1a	AP2[a]	GCAGTGGAGTTGTTGCTGTTGTTGGTG
1a	AP1[a]	CTTGCCAGGCTCCCAGGGTTTGGATTCCTT
1a–2	CGCAAAGGGGAAAGCCGAGCAAAGGA	TGCTATTATTACCCATGTGCAG
1b1–1b2	AP2[a]	ACCGGGGTTGTGCTGAAGAGGTTGTTTA
1b1–2	AP1[a]	TGCTATTATTACCCATGTGCAG
1b2–3	TTGCGACTCATAAACAACCTCTTCAG	CTTGTCACTGGAGGTGGCAGATGGAATC
2–4	AGGACTTAATGGCACTATAGAA	GAACAGGAAAATCCCACGAAGC
4–5	TGTATTATATGGCACAAGATTC	AATTGTGTCCCAGGCTCTGCT
5–6	ATATCCAGCCCTAGTTTTCCTA	AGCACTAAATCCACGGTATCGA
6–12	TATACCACCACCAATTATCAGC	TGTCAAGGTATCATAGCCAATC
11–12	TTTACATCTCCCAACTTTCC	TGTCAAGGTATCATAGCCAATC
12–18	CAATTGGGATGGGGGGGAAGT	TGACAGAAGATTTGGCCCATCA
18–20	TGATGGGCCAAATCTTCTGTC	CGGGTAACCAGGTGATAAGATT
20–22	AGGAGATGTTAGAGGGCCTAGT	ATGCCAGGATCTTCACAAGCTT
21–24	GCTCAATTGCGTTTCATTTCA	AAGCCTTCATCTGTCCCTTCAG
24–25	TCTATGCGCGGACTGACACTTAGT	AAGGCTACTTCCGTGGAGAGTG
25–27	ATACAAGATCAGTGACCAAGGC	TTACTATATTGAGGGTGCTATG
27–30	CATGATATACTCCGAGTCTGGG	CATCACTCGGAAAAGCCAAATG
28–32	TGCCACTGCGTGTCGTGACCCA	AAACACAATTATGTCCCACACT
32–36	CGCCCTGTGGAAGTCGTTCAAC	CAGCTGCCTCTGGCACTGTGAT
36–41	TTTTAACTAAGCGCAAAGGGAC	GAAACCCAGGACTGAGGATCAC
41–43	TCAGTGTGGTGGTGCTATGTCA	CTCCGTGAAGGCATGTGAGAGC
43–45	TGATTTTACTGTGGGTCAAACCA	GCCACTTGTTGTGAAATCACTG
45–47	AATACCGCTTTGGAATCAGTCT	CCAACTAAAGTAAATCCTGGAA
46–48	CCCAATGCTGAAATTTTGACGGAAGATGAT	TGAAGATTTGGGTAACTGTCAG
48–50	GAGTCATATTGAGCCCTGGAT	CACTGTTAAGCTGCCCACCTGT
50–53	ACACCAGAATCCCCACCTCATG	CTGAGGAAAGGCATTCCCTTAC
51–54	GGAACGCGAGTTACCTATTTTT	CACAGGATGGGAGCTGACCAGA
54–56	AATTATGGATATAGAGACACAG	AGGATTGCAGTCATAGAATAC
56–61	ATTTTACTTAGGGCACTGTGGT	TAGAGATCTGGGGAGGAGTTG
60–62	TTACATGTGCCAGCCAGGCTAC	CTATGCAGTGAGGTGATGAACCACTCCAAG
61–63	GAGTGGTGAAGTACCGCAGTGC	GGCACACCTGGGTTTTCACAAG
62–65	TTCCTTTCATATTAGTGGGATC	CTTTTCCACTCCAGGTGTTATC
65–71	CTGTAAACAGCCAGAAACTCCT	TACCCCATAGCTCACTGAATAA
71	ATCACGCCCACAAATCTGTCTT	TAAATAACTGATGGCATAAAAT
71	CTGCTTGGCTCTATCTCTACAACATTTA	AP1[a]
71	TTTTAAATTTTATGCCATCAGTTATT	AP2[a]

[a] The sequences of AP1 and AP2 which were used for 5′- and 3′-RACE are described in Materials and methods.

Table 5
Primers used for mutation search

Exon	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
1a	CCGATTCATTATCGTCACGGTTT	GAACGAGCTCTGAATCAACTCCTTTAGTAT	803
1b2	CGCAGCGCACACACCTTTAGTTT	GGGTTGGAATGTACAAGACACTTAAGAGTA	502
2	AATTATTGTTAACTTTGGTCTTGGAAATGC	GGTGAATACAACTTTATTATAATTGCATCA	450
3	TGTTGGCTGGATAGCATTCCTTCTGGAAAT	GAGACAACATGATGTTGTCTCAAATGTTGA	358
4	TGACTCTTCTCCAAAGAAATAAGAATATCC	CTCTATCCTTTAGATTCCGTAGAAGAAACA	411
5	TATTATGCACTTGGTATCACATTTGAACTG	CAACCTCTTCACATAACTTTCCTGTAAGAT	468
6	ATGAATATAGTTGGACATTTGTTTGGTCCC	CTCAGCAACAGCATGACCATTTTTTTCAAT	351
7	TCTTCATGTTGATTATCCTTAAGGTAATAG	GGCTCTCTTTCAGCTCATTCTCTAATTAGA	523
8	TAAGAAAGTGGTTTCTTTATCTTGCATAAG	GCATGTTACACAGATATTAATGCAGTTAAT	325
9	TAACAAATGCTTAAATGACAGTGATTATAG	GTTTGATGCCACTAAGACTTCATCTTTATA	331
10	TACATATTTGACCTATTGGATATTGCCAGC	GCACAATCTTTCCTTAAAGATTATATTACA	340
11	AATGTTGAAAATATCGAGCTTGTGAATCCC	CCCAAATTTCTCATATAAATCTGGTTTATA	491
12	TATTCTAACATGACTTGCTGCCATACTATG	GGAGCGATCAATGTAAAACAATAATTTGAA	391
13	TCAACACATACTCCTCTATGTTTATCTGAC	GGAGGAAATGATCTTTGTTGAAATGTAGCA	386
14	TATCTCCACAACAAAGAGCATATATCTACC	GCAGCTCTTTGCAATTAATTACAAAAGCAA	368
15	TAGAGCCTTAAGTTTATCCAAAGTACTAGT	GGCACAATGAGATTTACAGCTTTTACAAAT	660
16	TCTCTCTTTTCCAAAATATAGAAGGATCTC	CTTGAACCTTGTTGACTATATTTAATTGGA	796
17	ACAGAAGTAATTGACTAGATGTACCATTGC	GGAAGCATTCAAAGCACAGCAATTGACTAT	428
18	AATTTAAATAGCAGTCCAAAGCATTATTTC	GCAGAATTTACTAAAACACACACCTTATAA	437
19	TCATTGCTTGTAATTGTTTGAAGAACTAGG	GGTTTGATAACATAAATTTGCCAGAAATCA	429
20	AGATAATGCGACTGCTCAATTCAAGAGAGG	GCTCATGCAAAATAACATGTAAAGGAAAGT	312
21	TATTTGGAATATTTTATAGCAATGCCTCTC	CCAGCTATTTTTCACTGTTTTATCATCAGT	460
22	TACGTTTCTGATAGCTATTCATATAGTCCCT	GCAGAAATTCAACAGATTATAAAGAACGAA	432
23	AATGCATAACACTTCCATACATGATATTGC	GTGAATGGTAGAATCAACTTATGGTTAATA	389
24	TTAGTTGCCCTTTGGAAATGTACTGATCTG	GGATATCATTTCAATTTGTGCAATGTAATT	378
25	TATGATACGAAAATTAAATATGGTAAGGGC	CAAGCTATGAGTTCATTAATTTCTCGATAT	472
26	TTCTTTTGTCTACTCATGAGAAATTTGTAG	GGGTAATAATTTGCAAAACTCTGTATATCA	635
27	ATAGCAAAAAAGAACTATCTAGTAAAAGGC	CCTTTGCTGCAGAAATATAGGAAGACTAGA	460
28	ATACTATGCCACTTTTTCAAATAAGTTAGC	GACTCTCATATAGAATCTAGTCTAGAATAT	482
29	GCCTCATAAGCTCTTTTAGAGACGTGAT	AATGCTAAAGCAAAATGGCCTAACAATATA	304
30	GTTGCCAGGTGATTTTTTATATTAGCTTAT	ATACTAATTGTTAATTTGTTTGCCTGATAC	591
31	TTTTAAAGCAATTAATGTTATACTAACACT	TAGTACGACACTTGTAGATTGTATGTGGAA	353
32	AATAAATTATACACCTTAAATTCCATTAGC	ATGTAGTGAAAAATAAAGCATGTCTG	297
33	TTATGACCTGAAGTACATGTGTCAGTAA	TTCAGTTTGCTTTATTTCTTATATTGATGA	417
34	TGCAATACAAGTTGAACCCAAAGGA	GAGGGACAAAATAAAACTGCTTAAAAACA	397
35	AATGTATTCAAATTGTTAATTCAAGTTCTG	AAATCCATATAAATCAGTAAATGAGGAAAT	585
36	TGTCTAAGATGTTATCTCTGACCTTTACTC	CACTTGCTAAACTTTTAGAAATAATGTTCA	310
37	GGATTTGAACTCTCTTTTCCAAGGGGGATT	AATTAAAACTACCAAGTAAGAGTTGTAGAT	291
38	AACTTGAATAATGCATATTTTTAGTGGTAT	TGTAATTTTGCAATTGAACTGAAGTAATAG	385
39	GAGACACTGATATTCAATTTTAGCAACTTA	CAATGTTGTAAAACCCGTGGCTTATCAATC	309
40	AATATGTGTATAAAAATAAAGCCATGTACT	ATTTCCATAAGATCTGGTAAAAGGCAAACT	347
41	ATTCCTAGTGTATAGTGAGTGAACAGACAA	AAGGCTACATTAATAAATATTGAAAGCTCT	323
42	TAAATGCAAACTTATCTAAGTACATTGATG	CTAAAAATCTTCCTAATTGAAGCTCACCTT	466
43	AAGTTGCAAAATTGCTGTAGTACCCTATCT	ATTGGAAGAGGAAGACAAGTAAAAAGTGCC	360
44	ACAAGGCATAAATGTGTTTCATAATGCTTA	TTTATTCCAACCTTGTTTTACTGTGTATAT	358
45	AATATATGCAATACTTTTATTAAATACGGT	TTTCCAGGACAAATTAAACAGTAGCAAATG	293
46	TTTTGAACCACATAACAAGAAGGACTAGAA	GAATGAGGGTCTCAAGACAGGCTAT	443
47	CTGATGGGTCAATGAGTATTATATACTG	TTATAAAATGTATAGCATTGTTTCCAGTTA	435
48	GAACATGAAAGCAGAATACTCAGTATGTAA	GTATAATTTGTAAAATAATAGTGGAGCTAA	474
49	AGCAATTTAAGCATATTTAATACTATTCTA	AAATGCTTTTAGGACATATAGGATAATTTA	524
50	AGCACTAGTCGCTGTATAGGTGTTTATCAC	AAAACGTTAAATGGTGCTAATCTCACATTC	341
51	TCAGAGGAATTGATAAAAATCCCCGTTAGT	AGACATCACATTTCAGTTTAGGGATTGGTT	418
52	TCAGTGGAGATGTAAAGAATGGAATAGACG	GATTCAAACAATTCGATAACCAAACATAGC	337
53	TGTAAATACATCAAATCATAGGAAGCTCAA	AGTATTTTCTGGTTTTTAACATACTTACTG	414
54	CATATAGTTATAATTGAATTATTTGTTGCT	ATAACTGAAATTCTCTTGAACATATTCCAC	497
55	CGTATATTTCCAGATAAAAAGTAGTCTTCT	TCTGCTCAAGCTGTTTTAAGTGTGTCACTG	397
56	TCTTCTGTATTTTCTACCACATAGATGTCA	ACAATAAAATCAATATGCTTCTCTGTGATA	496
57	ATCCTCGGAATGAGTAAGTTAACAGAACAG	ATTGTTTTGTGTATACGTTGCTAGTACTAC	470
58	ATGAATTACAATTTTACTCCATCTGTGCTA	GTAGTACTCATCTGGATTTAGATACACACT	393
59	TGAATAATTTGAAAGAATCGTTTAGTCATT	GCCATGGTAACTACTATAATCCTTTAATCT	427
60	ATTAAGTATGCCATATGCTTACTACTCCTC	AGTACTAGAAAAATAAATGCAACCTGGAAC	392
61	ATGTCACAACATATTGTCCTCGAAAAGGTT	AGGCATTGAAGGAAGCTTAACAATCTCATA	391
62	ATTCAGTATTTATCTGCACATAAGCTAAAA	AAATGTCACCCCTATTAATTACACAGTTAG	409

Table 5 (continued)

Exon	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
63	TTGCTTCAAGATTAACAGGCTTCTGTCCAT	GAACCAAAGACGCATTCTGACCTAGTATGA	307
64	GATAACATTTTCTCACTCAAGCTCTGTTTG	AAAATGCATATAAATTCTTACCCATGATAG	254
65	TATATTTGCATTGAAACATTTTTGTGATTT	AGGAAAGCAATAGTGCAATCTCTTGTGTTA	397
66	GCATCAACTTTTGTAAACACCTTTATTTCT	ACTATGAACAATACTTTTGGAATACTAACA	306
67	TAAACCTAATATCTTTAACTGGTGTTTTCT	AAACATTTCACCATATAAAAAGCATTACTA	511
68	TAATTTAAACCCCACAACTAAGAAATAAGT	GACAAAAGTAAGCAAAACACATCAGAATCC	543
69	TAAAATTGTGTATTTATTAAGGCTGGGAAA	TTTATAATGCAGTAAGTGTACCTAATGTAC	273
70	TATTTTTGTTTACTATGTGATTTCCAGGTA	TATGTTGTATGTAATGTTGCAGGGCTGGTT	452
71	TATTTTTAAGTTACATTATCTGCTAACGAT	AAAGGTGACCCAGCAAGTCCTGAAAAGTGT	382

AP005017, AP002979, AC087710, AC024996, and AC007719. Accession numbers of cDNA sequences of the CSMD3 gene are AB114604 (CSMD3a) and AB114605 (CSMD3b).

Results

Identification of CSMD3 gene

We have sequenced and analyzed a BAC contig of 30 Mb corresponding to the 8q22–q24.1, within which the genetic locus of an epilepsy BAFME1/FAME has been mapped (Figs. 1A and B). Extensive computer-aided analysis with exon prediction programs revealed many potential exons, some of which encoded CUB and/or sushi domains (Fig. 1B). We integrated these predicted exons and formulated initially eight putative genes (CUB1–CUB8, data not shown). CUB1 showed significant homology to a cDNA clone KIAA1894, but no other putative genes had any significant homology to the cDNA sequences deposited in GenBank nr database. We then designed a series of PCR primer pairs to amplify every exon of the eight putative CUB-containing genes and attempted PCR amplification using human cDNAs of adult brain, fetal brain, and testis. Moreover, we examined possible connection among these putative CUB-containing genes by PCR and found that all the neighboring two genes were actually connected to each other to form a single gene. Consequently, all the partial cDNA fragments were integrated into a single contiguous cDNA sequence of 13 kb (Fig. 1B, Tables 1 and 2). At this stage of analysis, we realized that this is a giant gene homologous to CSMD1 gene consisting of CUB and sushi multiple domains which was on human chromosome 8p23 [10] and hence we tentatively named it CSMD1L gene. However, as described later, we identified another CSMD1-like gene on human chromosome 1p34 and this new gene was more closely related to CSMD1 gene. We therefore designated this gene as CSMD2 and renamed CSMD1L as CSMD3 gene.

PCR amplification analysis using various tissue cDNAs indicated that there are two alternative first exons, namely exons 1a and 1b. In fact, two remarkable CpG islands were found in the genomic sequence of corresponding region (Fig. 1C). Transcripts in which exon 1a was connected directly with exon 2 were found in adult and fetal brains, whereas transcripts in which exon 1b was connected with exon 2 were found in testis. However, no cDNA fragments connecting exons 1a and 1b were amplified from any tissues in the MTC panel, and hence we concluded that exons 1a and 1b act as an independent first exon, generating two distinct transcripts. The first base of exon 1a was determined by sequence analysis of the longest 5′-RACE product from fetal brain cDNAs. We also analyzed 5′ end of exon 1b using testis cDNAs and unexpectedly we found an additional exon very close to exon 1b. Therefore, we named the original exon 1b as "exon 1b2" and the new exon as "exon 1b1", respectively. Thus, this new gene consists of 73 exons and produces two transcripts from two independent first exons (1a and 1b1) (Fig. 2A). We also found in the transcript of brain that exons 32 and 35 were connected and therefore two exons 33 and 34 were skipped, generating a shorter transcript. In addition, we found that exon 7 was spliced out in the transcripts of testis. In adult and fetal brains, two transcripts with or without exon 7 were observed. The longest coding sequences are estimated to be 11,124 bp for the transcript with exon 1a and 11,004 bp for the transcript with exon 1b. All the exon–intron junctions follow the GT–AG rule.

Protein structure of CSMD3

The longest open reading frame of 11,124 bp in this new gene CSMD3 encodes a protein of 406 kDa consisting of 3707 amino acids (Fig. 1F). Examination with SMART program revealed multiple units of CUB and sushi domains. The protein deduced from a transcript variant without exons 33 and 34 lost 10th CUB domain and therefore the predicted molecular mass was decreased to 398 kDa.

There are 14 CUB domains which are located from N-terminus and intervened by 13 sushi domains (Fig. 1F). There are additional 15 sushi domains which are arranged in tandem starting from the last CUB domain toward the C-terminus. A transmembrane domain was predicted at a position after the last sushi domain. The transcript which started at exon 1b caused

no change in domain structure (Fig. 1D). These analyses indicate that CSMD3 protein is a transmembrane protein composed of a long N-terminal polypeptide with CUB and sushi multiple domains exposing outside the cell, a single transmembrane domain, and C-terminal domain of 55 amino acid residues located in the cytoplasm.

Expression of CSMD3

Northern blot hybridization was performed using human adult and fetal multiple-tissue blots with 5′-partial cDNA of CSMD3 (containing exons 2–4) as a probe (Fig. 2A). The Northern blot analysis indicated that the longest transcript of about 13 kb is detected mainly in the adult brain, fetal brain, and testis (Fig. 2B). Some shorter transcripts were also found in the testis and fetal brain. The 13-kb transcript was not clearly detected in other tissues, but smears were seen in some tissues such as pancreas and spleen.

We then made further expression analysis by PCR amplification of cDNA fragments in the MTC panel. For this, we used three sets of PCR primers, which are designed to detect any cDNA fragments with the sequences covering exons 1a–3 (primers A), exons 1b2–3 (primers B), and exons 68–71 (primers C) (Fig. 2C). The

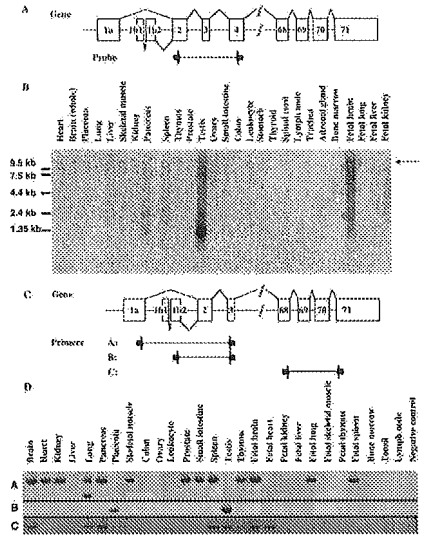

Fig. 2. Expression of human CSMD3 gene in various tissues. (A) The position of a cDNA probe used for Northern blot hybridization. It detects exons 2–4 of CSMD3 gene corresponding to the N-terminal CUB and sushi domains. (B) Northern blot analysis of CSMD3 gene expression using human multiple-tissue Northern blots of 27 tissues. Arrow indicates the longest transcript of CSMD3 gene. (C) Three sets of PCR primers (A, B, and C) used for expression analysis of CSMD3 gene. (D) PCR products amplified from each tissue in the MTC panels with three sets of primers. A second band in the lung with primer set A may represent an alternatively spliced transcript variant.

with BAFME1/FAME and identified three types of single nucleotide changes, such as 6204T/C, 10861A/C, and 657A/G in the coding sequences (Table 3).

The 6204T/C transition was found at the third letter of Tyr codon, but caused no amino acid substitution. This single nucleotide polymorphism (SNP) was a new type and was not deposited in the NCBI SNP database. The 10861A/C transversion caused amino acid substitution from Asn to His (N3621H). Interestingly, two patients from two unrelated families showed homozygous change of this type 10861A/C, however such a homozygous change is unlikely in the case of autosomal dominant disease like epilepsy BAFME1/FAME. The 657A/G transition caused amino acid change from Ile to Met (I219M). We expanded the mutation analysis of 6204T/C and 657A/G for additional 18 members of one of the patient families. However, these changes were not associated with the patients in the family and in fact these substitutions, 10861A/C and 657A/G, were found in the SNP database. Thus, we conclude, these three types of base-substitution are not responsible for pathogenesis of the BAFME1/FAME.

During the course of mutation search, we identified 24 potential SNPs including 18 new SNPs for flanking sequences of 72 exons of CSMD3 gene. These SNPs should be useful for further genetic analysis, particularly for linkage disequilibrium mapping of the region.

Comparison of CSMD genes among human, mouse, and fugu

We performed a homology search to the genomic sequence databases NCBI and Ensembl using the sequence of CSMD1 [10] and CSMD3. We identified an additional CSMD gene (CSMD2) on human chromosome 1p34. We also found three CSMD homologs in the mouse genome and four CSMD genes in the fugu genome. Thus, we could construct putative structure of human CSMD2, mouse CSMD2, and 3. Fig. 1F shows protein structures of human CSMD protein family. The numbers and positions of CUB and sushi domains and transmembrane domain are completely conserved among the human CSMD family. Fig. 1E shows comparison of exon structures and corresponding protein domains among human CSMD family genes. The exon structures are also well conserved except for exon 7 and last exon of CSMD2 and exon 7 and 8 of CSMD3. The exons 7 and 8 correspond to a portion of CSMD3 protein between second CUB and sushi domains. Consequently, CSMD3 protein is longer than CSMD1 and CSMD2 proteins. Interestingly, exon 7 of CSMD3 gene was subject to alternative splicing. We could not construct the entire structure of fugu CSMD because information on fugu genome sequence is incomplete. Nevertheless, using the sequences of human and mouse CSMD gene family and all available fugu sequences, we could identify the fifth unit (designated E unit) of CUB and sushi domain in the CSMD genes of human, mouse, and fugu (Fig. 3). Using the deduced amino acid sequences of the E unit of CUB and sushi domain, we were able to draw a phylogenetic tree of CSMD gene family. A relationship between CSMD1 and CSMD2 was found to be much closer than CDMD3. In addition, we found a fourth CSMD gene in fugu genome which could be derived from fugu CSMD3 gene.

Discussion

We identified a novel giant gene encoding a transmembrane protein with CUB and sushi multiple domains on the human chromosome 8q23.3–q24.1 in which benign adult familial myoclonic epilepsy type 1 (BAFME1/FAME, OMIM:601068) has been mapped. This giant gene consists of 73 exons and spans over 1.2 Mb on the genomic DNA region. Deduced amino acid sequence of the protein showed high homology to two other CSMD genes (CSMD1 on 8p23 and CSMD2 on 1p34) and hence this new gene was named as CSMD3. The CSMD3 gene was expressed mainly in adult and fetal brains as several forms of transcript variants. Comparative genomic analysis revealed the conserved family of CSMD genes in the mouse and fugu genomes.

There is little information about the function of CUB-containing proteins, although it has been postulated that they would be mainly involved in developmental process [11]. On the contrary, some sushi repeats are known as CCP domain, which exists in a wide variety of complement and adhesion proteins and is known to form a β-sandwich arrangement [12].

Epileptic seizures are induced by abnormal electrical discharges in the brain. Recent studies have revealed that membrane proteins for potassium, sodium, and calcium ion channels are the "gates" to regulate neuron signaling and are considered to be involved in some types of epilepsy. For example, it is reported that mutation of potassium gate proteins KCNQ2 and KCNQ3 causes benign familial neonatal convulsion [13,14]. Since ion channels are fundamental in generation of membrane potential, mutation of genes encoding some types of ion channel proteins may cause epilepsy. However, mutation of non-gate proteins also causes epilepsy or seizures in some cases. For instance, cystatin B, which is a widely expressed cysteine protease inhibitor, is responsible for a severe neurological disorder known as progressive myoclonus epilepsy of the Unverricht–Lundborg disease (EPM1) [15]. More recent study shows that SEZ-6 containing both CUB and sushi domains is involved in signaling and cell-cell adhesion. The SEZ-6 was first identified as a seizure related gene by differential screening of mRNA from cortical neurons

cDNA fragments covering exons 1a–3 were detected in many tissues including adult and fetal brains, whereas those covering exons 1b2–3 were detected only in placenta and testis (Fig. 2D). Interestingly, cDNA fragments covering exons 1a–3 were not found in placenta and testis, indicating the alternative use of first exons 1a and 1b. In seven tissues including adult brain, fetal brain, and testis, cDNAs covering from 5′-end through 3′-end were detected. These results are not perfect match with the Northern blot data, but strongly support that CSMD3 gene is expressed mainly in adult and fetal brains. The expression in the adult brain led us to examine CSMD3 gene as a candidate gene for the BAFME1/FAME.

Mutation analysis

We investigated the possible sequence variations in the coding regions of CSMD3 gene by PCR-based sequence analysis. Based on the exon/intron structure of CSMD3 gene, we designed sets of PCR primers to amplify every 72 exons except 1b1. For some larger exons, multiple overlapping PCR primers were generated. Using these PCR primers, we analyzed seven patients from five families

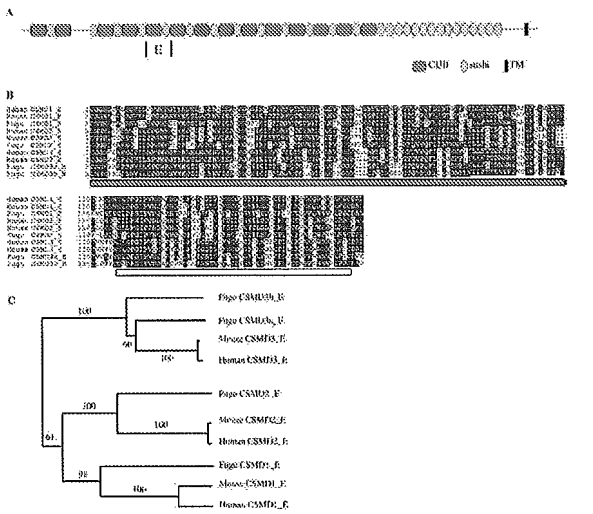

Fig. 3. Comparison of CSMD3 gene families among human, mouse, and fugu. (A) The position of the 5th unit of CUB and sushi domain (E unit) in the CSMD3. (B) Alignment of the E units of CUB-sushi domain in the CSMD gene families of human, mouse, and fugu. Alignment was performed by using the BCM implementation of Clustal X. The CUB and sushi domains are underlined with thick gray and white bars, respectively. Black background indicates that these amino acid residues are identical for more than half of the aligned amino acid residues. Three "X" letters at amino acid residues 108–110 in fugu CSMD2_E indicate undetermined amino acid sequence remaining in the fugu genome scaffold sequences. (C) Evolutionary trees of CUB-sushi unit E among human, mouse, and fugu CSMD. The numbers labeled with the bootstrap nodes indicate percentage of reliability of this tree deduced from 1000 bootstrap replicates.

treated with pentylenetetrazole (PTZ), which is a drug to induce epileptic seizures. The SEZ-6 is strongly expressed in the developing forebrain and necessary for the formation of neural network [16]. These findings led us to examine CSMD3 as a candidate gene.

Although our initial trial to identify causative mutations in the CSMD3 gene was unsuccessful, a possibility of CSMD3 gene as a candidate gene for BAFME1/FAME still remains because many other types of mutations have not been analyzed due to unusually large size of CSMD3 gene. As a similar example, majority of mutations in the Parkin gene, which is one of the major familial Parkinson's disease genes, are exonic deletions, and such exonic deletions are often difficult to find, particularly in the case of patients with compound heterozygosity, when simple PCR-based method was applied [17]. The size of CSMD3 gene is as large as Parkin and dystrophin, therefore it is likely that exonic deletions might frequently occur in the CSMD3 gene as observed in those giant genes. Because BAFME1/FAME is an autosomal dominant inheritance, and judging from the pedigrees of patients, it is very unlikely that both alleles harbor causative mutations.

Further genetic linkage analysis with newly identified SNPs will be necessary to identify the causative mutations for BAFME1/FAME.

Acknowledgments

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Original Article

Association of a GNAS1 Gene Variant with Hypertension and Diabetes Mellitus

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Previous studies have shown that the T allele of the GNAS1 T393C polymorphism is associated with poor responsiveness to β-blockade and that the T393C polymorphism interacts with cigarette smoking and alcohol consumption in the pathogenesis of hypertension. Thus, the T393C polymorphism is likely to interact with β-adrenoceptor (β-AR) stimulation in the pathogenesis of hypertension. Although this interaction might be caused by a direct effect of Gs proteins on the cardiovascular system, it could also result from an indirect effect of Gs proteins mediated by glucose metabolism. Moreover, association studies are often irreproducible. We therefore examined the possible interaction between the T393C polymorphism and γ-glutamyl transpeptidase (GGT), which is an established biomarker of alcohol consumption, in the association with glucose metabolism as well as with hypertension in a Japanese population. Genotyping for GNAS1 was performed by using the polymerase chain reaction-restriction fragment length polymorphism method in all 821 samples. The present study showed a significant interaction between the T393C polymorphism and GGT in the association with hypertension ($p=0.033$). This interaction was even more significant after adjustment for all confounding factors ($p=0.0025$). In contrast, analysis of the possible interaction of the T393C polymorphism with GGT in the association with diabetes mellitus or fasting plasma glucose failed to show a significant result. These results did not support the hypothesis that the interaction between the T393C polymorphism and GGT in the association with hypertension could be caused by an indirect effect of Gs proteins mediated by glucose metabolism. (*Hypertens Res* 2004; 27: 919–924)

Key Words: **G proteins, glucose, hypertension, polymorphism, sympathetic nervous system**

Introduction

Hypertension is considered to be a complex trait to which genetic, environmental, and demographic factors contribute interactively (*1*). The β-adrenoceptor (β-AR)-stimulatory guanine nucleotide-binding protein (Gs) system has been shown to play important roles in the cardiovascular system. Recently, based on several lines of biological evidence suggesting an association of the α-subunit of Gs proteins with hypertension (*2–4*), an initial study examined the association between a common silent polymorphism (T393C) in GNAS1 and hypertension (*5*). This study showed that the T393C polymorphism was significantly associated with hypertension and with poor responsiveness to β-blockade. Subsequently, we also studied the association in a large Japanese population, resulting in replication of the association between the T393C polymorphism and hypertension (*6*). Additionally, in the same population, we showed that the T393C polymorphism interacted with cigarette smoking and with alcohol consumption in the pathogenesis of hypertension (*6, 7*). Taken together, these results indicate that the T393C polymorphism is likely to interact with β-AR stimulation in the pathogenesis of hypertension. Although this interaction could be caused by a direct effect of Gs proteins on the cardiovascular system, it could also result from an indirect effect of Gs proteins mediated by glucose metabolism. However, we were unable to assess these two possibilities in the population previously analyzed due to a lack of information on glucose metabolism. In this regard, information on glucose metabolism as well as a well-established biomarker of alcohol consumption, γ-glutamyl transpeptidase (GGT) (*8–11*), was available in another population. Moreover, association studies are often irreproducible. We therefore examined the possible interaction between the T393C polymorphism and GGT in the association with glucose metabolism as well as with hypertension.

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Table 1. Characteristics of Participants According to Hypertension Status

Variable	Normotensive ($n=554$)	Hypertensive ($n=267$)	p value
Sex (male %)	78.3	89.0	<0.001
Age (years)	52.8±8.6	57.4±8.4	<0.001
BMI (kg/m²)	22.6±2.9	23.9±2.9	<0.001
SBP (mmHg)	115.1±11.6	143.9±16.6	<0.001
DBP (mmHg)	72.2±8.9	88.9±9.9	<0.001
T-Cho (mg/dl)	197.7±31.2	202.9±37.1	NS
HDL-Cho (mg/dl)	54.2±14.6	52.1±14.1	NS
TG (mg/dl)	116.3±80.9	151.6±129.2	<0.001
FPG (mg/dl)	101.2±17.0	105.7±18.5	<0.001
AST (U/l)	23.3±26.0	25.1±14.3	NS
ALT (U/l)	20.9±33.8	21.4±14.9	NS
GGT (U/l)	31.3±34.8	43.3±45.4	NS

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-Cho, plasma total cholesterol; HDL-Cho, plasma high density lipoprotein cholesterol; TG, plasma triglyceride; FPG, fasting plasma glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase. Data are mean±SD. Blood pressure readings prior to the start of antihypertensive medication were not available for 113 hypertensive subjects whose values were measured under treatment.

Methods

Subjects

According to the criteria described below, 267 hypertensive subjects and 554 normotensive subjects were selected from a population in the Hyogo region of Japan (Table 1). All subjects were Japanese. They had participated in a medical check-up, and the values of variables in their personal health records were used in the analyses. All subjects provided informed consent to participate, and 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. Diabetic subjects were diagnosed according to the WHO98 definition of type 2 diabetes (*12*). Subjects were considered to have diabetes mellitus if their fasting plasma glucose (FPG) concentration was ≥126 mg/dl.

DNA Analysis

Polymerase chain reaction (PCR) was used to detect the GNAS1 T393C polymorphism (*5*). The sense oligonucleotide primer was 5′-CTCCTAACTGACATGGTGCAA-3′ and the antisense primer was 5′-TAAGGCCACACAAGT CGGGGT-3′. The amplified PCR products were digested with 3 U of the restriction enzyme, FokI. A thymine at nucleotide position 393 was shown by a fragment of 345 bp, whereas a cytosine at nucleotide position 393 was shown by two fragments of 263 bp and 82 bp. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated.

Statistical Methods

Analysis of variance was used to assess differences in the means and variances of continuous variables. Logistic regression models were used to assess whether the GNAS1 T393C polymorphism made a statistically significant contribution to prediction of hypertension or diabetes mellitus, with consideration of the interactions between the T393C polymorphism and GGT. Sex, age, body mass index, plasma total cholesterol, high-density lipoprotein-cholesterol, and triglyceride levels were considered to be confounding factors (Table 1). Because the distributions of plasma triglyceride and GGT values were skewed, their logarithmically transformed values were used in the analyses. General linear regression models were used to assess whether the T393C polymorphism made a statistically significant contribution to prediction of blood pressure or of FPG, with consideration of interactions between the polymorphism and GGT. p values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software.

Table 2. GNSA1 Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype	Genotype frequency: Normotensive ($n=554$)	Genotype frequency: Hypertensive ($n=267$)	p value*	OR*	95% CI*
GNAS1 genotypes					
TT (%)	187 (33.8)	87 (32.6)			
TC (%)	254 (45.8)	126 (47.2)			
CC (%)	113 (20.4)	54 (20.2)	0.954*	1.01*	0.70–1.45*
GNAS1 alleles					
T (%)	628 (56.7)	300 (56.2)			
C (%)	480 (43.3)	234 (43.8)	0.849	0.98	0.79–1.21

*p value, OR and 95% CI are for TT+TC *vs.* CC. OR, odds ratio; CI, confidence interval.

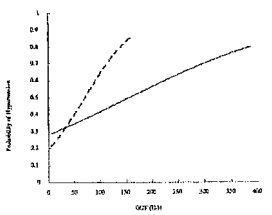

Fig. 1. *Genotypic variations in the positive relationship between GGT and the probability of having hypertension. The solid line indicates the TT and TC genotypes; the dotted line indicates the CC genotype. The regression between GGT and the probability of having hypertension in T carriers was represented by the equation, $Y=\exp(0.006X-0.946)/[1+\exp(0.006X-0.946)]$. The equation was $Y=\exp(0.020X-1.450)/[1+\exp(0.020X-1.450)]$ in CC homozygotes. CC homozygotes show a steeper slope than T carriers ($p=0.033$; after adjustment for all confounding factors, $p=0.0025$).*

Results

Association of GNAS1 T393C Polymorphism with Hypertension

A total of 821 Japanese individuals from the Hyogo region were categorized as hypertensive or normotensive and genotyped for the T393C polymorphism (Table 2). The frequencies in both hypertensive and normotensive subjects were in Hardy-Weinberg equilibrium. Statistical analysis failed to show a significant difference in the frequencies of the alleles ($p=0.954$) and genotypes ($p=0.849$ for TT+TC *vs.* CC) between the hypertensive and normotensive subjects (Table 2). Moreover, there was no significant difference in GGT and FPG between the genotypes (TT+TC *vs.* CC) (data not shown).

Interaction of GNAS1 T393C Polymorphism with GGT in Association with Hypertension and Diabetes Mellitus

We next analyzed the possible interaction of the GNAS1 T393C polymorphism with GGT in the association with hypertension in a logistic regression model. This analysis showed a significant interaction ($p=0.033$) (Fig. 1). This interaction was even more significant after adjustment for all confounding factors ($p=0.0025$). Because, all nine of the subjects with a GGT level above 150 U/l of GGT had the T allele(s), and despite the fact that the T393C polymorphism was not associated with GGT ($p=0.686$), we also examined this interaction using GGT stratified by quartiles (13, 22, and 42 U/l). The results showed that the p value for the interaction was 0.034. The p value was 0.0013 after adjustment for all confounding factors. In contrast, analysis of the possible interaction of the T393C polymorphism with GGT in the association with diabetes mellitus failed to show a significant relation ($p=0.492$).

We further analyzed the interaction between the T393C polymorphism and GGT in the association with blood pressure in general linear regression models (Table 3 and Fig. 2). This analysis showed a marginally significant interaction between the T393C polymorphism and GGT in the association with DBP ($p=0.049$). This interaction was more significant after adjustment for all confounding factors ($p=0.0027$). The analysis also showed a non-significant trend supporting the presence of an interaction between the T393C polymorphism and GGT in the association with SBP ($p=0.198$). However, this interaction was significant after adjustment for all confounding factors ($p=0.011$). Analyses of these interactions using GGT stratified by quartiles showed that the p values for these interactions were 0.039 and 0.183 for DBP and SBP, respectively. The p values were 0.0011 for DBP and 0.0041 for SBP after adjustment for all confounding factors. In contrast, analysis of the possible interaction of the T393C polymorphism with GGT in the association with FPG failed to show a significant result ($p=0.998$) (Table 3).

Table 3. General Linear Model for Regression of GGT in Association with Blood Pressure and FPG According to Genotype

Phenotype	Genotype (n)	Coefficient	Constant	p value for regression	Determination coefficient	p value for interaction
SBP (mmHg)	TT+TC (654)	0.05	123.0	0.037	0.027	
	CC (167)	0.10	119.6	0.006	0.077	0.198
DBP (mmHg)	TT+TC (654)	0.03	76.5	0.003	0.034	
	CC (167)	0.09	74.1	0.003	0.122	0.049
FPG (mg/dl)	TT+TC (654)	5.59	85.0	<0.001	0.065	
	CC (167)	5.58	84.0	<0.001	0.071	0.998

GGT, γ-glutamyl transpeptidase; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure.

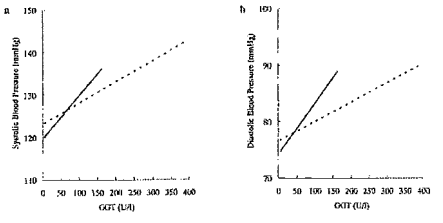

Fig. 2. *Genotype-specific regression slopes of blood pressure on GGT. The solid line indicates the TT and TC genotypes; the dotted line indicates the CC genotype. a: The regression between GGT and SBP in T carriers was represented by the equation, $Y=0.101X+119.6$. The equation was $Y=0.090X+74.1$. in CC homozygotes. CC homozygotes showed a steeper slope than T carriers ($p=0.198$; after adjustment for all confounding factors, $p=0.011$). b: The regression between GGT and DBP in T carriers was represented by the equation, $Y=0.034X+76.5$ in CC homozygotes. CC homozygotes showed a steeper slope than T carriers ($p=0.049$; after adjustment for all confounding factors, $p=0.0027$).*

Discussion

The present study showed a significant interaction of the GNAS1 T393C polymorphism with GGT in the association with hypertension in a Japanese population. In subjects with lower GGT, the CC genotype appeared to have a protective effect against the development of hypertension, whereas the TT and TC genotypes appeared to have a risk-increasing effect (Figs. 1 and 2). However, CC homozygotes were more sensitive to an increment of GGT than T carriers in the association with hypertension. Consequently, in subjects with higher GGT, the TT and TC genotypes appeared to have a protective effect against the development of hypertension, whereas the CC genotype appeared to have a risk-increasing effect. These relations and directions were very similar to those in the interaction of the T393C polymorphism with cigarette smoking and alcohol consumption in the association with hypertension (*6, 7*). Given that both cigarette smoking and alcohol consumption could affect blood pressure through the β-AR–Gs protein system (*13–16*), and because GGT is a well-established biomarker of alcohol consumption (*8–11*), the significant interactions of the T393C polymorphism with cigarette smoking, alcohol consumption, and GGT in the association with hypertension may each indicate the presence of an interaction between the T393C polymorphism and β-AR stimulation in the pathogenesis of hypertension from different angles. Indeed, the T393C polymorphism has been shown to be associated with blood pressure response to β-blockers in a Caucasian population (*5*). However, more accurate assessment will require additional information on drinking history.

The present study failed to show a significant association between the T393C polymorphism and hypertension. How-

ever, given the above interactions, this failure may not be surprising, because an association between a polymorphism and hypertension could be masked in the presence of gene-environmental interactions even when analyzed in subjects with matched confounding factors (6). In this context, the significant association shown in the population previously analyzed was largely dependent on the fact that hypertensive subjects had a significantly higher frequency of the TT and TC genotypes than normotensive subjects both in non-heavy smokers and in non-drinkers or light drinkers. In the present population, a possibly lower β-AR stimulation might have resulted in failure to show a significant association between the T393C polymorphism and hypertension, although the extent of β-AR stimulation is difficult to estimate.

The precise mechanism of the interaction between the T393C polymorphism and β-AR stimulation in the pathogenesis of hypertension is elusive and remains to be investigated. Previous studies have shown that the T allele of the T393C polymorphism is associated with poor responsiveness to β-blockade (5) and that the T393C polymorphism interacts with cigarette smoking and alcohol consumption in the pathogenesis of hypertension (6, 7). Based on this evidence, we previously speculated that the TT and TC genotypes or genotypes in linkage disequilibrium with them might produce a constant amount of α-subunit of Gs proteins independent of activation of the sympathetic nervous system (6). In contrast, the CC genotype or genotype in linkage disequilibrium with it might produce a controlled amount of α-subunit of Gs proteins. In this context, it is noteworthy that CC homozygotes tended to be more strongly affected by an increment of GGT than T carriers in the association with hypertension (Figs. 1 and 2). Thus, the above explanation appears also to be applicable to the interaction between the T393C polymorphism and GGT in the association with hypertension.

Another point of view in regard to the interaction between the T393C polymorphism and β-AR stimulation in the association with hypertension is that, depending on the genotype, β-AR stimulation could influence glucose metabolism, which in turn could influence blood pressure (6). In order to assess this possibility, we examined the possible interaction between the T393C polymorphism and GGT in the association with diabetes mellitus, and failed to show a significant interaction. Analysis of the possible interaction between the T393C polymorphism and GGT in the association with FPG also failed to show a significant interaction. These results appeared to contribute evidence against the hypothesis that the interaction between the T393C polymorphism and GGT in the association with hypertension could be caused by an indirect effect of Gs proteins mediated by glucose metabolism. Further extending this, the interaction between the T393C polymorphism and β-AR stimulation in the association with hypertension might not result from an indirect effect of Gs proteins mediated by glucose metabolism. However, because data on insulin levels, HbA1c, and oral glucose tolerance test were not available in the present population, a further study including such data should be performed.

Taking advantage of the fact that GGT is a good biomarker of alcohol consumption, the present study provided an additional piece of evidence supporting the presence of an interaction between the GNAS1 T393C polymorphism and β-AR stimulation in the pathogenesis of hypertension. Moreover, by analyzing the possible interaction between the T393C polymorphism and GGT in the association with diabetes mellitus and with FPG, the present study provided evidence against the possibility that the interaction may be caused by an indirect effect of Gs proteins mediated by glucose metabolism. However, the present study did not assess gene-gene interaction, which is a candidate factor for modifying the evaluation of an association. In this context, interaction analyses of the GNAS1 gene with other genes involved in the β-AR–Gs protein system may be helpful to improve understanding of the relation between the β-AR–Gs protein system and hypertension. Furthermore, in order to establish an association, it is also important that associated alleles affect the gene product in a physiologically meaningful way. In this context, thus far, there is no evidence showing that the T393C polymorphism affects the gene product in a physiologically meaningful way. Therefore, it is necessary to investigate the possible biological change of the gene product by the T393C polymorphism or another variant in linkage disequilibrium with it.

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Genetic Predisposition to Neurological Symptoms in Lacunar Infarction

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Key Words
Polymorphism · Angiotensin-converting enzyme · Angiotensinogen · Type 1 angiotensin II receptor · Lacunar infarction · Magnetic resonance imaging

Abstract
Objective: Lacunar infarction is a unique stroke entity with characteristic symptoms. However, it is often silent clinically. The possible genetic predisposition to symptoms of lacunar infarction was investigated. ***Methods:*** One-hundred and fifty-one patients with lacunar stroke were consecutively recruited. Lacunar stroke was diagnosed based on both neurological symptoms and lacunar lesion(s), demonstrated by MRI, that were responsible for the symptoms. One-hundred and fifty control subjects with MRI-proven lacunar lesions without neurological symptoms served as controls. There was no significant difference in age, sex and prevalence of known risk factors between cases and controls. Insertion and deletion polymorphisms of the angiotensin-converting enzyme gene (ACE), M235T substitution of the angiotensinogen gene (AGT), and A1133C substitution of type 1 receptor of the angiotensin II gene were determined. ***Results:*** The frequency of ACE D allele was significantly higher in symptomatic patients compared with asymptomatic subjects (0.44 vs. 0.36, $p < 0.05$). The genotype distribution of AGT was significantly different between symptomatic and asymptomatic patients ($\chi^2 = 6.6$, $p = 0.037$). Multiple logistic regression analysis revealed that ACE gene and AGT genotypes were independently associated with the neurological manifestation of lacunar infarction. In subjects with 1 lacuna, the odds ratio of the ACE DD genotype for symptomatic manifestation was 4.98 (95% CI 1.25–19.9). In subjects with 4 or more lacunae, the odds ratio of the ACE II genotype for symptomatic manifestation was 0.24 (95% CI 0.10–0.56). Furthermore, the ACE gene polymorphism was significantly different between symptomatic patients with a single lacuna and asymptomatic subjects with 4 or more multiple lacunar infarctions ($\chi^2 = 10.6$, $p = 0.005$). ***Conclusion:*** These findings suggest that 2 subtypes of lacunar infarction, single symptomatic lacuna and multiple asymptomatic lacunae, may possess different genetic backgrounds. Subjects with the ACE DD genotype could be more predisposed to be symptomatic in first-ever lacunar stroke, while the ACE II genotype may convey resistance to symptoms even after multiple lacunar strokes. Polymorphism of genes of the renin-angiotensin system could be involved in the manifestation of neurological symptoms of lacunar infarction.

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Introduction

The candidate gene approach is the mainstay of genetic study of ischemic stroke [1]. Among numerous candidate genes, insertion and deletion polymorphism of the angiotensin-converting enzyme (ACE) gene is the most frequently studied in the field of cardiovascular diseases including stroke [1–8]. However, conflicting results have been reported [1–8].

The lack of precise phenotyping of ischemic stroke is thought to be a major problem leading to the conflicting results. Many studies have evaluated ischemic stroke cases with diverse clinical manifestations including atherothrombotic as well as lacunar infarctions [2, 3, 5, 6]. Since the pathophysiological backgrounds and mechanisms are significantly different among subtypes of ischemic stroke, a more precise approach with accurate phenotyping of the stroke subtypes should be taken. Among subtypes of ischemic stroke, many studies have reported the strongest association of the ACE genotype with lacunar stroke [1–3]. However, the number of cases in these studies was too small to reach a conclusion.

Lacunar infarction is a common form of stroke, accounting for 10–40% of stroke cases [9–12]. Lacunar infarction possesses several noteworthy characteristics including low mortality rate [12–14]. Although its symptoms are well known as lacunar syndromes, lacunar infarction is more often silent [15, 16]. The prevalence of asymptomatic lacunar lesions has been shown to increase with hypertension and aging [15–17]. Since a previous study showing a positive association between lacunar infarction and ACE polymorphism analyzed both symptomatic and asymptomatic lacunar subjects together [3], phenotyping of lacunar stroke has not been completely accurate.

Furthermore, asymptomatic lacunar infarctions are often multiple [15, 16]. This other feature of lacunar infarction raised the possibility of two distinct clinical entities of lacunar infarction: single symptomatic lacunar stroke and asymptomatic multiple lacunar infarctions [18–20]. Based upon these findings, we hypothesized that there is a genetic predisposition to the manifestation of neurological symptoms of lacunar infarction. However, there has been no study investigating the genetic background of lacunar infarction including these clinical characteristics.

In the present study, we performed an association study of genes of the renin-angiotensin system (RAS) between symptomatic lacunar infarction patients and subjects with lacunar infarction without neurological symptoms. In this particular case-control matching, we revealed a genetic predisposition to the neurological symptomatic manifestation of lacunar infarction. We also compared subjects with first-ever lacunar infarction with neurological clinical manifestation as well as MRI documentation, and asymptomatic subjects with multiple lacunar infarctions, to determine whether there is any genetic difference in the two categories of lacunar infarction.

Subjects and Methods

Subjects

The cases were recruited from patients admitted to Ehime University Hospital, Katagi Neurosurgical Clinic or Kyoto Second Red-Cross Hospital in Japan with the manifestation of first symptomatic lacunar stroke between April 1998 and December 1999. The diagnosis of lacunar stroke was made when both neurological symptoms and lacunar infarctions were confirmed on an MRI scan by neurologists. Cases with a history of symptomatic lacunar stroke events with documentation in both the clinical records and MRI were also included [21].

Control subjects were recruited from consecutive subjects who visited the same institute for medical checkup. They had several risk factors for stroke and underwent brain MRI examination for evaluation of atherosclerotic conditions. The criteria for asymptomatic lacunar infarction were as follows: (i) lacunar lesion(s) demonstrated by MRI, (ii) absence of neurological signs and symptoms and (iii) no past history of stroke including transient ischemic attack.

Risk factors for stroke were determined for each participant. These included hypertension, diabetes mellitus, dyslipidemia and current smoking. Hypertension was defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg without medication in the outpatient clinic on at least 2 separate measurements or taking antihypertensive drugs. Diabetes mellitus was defined as fasting blood glucose ≥ 126 mg/dl (7 mmol/l) or nonfasting blood glucose ≥ 200 mg/dl (11.11 mmol/l) or use of medication for diabetes. Dyslipidemia was defined as total cholesterol ≥ 220 mg/dl (5.69 mmol/l) and/or HDL cholesterol ≤ 35 mg/dl (0.90 mmol/l) and/or triglyceride ≥ 150 mg/dl (1.71 mmol/l) or use of medication for dyslipidemia. Informed consent to the procedure was obtained from each participant. All procedures were approved by the Ethics Committee of the Ehime University School of Medicine.

Brain MRI Examination

The diagnosis of lacunar infarction was made by brain MRI examination [21–23]. MRI was performed with a superconducting magnet with a main field strength of 1.0–1.5 T. A lacuna was defined as an area of low signal intensity that measured > 3 mm and < 15 mm on T_1-weighted images and was also visible as a hyperintense lesion on T_2-weighted images. The number of lacunae was counted for each subject. Both symptomatic and asymptomatic patients were divided into 3 groups according to the number of lacunae: single lacuna, 2 or 3 lacunae and 4 or more lacunae. MRI was evaluated by 2 authors (K.K. and Y.Y.) who were not aware of the types of gene polymorphisms. An active lacuna lesion among multiple lacuna infarcts in symptomatic patients was determined by the sequential change in MRI findings and brain CT lesions during their course of the stroke.

Detection of Gene Polymorphisms

Genomic DNA was extracted from peripheral blood samples using an extraction kit (Qiagen GmbH, Hilden, Germany). Gene polymorphisms of ACE insertion/deletion, angiotensinogen gene (AGT) M235T and type 1 receptor of angiotensin II gene (AT1R) A1166C were determined by standard methods [24–27].

In brief, the insertion/deletion polymorphism of the ACE gene was identified by polymerase chain reaction (PCR) using a set of oligonucleotide primers flanking the polymorphic site in intron 16 (sense primer 5'-GCCCTGCAGGTGTCTGCAGCATGT-3' and antisense primer 5'-GGATGGCTCTCCCCGCCTTGTTCTC-3') [24]. To avoid mistyping, each sample found to have the DD genotype was subjected to a second, independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' and 5'TCG CCA GCC CTC CCA TGC CCA TAA-3') [25].

To identify the AGT M235T polymorphism, sense primer 5'-TGACAGGATGGAAGACTGGCTGCTCCCTGC-3' and antisense primer 5'-AGCAGAGAGGTTTGCCTTACCTTG-3' were used [26]. The PCR product (5 µl) was digested with 5 units *Msp*I for 1 h, and cleaved products were separated by electrophoresis.

The AT1R A1166C polymorphism was determined using sense primer 5'-TCCTCTGCAGCACTTCACTACCAAATGGGC-3' and antisense primer 5'-TTCATCGAGTTTCTGACATT-3' [27]. The PCR product (5 µl) was digested with 5 units *Hae*III for 1 h, and cleaved products were separated by electrophoresis.

Statistical Analysis

All values are expressed as means ± SD if not specified. Statistical analysis among genotypes was performed by ANOVA. Prevalence of genotypes and the Hardy-Weinberg equilibrium were analyzed by the χ^2 method. To assess the independent role of risk factors, multiple logistic regression analysis was performed with neurological symptoms as dependent variables, and age, sex, current smoking, hypertension, dyslipidemia, diabetes, total number of lacunae and genotypes as independent variables. The inheritance models of dominant (DD + ID vs. II), additive (DD vs. ID vs. II) as well as recessive (DD vs. ID + II) were all considered. For each odds ratio, the 95% confidence interval was calculated. A probability value less than 0.05 was considered statistically significant. All statistical analyses were performed using StatView package and JMP 4.0 (SAS).

Results

Demographic Characteristics of Cases and Controls

The clinical profiles of the two populations studied in the present study are summarized in table 1. There was no difference in age, sex and the frequency of risk factors including hypertension, dyslipidemia, diabetes mellitus and current smoking between the symptomatic and asymptomatic lacunar infarction groups.

The number of lacunae and their locations are also summarized in table 1. There was no difference in number of lacunae in the whole brain, deep white matter as well as brainstem between the symptomatic and asymptomatic lacunar infarction groups. However, the number and prevalence of lacunae in the basal ganglia were significantly higher in symptomatic patients compared with asymptomatic subjects. In the symptomatic lacunar infarction group, hemiparesis was the most common symptom, followed by sensory deficit.

Table 1. Clinical profile of cases and controls

	Lacunar infarction patients	
	symptomatic	asymptomatic
Number (male/female)	151 (88/63)	150 (82/68)
Age, years	68 ± 9	69 ± 9
Hypertension, %	118 (78)	120 (80)
Dyslipidemia, %	66 (44)	59 (39)
Diabetes mellitus, %	33 (23)	23 (15)
Current smoker, %	57 (38)	56 (37)
Number of lacunae		
Whole brain	2.9 ± 2.1	2.7 ± 1.8
Basal ganglia	1.9 ± 1.7*	1.1 ± 1.5
Corona radiata	0.9 ± 1.2	1.0 ± 1.4
Brainstem	0.1 ± 0.3	0.06 ± 0.8
Symptoms[1]		
Hemiparesis	115	
Sensory disturbance	19	
Ataxia	12	
Dysarthria	9	

* $p < 0.05$ versus asymptomatic lacunar infarction patients.
[1] Four patients had more than 1 symptom.

Table 2. Genotype and allele frequencies of ACE, AGT and AT1R in the study population

		Lacunar infarction patients	
		symptomatic (n = 151)	asymptomatic (n = 150)
ACE	II	49 (0.32)	59 (0.39)
	ID	71 (0.47)	74 (0.49)
	DD	31 (0.21)	17 (0.11)
	Allele D	0.44*	0.36
AGT	MM	2 (0.01)*	11 (0.07)
	MT	46 (0.30)*	42 (0.28)
	TT	103 (0.68)*	97 (0.65)
	Allele M	0.17	0.21
AT1R	CC	2 (0.01)	0 (0)
	AC	16 (0.11)	22 (0.15)
	AA	133 (0.88)	128 (0.85)
	Allele C	0.07	0.07

* $p < 0.05$ versus asymptomatic lacunar infarction patients. Figures in parentheses indicate ratio of genotype.

Table 3. Multiple logistic regression analysis with neurological symptoms as dependent variable in patients with lacunar infarction

Independent variables	χ^2	OR	95% CI	p value
Age	0.98	1.01	0.99–1.04	0.32
Sex (male)	0.06	1.07	0.60–1.93	0.81
Hypertension	0.42	0.83	0.46–1.48	0.52
Diabetes mellitus	1.84	1.54	0.83–2.87	0.17
Dyslipidemia	0.38	1.17	0.71–1.90	0.54
Current smoker	0.20	1.15	0.62–2.13	0.66
Number of lacunae (total)	2.58	0.86	0.72–1.03	0.11
Number of lacunae (basal ganglia)	7.39	1.39	1.10–1.76	0.007
ACE genotype (D recessive)	4.99	2.13	1.10–4.13	0.026
AGT genotype (T dominant)	4.39	5.28	1.11–25.04	0.036
AT1R genotype (C dominant)	0.16	0.87	0.42–1.77	0.69

ACE genotype (D recessive): 1 = II + ID, 2 = DD; AGT genotype (T dominant): 1 = MM, 2 = TT + MT. AT1R genotype (C dominant): 1 = AA, 2 = CC + AC.

Gene Polymorphism of RAS and Symptomatic Lacunar Infarctions

Table 2 summarizes the genotype and allele frequencies of ACE, AGT and AT1R. The distributions of ACE, AGT and AT1R genotypes observed in the study population were in agreement with the Hardy-Weinberg equilibrium. The frequencies of ACE genotypes were not different between symptomatic patients and asymptomatic subjects. However, the frequency of the D allele was significantly higher in symptomatic patients compared with asymptomatic subjects. The genotype distribution of AGT in symptomatic patients was also significantly different from that in asymptomatic patients.

To further investigate whether genotype was independently associated with the symptomatic manifestation of lacunar infarction, multiple logistic regression analysis was performed in all subjects with lacunar infarction (n = 301) with neurological symptoms as dependent variables (table 3). It revealed that the number of lacunae in the basal ganglia and ACE and AGT genotypes were independently associated with the manifestation of neurological symptoms. On the other hand, other risk factors including age, sex, hypertension, diabetes mellitus, dyslipidemia and smoking were not significantly related to the symptomatic manifestation of lacunar infarction.

Table 4. Single symptomatic lacunar infarction and multiple asymptomatic lacunar infarctions: distribution of genotypes encoding the renin-angiotensin system

		Patients with a single symptomatic lacuna (n = 54)	Patients with 4 or more multiple asymptomatic lacunar infarctions (n = 42)
ACE	II	18 (0.33)	28 (0.67)
	ID	24 (0.44)	10 (0.24)
	DD	12 (0.22)	4 (0.10)
		$\chi^2 = 10.60$, f = 2, p = 0.005	
AGT	MM	2 (0.04)	2 (0.05)
	MT	16 (0.30)	12 (0.29)
	TT	36 (0.67)	28 (0.67)
		$\chi^2 = 0.07$, f = 2, p = 0.96	
AT1R	CC	0 (0)	0 (0)
	AC	3 (0.06)	6 (0.14)
	AA	51 (0.94)	36 (0.86)
		$\chi^2 = 2.12$, f = 1, p = 0.15	

Lacunar Subtypes and RAS Genes

The genetic difference between 2 lacunar categories, single symptomatic lacunar infarction and asymptomatic multiple lacunar infarction, was further evaluated (table 4). There was a significant difference in ACE genotype distribution between the 2 lacunar subtypes. Multiple logistic regression analysis also showed that ACE genotype (D dominant) was independently associated with the manifestation of neurological symptoms in this population (odds ratio 11.09, 95% CI 2.0–14.63; p = 0.0009). Odds ratios of ACE genotypes for the manifestation of neurological symptoms according to the number of lacunae are shown in figure 1. The ACE DD genotype was significantly associated with the symptomatic manifestation of single lacunar infarction. On the other hand, the ACE II genotype was significantly associated with an asymptomatic state in patients with multiple lacunar infarctions.

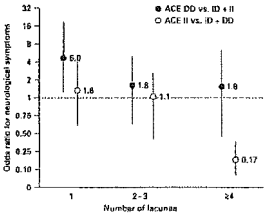

Fig. 1. Odds ratios of ACE genotypes for the manifestation of neurological symptoms of lacunar infarctions according to the number of lacunae. Closed circles indicate the odds ratio for ACE DD genotype compared with ACE ID + II, and open circles indicate the odds ratio for ACE II genotype compared with ACE DD + ID. Bars indicate 95% confidence intervals. Odds ratios were corrected for other risk factors including hypertension, diabetes mellitus, dyslipidemia, age and smoking.

Discussion

In the present study, polymorphisms of genes encoding the renin-angiotensin system were significantly associated with a symptomatic manifestation of lacunar stroke. Several studies on the genetic predisposition to stroke have been reported, with conflicting results on genes related to components of the renin-angiotensin system [1–8]. Many of these studies evaluated stroke patients combining different categories of infarction including lacunar stroke [2, 3, 5, 6]. Since the main underlying mechanism of lacunar infarction has been shown to be arteriosclerosis and lipohyalinosis in small arterioles such as perforating arteries [28], the etiology of lacunar infarction is quite different from that of atherothrombotic infarction as well as cardioembolic infarctions. Accordingly, the genetic background for lacunar infarction could also be different from that of atherothrombotic as well as cardioembolic infarction. However, no study has evaluated the genetic background of symptomatic lacunar infarction alone. The diversity of the background for the different categories of stroke could underlie the failure to detect an association in previous studies. Furthermore, the number of lacunar infarction patients evaluated according to genetic background was small in previous studies [4, 5, 7]. This could be another reason for the negative results. In the present study, we focused on lacunar stroke as a single category of ischemic stroke.

Fisher [28, 29] defined lacunes pathologically as areas of infarction of less than 2 cm in size. Lacunar stroke is more often asymptomatic than symptomatic, although the symptoms of lacunar infarction are recognized as classical lacunar syndromes. In the Cardiovascular Health Study, 3,660 elderly subjects aged ≥ 65 years underwent brain MRI examination [15]. Among them, 751 subjects without any history of TIA or stroke had MRI-proven lacunar lesions. The frequency of asymptomatic lacunar lesions was more than 30%. One third of lacunar lesions were multiple. Accordingly, an asymptomatic status as well as multiple lesions are clinical features of lacunar infarction. The genetic background of asymptomatic lacunar stroke in the Japanese population has been investigated [4, 8]. Although the number of patients with lacunar infarction was small, both studies failed to demonstrate an association with ACE gene polymorphism. In a community-based study, it has been shown that the number of asymptomatic lacunae was significantly associated with AT1 AC and AGT MT genotypes [4]. However, no study has ever investigated the genetic association with neurological symptoms in lacunar stroke. In the present study, we compared neurologically symptomatic lacunar infarction patients with asymptomatic patients. Background risk factors including age, prevalence of hypertension, diabetes mellitus and dyslipidemia as well as current smoking were not significantly different between the two groups. In this population, it was revealed that ACE and AGT genotypes were associated with symptomatic manifestation.

Fisher [29–31] distinguished 2 causes of local small-vessel obstruction: lipohyalinosis, mainly found in hypertensive patients with small, multiple and usually asymptomatic lacunes, and microatheromatous disease, which mainly occurred in patients with a larger, usually single symptomatic lacuna. However, recent study indicates that small-vessel atheromatous disease but not lipohyalinosis may be a primary mechanism for lacunar infarctions [32]. There have been suggestions that the profiles of risk factors for lacunae may differ between single and multiple lacunae, as well as silent and symptomatic lacunae [15, 18–20]. However, a study with a large cohort of elderly subjects failed to reveal significant factors to discriminate single and multiple, as well as symptomatic and asymptomatic lacunae [15]. To examine the possibility that the genetic background for multiple asymptomatic lacunar infarctions is different from that for single symptomatic lacunar infarction, we analyzed the gene polymorphisms in symptomatic lacunar stroke patients and asymptomatic lacunar infarction subjects, focusing on the number of lacunae. We observed a significantly higher prevalence of ACE DD in patients with single symptomatic lacunar infarction compared with subjects with multiple asymptomatic lacunar infarctions. Analysis of subjects with multiple lacunar infarctions revealed that those with the ACE II genotype might be resistant to being symptomatic even after multiple lacunar strokes. These findings have never been obtained in previous analyses between patients and normal controls. Our finding indicates that the ACE DD genotype predisposes to microatheromatous lacunae, since microatheromatous disease occurs in cases with a larger, usually single symptomatic lacuna [29–31, 33].

Recently, relatively high prevalences of distinct mechanisms for lacunar infarcts, cardioembolism [34] and carotid arterial stenosis [35] have also been reported. Since these reports studied symptomatic lacunar patients, it is conceivable that our symptomatic patients might also have had these underlying mechanisms. To address the mechanism-specific manifestation of lacunar infarcts more precisely, a more detailed determination of phenotype including carotid ultrasound as well as echocardiogram would be necessary.

In the present study, control subjects were recruited from patients who underwent brain MRI as an evaluation of atherosclerosis because of their risk factors. Although they did not have any neurological symptoms including lacunar syndromes, they might have had nonspecific symptoms such as headache. Furthermore, it is also reported that asymptomatic lacunae were associated with cognitive impairment [15, 36, 37], depressive mood [38] as well as autonomic abnormalities such as dysregulation of blood pressure [21, 37, 39]. Accordingly, we could not rule out the possibility that nonspecific symptoms could be associated with the present genetic finding rather than symptomatic stroke conditions. Two Japanese studies have reported no difference in ACE insertion/deletion genotype distribution between patients with asymptomatic cerebral infarction and subjects without a brain lesion demonstrated by MRI [4, 8]. These findings may indicate that the present findings are related to symptomatic lacunae rather than control-related conditions.

We could not exactly explain the mechanism by which the gene encoding ACE was associated with neurological manifestation. The site and size of the lacunae are responsible for the symptoms [33]. In the present study, there was no difference in the total number of lacunae between symptomatic and asymptomatic subjects. However, the number and prevalence of lacunae in the basal ganglia were significantly higher in symptomatic patients than asymptomatic subjects, suggesting that the site of infarction could be associated with genetic predisposition. However, our finding that ACE as well as AGT gene polymorphisms were significantly associated with the symptomatic manifestation of lacunar stroke was not due to a genotype-specific accumulation of lacunae in the region of the basal ganglia (data not shown). These findings indicate that ACE gene polymorphism could affect the size of lacunar infarctions. However, to reach the conclusion, studies with more precise determination of phenotype with pathological documentation as well as prospective studies with a larger population are needed.

In summary, ACE gene and AGT genotypes were associated with the manifestation of neurological symptoms in patients with lacunar strokes. Furthermore, the ACE DD genotype was an independent risk factor for being symptomatic with the first-ever lacunar stroke. On the other hand, patients with the ACE II genotype were less symptomatic even after multiple lacunar infarctions. These findings suggest the existence of diverse mechanisms in single symptomatic lacunar infarction and multiple asymptomatic lacunar infarctions.

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bjh research paper

TAFI polymorphisms at amino acids 147 and 325 are not risk factors for cerebral infarction

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Summary

Thrombin-activatable fibrinolysis inhibitor (TAFI) was reported as an anaphylatoxin-inactivating enzyme generated by proteolytic cleavage of its zymogen, and is the same enzyme as that first designated by our group as procarboxypeptidase R (proCPR). Its level in plasma appears to influence vascular disease. In addition, TAFI activity is strongly influenced by genetic polymorphism, especially at amino acids 147 and 325. We investigated whether these *TAFI* polymorphisms would act as a risk factor for cerebral infarction (CI) by examining 253 samples in which the diagnosis was cliniconeuropathologically confirmed. We found little that was statistically significant in terms of these polymorphisms among patients with no vascular problems or in a population-based control group. In the present study of an elderly Japanese group, our samples revealed a lower percentage of the Ile allele at Thr/Ile-325 compared with western counterparts. Although patients with severe infarcts had a lower percentage of the Ile allele (10%) at amino acid position 325 compared with the slightly and moderately affected patients and the population-based control group (15–18%), no statistical significance was found. None of our results showed any statistical correlation between *TAFI* polymorphisms and CI.

Keywords: carboxypeptidase R, thrombin-activatable fibrinolysis inhibitor, polymorphism, Thr/Ala-147, Thr/Ile-325.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is also termed procarboxypeptidase R (proCPR), procarboxypeptidase U and plasma procarboxypeptidase B.

We were the first to identify this enzyme, which removes carboxyterminal arginine of complement (C) 3a and C5a, as a plasma carboxypeptidase distinct from carboxypeptidase N (Campbell & Okada, 1989). Six years later, Bajzar *et al* (1995) reported this protein as TAFI, since when activated, it inhibited the lysis of clots formed during thrombin activation. In addition, we showed that following the activation of proCPR by thrombin (T) and thrombomodulin (TM) complexes (T/TM complexes), carboxypeptidase R (CPR) removed carboxyterminal lysine residues from plasminogen-binding sites, as did activated TAFI (TAFIa) (Redlitz *et al*, 1995; Bajzar *et al*, 1996; Sakharov *et al*, 1997).

The TAFI levels in plasma and its enzymatic activity suggest that this enzyme is an important regulator of fibrinolysis. Disturbances in TAFI levels and activity may represent a risk factor in vascular disorders and several reports have been published on the relationship between TAFI and deep vein thrombosis (van Tilburg *et al*, 2000; Kostka *et al*, 2003), disseminated intravascular coagulation (Watanabe *et al*, 2001) and coronary artery disease (Juhan-Vague *et al*, 2000; Silveira *et al*, 2000; Schroeder *et al*, 2002; Zorio *et al*, 2003). In patients with diabetes mellitus (DM), which is often accompanied by angiopathic complications, the plasma concentration and activity of TAFI were significantly higher than in healthy controls. Furthermore, these parameters were significantly elevated in obese DM patients compared with non-obese DM patients and non-obese healthy subjects (Hori *et al*, 2002).

Levels of circulating TAFI are strongly influenced by polymorphisms in the promoter and the 3'UTR of the TAFI gene (Henry *et al*, 2001) and may have an effect on the risk of venous thrombophilia (Franco *et al*, 2001). Several investigators have reported a functional polymorphism in the promoter region as well as in the exon at amino acid positions 147

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(Zhao *et al*, 1998) and 325 (Schneider *et al*, 2002). Schneider *et al* (2002) examined the difference in thermal stability and antifibrinolytic activity between Thr-325 and Ile-325.

A multicentre European study was performed to clarify the relationship between *TAFI* polymorphism and myocardial infarction (MI) (Juhan-Vague *et al*, 2002; Morange *et al*, 2002). There are many clinical signs that make MI relatively easy to diagnose. However, it is a little more difficult to diagnose a case of cerebral infarction (CI) using only clinical symptoms and computed tomography (CT) of the brain. A clinicopathological confirmation is essential. In this report, we used autopsy samples from 253 patients that were stored in the Fukushimura Brain Bank. These patients were confirmed to have had no infarcts, microinfarcts or severe infarcts, and *TAFI* polymorphisms were analysed at Thr/Ala-147 and Thr/Ile-325. We estimated the extent of arteriosclerosis and the clinicopathological CI grading using clinical history, neurological symptoms, brain CT scans and macroscopic/microscopic pathological findings.

Table I. Sex distribution of the 253 FBB samples/108 PBC and Thr/Ala-147 and Thr/Ile-325 polymorphisms.

	Males (FBB/PBC)	Females	Total
Number	121/22	132/86	253/108
Age (years)	80·6 ± 8·6/	83·8 ± 8·4/	82·4 ± 8·5/
	80·5 ± 8·0	81·9 ± 6·7	81·6 ± 6·9
Thr147Ala			
Thr/Thr	5/0	10/3	15/3
Thr/Ala	50/9	52/38	102/47
Ala/Ala	66/13	70/45	136/58
Allele Thr	60/9	72/44	132/53
Allele Ala	182/35	192/128	374/163
Thr325Ile			
Thr/Thr	96/17	94/64	190/81
Thr/Ile	23/4	35/17	58/21
Ile/Ile	2/1	3/5	5/6
Allele Thr	215/38	223/145	438/183
Allele Ile	27/6	41/27	68/33

FBB, Fukushimura Brain Bank, PBC, population-based controls. Of the 253 patients, 46% were 80–89 years old.

Materials and methods

Patients

All 253 patients had died while hospitalized, and a high percentage of these have already been included in a previous neuropathological evaluation (Akatsu *et al*, 2002). We had records of their past history, and reports of interviews employing a comprehensive questionnaire concerning psychological and medical symptoms, chronic conditions, treatment and activities of daily life. All had undergone CT scanning of the brain. We excluded patients who had been diagnosed with, or taken medication for DM, valvular problems, atrial fibrillation (AF) or hyperlipidaemia (HL) because these problems pose a high risk of thrombosis. Four patients who had experienced a subarachnoid haemorrhage (SAH) were also excluded because these would manifest cerebrovascular and other anatomical problems. However, hypertension (HT) is also a thrombosis risk factor, and as the elderly tend to exhibit increases in blood pressure, it was not surprising that 92 of our patients (36%) had a history of HT. We evaluated the contribution of *TAFI* polymorphism to the risk of developing CI using 189 neuropathologically diagnosed cases including those with HT; 180 cases had evidence of macroscopic arteriosclerosis, while in nine cases, no vascular sample was available.

Dissections were carried out at the Choju Medical Institute (Fukushimura Hospital, Japan) from 1993 to 2002. These were performed after obtaining the agreement of the patients' guardians for diagnosis, and biochemical, molecular biological and genomic research. This study was approved by the Ethics Committee of the Choju Medical Institute on 24 February 2003, and assigned application number 91.

To obtain population-based controls as a non-demented group, elderly individuals were recruited from Ehime University School of Medicine (Touon-shi, Ehime, Japan) and evaluated by a questionnaire that included questions regarding past and present illnesses. Written informed consent was obtained from each individual according to a protocol approved by the Genome Ethical Committee of Ehime University School of Medicine. These population-based non-demented controls were composed of 86 females and 22 males, with a mean ± SD age at blood drawing of 80·5 ± 8·0 years and 81·9 ± 6·7 years, respectively (range, 70–101 years) (Table I).

Autopsy and sampling of brain tissues

Each brain was removed at autopsy, weighed, cut midsagittally and examined for vascular and other macroscopically detectable lesions. Specimens for diagnostic examination were taken from the abnormal hemisphere, as determined by CT scanning, or from the left hemisphere, if no difference was observed between the left and right, and fixed in 4% paraformaldehyde (PFA). The other hemisphere was divided into several regions. Some samples were frozen for further analyses and stored at −80°C, while others were fixed in 4% PFA for immunohistochemical analysis.

Samples for diagnostic purposes were taken from the frontal, temporal, parietal and occipital lobes, hippocampal formation, amygdala, basal ganglia, thalamus and the midbrain including the substantia nigra, pons, medulla and cerebellum after separating the anterior, middle, and posterior cerebral arteries, the internal carotid and basilar arteries. The specimens were embedded in paraffin and processed into 8 μm sections for conventional histological and immunohistochemical examination.

Neuropathological evaluation of cerebral infarcts and other diagnostic signs of neurodegenerative disease

For macroscopic analysis of CI, the fixed half of specimens and separated arteries were examined in detail by a neuropathologist. To assess the extent of arteriosclerosis, the investigators, neuropathologist and several medical doctors evaluated the degree of blockage in each artery and arrived at an average. Grading was as follows: no blockage, no arteriosclerosis; 30% blockage, slight arteriosclerosis; 50% blockage, mild arteriosclerosis; and over 70% blockage, severe arteriosclerosis. The fixed specimens were cut into 1 cm thickness and carefully examined by touch and observation.

For microscopic examination, samples were embedded in paraffin and processed into 8 μm sections for conventional histological and immunohistochemical examination. Specimens were stained using haematoxylin–eosin (HE) and Klüver–Barerra (KB) staining methods. Methenamine silver (MS) staining, Congo red (CR) staining and immunostaining were used when necessary. For diagnosis of neurodegenerative diseases, we used our previously reported criteria (Akatsu *et al*, 2002).

For cliniconeuropathological classification of infarctions, the patient group consisted of 86 male and 103 female patients aged 44–102 years. CIs were classified as given below.

A 'large infarct' was marked by neurological findings, a clinical history of a stroke, involvement of a large, low-density (in total, over 20% of the hemisphere) area on the brain CT, severe macroscopic arteriosclerosis (if reported), and a widespread area of infarction (in total, 20% of the hemisphere) on macroscopic and microscopic analysis.

A 'small infarct' was characterized by a small, low-density (in total, <20% of the hemisphere) area on the brain CT, mild macroscopic arteriosclerosis (if reported), and a small infarction (in total, <20% of the hemisphere) on both macroscopic and microscopic analysis.

'No infarction' was presumed when there was no low-density area on the brain CT scan, no macroscopically detected infarction and no macroscopic arteriosclerosis (if reported). Cases with only microscopic microinfarcts were included in this group.

Chemicals

For brain tissue fixation, PFA was purchased from Merck (Darmstadt, Germany) and for dehydration, xylene and ethanol were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Reagents for HE, KB, CR and MS staining were from Wako Pure Chemical Industries Ltd. Boric acid, sodium sulphate, acetic acid and citric acid used in staining were from Sigma Chemical Co. (St Louis, MO, USA). The glass slides and cover glasses were from Matsunami Glass Industry Ltd (Osaka, Japan).

For investigation of genomic polymorphism, Taq DNA polymerase was obtained from Takara (Kyoto, Japan). Restriction enzymes, BbvI and SpeI, were from New England Biolabs (Beverly, MA, USA). Seakem GTG agarose was purchased for electrophoresis from FMC Bioproducts (Rockland, ME, USA).

Genomic analysis of TAFI Thr/Ala-147 and Thr/Ile-325

Genomic DNA was extracted using the phenol-chloroform method. *TAFI* gene mutations could easily be detected by polymerase chain reaction–restriction fragments length polymorphism (PCR-RFLP) analysis using the restriction enzyme BbvI for TAFI-147 and SpeI for TAFI-325. Sequences of the TAFI-147 and TAFI-325 regions were retrieved from GenBank (accession numbers AL137141 and AL157758). PCR was carried out in a 25 μl reaction volume containing a standard reaction buffer (1·5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH8·3), 200 μmol/l of each dNTP, 10 μmol/l of each primer, 0·5 U *Taq* DNA polymerase) and 50 ng genomic DNA as a template. The primers were TAFI147-F (5'-TTGAAACTTCCACATGCAGC-3'), TAFI147-R (5'-ATCTTGGGCACCATTTTGAG-3'), TAFI325-F (5'-CACAAAGAAAAACAGATCACACAG-3'), TAFI325-R (5'-AAAGCCACCCAATTGTGATT-3'). The protocol consisted of 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The TAFI-147 PCR product size was 456 bp, and the G (Ala) allele was digested by BbvI into 28 + 124 + 304 bp, whereas the A (Thr) allele was digested into 28 + 428 bp. The TAFI-325 PCR product was 363 bp, and the C (Thr) allele was digested by SpeI into 118 + 245 bp whereas the T (Ile) allele was not digested at all by SpeI. PCR products were digested with each enzyme, resolved on 2% agarose gels and visualized by ethidium bromide staining (Fig 1).

Statistical analysis

Statistical analysis was carried out on a personal computer running the Windows XP system. The significance of difference for each genotype was examined using both the chi-squared test with Yates's correction and Fisher's exact test using 2 × 2 tables. The level of significance was taken at $P < 0·05$.

Results

The 253 patients examined consisted of 121 males and 132 females with an average age of 82·4 ± 8·5 years (mean ± SD) at the time of death, and 46% (117 cases) were between 80 and 89 years of age. Among these 253 patients, those at risk of thrombosis or infarction because of a diagnosis of DM, VP or AF, HT and HL numbered 21 (8%), 26 (10%), 92 (36%) and six patients (2%) respectively. Several patients had two or three diseases that placed them at risk. Four patients with SAH were also omitted, because this condition constitutes a complicating factor. Nine patients (4%) received a pathological diagnosis of amyloid angiopathy and these were excluded as well as this condition also poses a vascular risk. This left 189 patients

