minor groove binding DNA oligonucleotides (MGB probes), Hum. Mutat. 19 (2002) 554–559.
[22] A.M. Dart, B.A. Kingwell, Pulse pressure – a review of mechanisms and clinical relevance, J. Am. Coll. Cardiol. 37 (2001) 975–984.
[23] N. Kuto, T. Sugiyama, H. Morita, H. Kurihara, Y. Yamori, Y. Yazaki, G protein beta3 subunit variant and essential hypertension in Japanese, Hypertension 32 (1998) 935–938.
[24] K. Ishikawa, Y. Imai, T. Katsuya, T. Ohkubo, I. Tsuji, K. Nagai, S. Takami, Y. Nakata, H. Satoh, S. Hisamichi, J. Higaki, T. Ogihara, Human G-protein beta3 subunit variant is associated with serum potassium and total cholesterol levels but not with blood pressure, Am. J. Hypertens. 13 (2000) 140–145.
[25] R.A. DeFronzo, E. Ferrannini, Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease, Diabetes Care 14 (1991) 173–194.
[26] K. Lemura, J. Nakura, K. Kohara, T. Miki, Association of ACE I/D polymorphism with cardiovascular risk factors, Hum. Genet. 107 (2000) 259–262.
[27] D.W. Moskowitz, From pharmacogenomics to improved patient outcomes: angiotensin I-converting enzyme as an example, Diabetes Technol. Ther. 4 (2002) 519–532.
[28] D.W. Moskowitz, Is angiotensin I-converting enzyme a "master" disease gene?, Diabetes Technol. Ther. 4 (2002) 683–711.
[29] T. Iiri, Z. Farfel, H.R. Bourne, G-protein diseases furnish a model for the turn-on switch, Nature 394 (1998) 35–38.
[30] H.L. Su, C.C. Malbon, H.Y. Wang, Increased expression of Gi alpha 2 in mouse embryo stem cells promotes terminal differentiation to adipocytes, Am. J. Physiol. 265 (1993) 1729–1735.
[31] H.Y. Wang, C.C. Malbon, The Gi alpha/Gi alpha 2 axis controls adipogenesis independently of adenylylcyclase, Int. J. Obes. Relat. Metab. Disord. 20 (1996) 526–531.
[32] S. Gasic, B. Tian, A. Green, Tumor necrosis factor alpha stimulates lipolysis in adipocytes by decreasing Gi protein concentrations, J. Biol. Chem. 274 (1999) 6770–6775.
[33] H. Hauner, K. Rohrig, W. Siffert, Effects of the G-protein beta3 subunit 825T allele on adipogenesis and lipolysis in cultured human preadipocytes and adipocytes, Horm. Metab. Res. 34 (2002) 475–480.
[34] G.S. Hotamisligil, N.S. Shargill, B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance, Science 259 (1993) 87–91.
[35] M.B. Anand-Srivastava, Enhanced expression of inhibitory guanine nucleotide regulatory protein in spontaneously hypertensive rats. Relationship to adenylate cyclase inhibition, Biochem. J. 288 (1992) 79–85.
[36] C. Thibault, M.B. Anand-Srivastava, Altered expression of G-protein mRNA in spontaneously hypertensive rats, FEBS Lett. 313 (1992) 160–164.
[37] M.B. Anand-Srivastava, J. de Champlain, C. Thibault, DOCA-salt hypertensive rat hearts exhibit altered expression of G-proteins, Am. J. Hypertens. 6 (1993) 73–75.
[38] F. Di Fusco, M.B. Anand-Srivastava, Enhanced expression of Gi proteins in non-hypertrophic hearts from rats with hypertension-induced by L-NAME treatment, J. Hypertens. 18 (2000) 1081–1090.
[39] Y. Li, M.B. Anand-Srivastava, Inactivation of enhanced expression of Gi(i) proteins by pertussis toxin attenuates the development of high blood pressure in spontaneously hypertensive rats, Circ. Res. 91 (2002) 247–254.
[40] R. Fogari, A. Zoppi, F. Tettamanti, L. Poletti, P. Lazzari, C. Parotti, L. Corradi, Beta-blocker effects on plasma lipids in antihypertensive therapy: importance of the duration of treatment and the lipid status before treatment, J. Cardiovasc. Pharmacol. 16 (1990) S76–S80.
[41] R. Fogari, A. Zoppi, L. Corradi, P. Preti, A. Mugellini, P. Lusardi, Beta-blocker effects on plasma lipids during prolonged treatment of hypertensive patients with hypercholesterolemia, J. Cardiovasc. Pharmacol. 33 (1999) 534–539.
[42] C.M. Moxham, C.C. Malbon, Insulin action impaired by deficiency of the G-protein subunit Gi alpha2, Nature 379 (1996) 840–844.
[43] J.F. Chen, J.H. Guo, C.M. Moxham, H.Y. Wang, C.C. Malbon, Conditional, tissue-specific expression of Q205L G alpha i2 in vivo mimics insulin action, J. Mol. Med. 75 (1997) 283–289.
[44] J. Tso, C.C. Malbon, H.Y. Wang, G alpha (i2) enhances insulin signaling via suppression of protein-tyrosine phosphatase 1B, J. Biol. Chem. 276 (2001) 39705–39712.

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

Yasuharu TABARA, Katsuhiko KOHARA*, Yoshikuni YAMAMOTO*, Michiya IGASE*, Jun NAKURA*, Ikuko KONDO, and Tetsuro MIKI*

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 at 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, 168±36 ng/ml; GG+AG, 184±56 ng/ml; $p=0.036$). 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 (*7–10*). 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

From the Department of Medical Genetics and *Department of Genome Medicine, Ehime University School of Medicine, Ehime, Japan.
This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health, Labor, and Welfare, and a grant from the Japan Atherosclerosis Prevention Fund.
Address for Reprints: Katsuhiko Kohara, M.D., Department of Genetic Medicine, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan. E-mail: kohara@gen.ehime-u.ac.jp
Received March 5, 2003; Accepted in revised form May 1, 2003.

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 oscillometric 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 Pvu II and separated by 3% agarose gel electrophoresis. If the polymorphism was −2518A, a unique Pvu II restriction site would be eliminated from this segment of the 5′-flanking region. The DNA segment from G/G 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/m²)	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.

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/m²)	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.

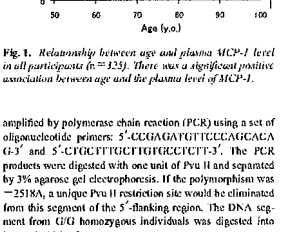

Fig. 1. *Relationship between age and plasma MCP-1 level in all participants (n=325). There was a significant positive association between age and the plasma level of MCP-1.*

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.

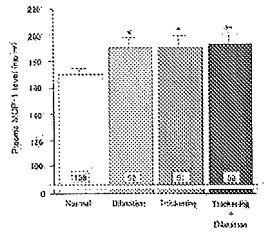

Fig. 2. *Plasma MCP-1 level in subjects divided into four groups according to the presence of carotid intima-media thickening and dilatation. Carotid arterial dilatation was defined as carotid arterial internal diameter >7.0 mm, and carotid thickening was defined as carotid intima-media thickness >0.85 mm. The plasma level of MCP-1 was significantly different among the four groups (F(3, 321)=4.30, 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*).

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

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, IMT, total-cholesterol, HDL-cholesterol, glucose, and medication status were not entered in the equation.

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 (*33*). 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.

References

1. Schmidt AM, Stern DM: Chemokine on the rise: MCP-1 and restenosis. *Arterioscler Thromb Vasc Biol* 2001; **21**: 297–299.
2. Rollins B: MCP-1: a potential regulator of monocyte recruitment in inflammatory disease. *Mol Med Today* 1996; **5**: 198–204.
3. Rollins B: Chemokines. *Blood* 1997; **90**: 909–928.
4. Luster A: Chemokines: chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**: 436–445.
5. Landry DB, Couper LL, Bryant SR, Lindner V: Activation of the NF-kappa B and I kappa B system in smooth muscle cells after rat arterial injury: induction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. *Am J Pathol* 1997; **151**: 1085–1095.
6. Wysocki SJ, Zheng MH, Smith A, *et al*: Monocyte chemoattractant protein-1 gene expression in injured pig artery coincides with early appearance of infiltrating monocytes/macrophages. *J Cell Biochem* 1996; **62**: 303–313.
7. Yla-Herttuala S, Lipton B, Rosenfeld M, *et al*: Expression of MCP-1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci USA* 1991; **88**: 5252–5256.
8. Nelken N, Coughlin S, Gordon D, Wilcox J: MCP-1 in human atheromatous plaques. *J Clin Invest* 1991; **88**: 1121–1127.
9. Yu X, Diaz S, Graves D, *et al*: Elevated expression of MCP-1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc Natl Acad Sci USA* 1992; **89**: 6953–6957.
10. Kuwata M, Reece R, Beyer S, Gu C, Valentine M: Characterization of atherosclerosis in LDL receptor knockout mice: macrophage accumulation correlates with rapid and sustained expression of aortic MCP-1/JE. *Atherosclerosis* 2000; **149**: 323–330.
11. Matsumori A, Furukawa Y, Hashimoto T, *et al*: Plasma levels of monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 are elevated in patients with acute myocardial infarction. *J Mol Cell Cardiol* 1997; **29**: 419–423.
12. Soejima H, Ogawa H, Yasue H, *et al*: Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol* 1999; **34**: 983–988.
13. Aukrust P, Berge RK, Ueland T, *et al*: Interaction between chemokines and oxidative stress: possible pathogenic role in acute coronary syndromes. *J Am Coll Cardiol* 2001; **37**: 485–491.
14. van Aken BE, den Heijer M, Bus GM, van Deventer SJ, Reitsma PH: Recurrent venous thrombosis and markers of inflammation. *Thromb Haemost* 2000; **83**: 536–539.
15. Jibiki T, Terai M, Shima M, *et al*: Monocyte chemoattractant protein 1 gene regulatory region polymorphism and serum levels of monocyte chemoattractant protein 1 in Japanese patients with Kawasaki disease. *Arthritis Rheum* 2001; **44**: 2211–2212.
16. Ni W, Egashira K, Kataoka C, *et al*: New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 2001; **103**: 2096–2101.
17. Stork S, Baumann K, von Schacky C, Angerer P: The effect of 17 beta-estradiol on MCP-1 serum levels in postmenopausal women. *Cardiovasc Res* 2002; **53**: 642–649.
18. Ruvin B, Lu L, Saxena R: A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. *Biochem Biophys Res Commun* 1999; **259**: 344–348.
19. Szalai C, Duba J, Prohaszka Z, *et al*: Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD): coincidence of elevated Lp(a) and MCP-1 −2518 G/G genotype in CAD patients. *Atherosclerosis* 2001; **158**: 233–239.
20. Aguilar F, Gonzalez-Escribano MF, Sanchez-Roman J, Nunez-Roldan A: MCP-1 promoter polymorphism in Spanish patients with systemic lupus erythematosus. *Tissue Antigens* 2001; **58**: 335–338.
21. Yamamoto Y, Kohara K, Tahara Y, Miki T: Association between carotid arterial remodeling and plasma concentration of circulating hepatocyte growth factor. *J Hypertens* 2001; **19**: 1975–1979.
22. Jiang YN, Kohara K, Hiwada K: Low wall shear stress in carotid arteries in subjects with left ventricular hypertrophy. *Am J Hypertens* 2000; **13**: 892–898.
23. Kohara K, Jiang Y, Igase M, Hiwada K: Effect of reflection of arterial pressure on carotid circulation in essential hypertension. *Am J Hypertens* 1999; **12**: 1015–1020.
24. O'Brien E, Mee F, Atkins N, Thomas M: Evaluation of three devices for self-measurement of blood pressure according to the revised British Hypertension Society Protocol: the Omron HEM-705CP, Philips HP5332, and Nissei DS-175. *Blood Press Monit* 1996; **1**: 55–61.
25. Uemura K, Nakura J, Kohara K, Miki T: Association of ACE I/D polymorphism with cardiovascular risk factors. *Hum Genet* 2000; **107**: 239–242.
26. Cipollone F, Marini M, Fazia M, *et al*: Elevated circulating levels of monocyte chemoattractant protein-1 in patients with restenosis after coronary angioplasty. *Arterioscler Thromb Vasc Biol* 2001; **21**: 327–334.
27. Kumar A, Ballantyne C, Michael L, *et al*: Induction of MCP-1 in the small veins of the ischemic and reperfused canine myocardium. *Circulation* 1997; **95**: 693–700.
28. Taubman M, Rollins B, Poon M, *et al*: JE mRNA accumulates rapidly in aortic injury and in PDGF-stimulated vascular smooth muscle cells. *Circ Res* 1992; **70**: 314–325.
29. Oshima S, Ogawa H, Hokimoto S, *et al*: Plasma monocyte chemoattractant protein-1 antigen levels and the risk of restenosis after coronary stent implantation. *Jpn Circ J* 2001; **65**: 261–264.
30. Olson T: The secondary and tertiary inflammatory cellular reaction in arterioles damaged by acute angiotensin-hypertension in rats. *Acta Pathol Microbiol Scand A* 1970; **78**: 451–457.
31. Lull FC, Mervaala E, Muller DN, *et al*: Hypertension-induced end-organ damage: a new transgenic approach to an old problem. *Hypertension* 1999; **33**: 212–218.
32. Tahara Y, Kohara K, Nakura J, Miki T: Risk factor–gene interaction in carotid atherosclerosis: effect of gene polymorphisms of renin-angiotensin system. *J Hum Genet* 2001; **46**: 278–284.
33. Kohara K, Tahara Y, Yamamoto Y, Miki T: Orthostatic hypertension: another orthostatic disorder to be aware of. *J Am Geriatr Soc* 2000; **48**: 1538–1539.
34. Kohara K, Tahara Y, Yamamoto Y, Igase M, Nakura J, Miki T: Genotype-specific association between circulating soluble cellular adhesion molecules and carotid intima-media thickness in community residents: J-SHIPP study: Shimanami Health Promoting Program. *Hypertens Res* 2002; **25**: 31–39.
35. Yamamoto Y, Kohara K, Tahara Y, Igase M, Nakura J, Miki T: Plasma hepatocyte growth factor and the relationship between risk factors and carotid atherosclerosis. *Hypertens Res* 2002; **25**: 661–667.
36. Kohara K, Tahara Y, Yamamoto Y, Igase M, Miki T: Chlamydia pneumoniae seropositivity is associated with increased plasma levels of soluble cellular adhesion molecules in community-dwelling subjects: the Shimanami Health Promoting Program (J-SHIPP) study. *Stroke* 2002; **33**: 1474–1479.
37. Rossi A, Baldo-Enzi G, Calabru A, Snechetto A, Pessina AC, Rossi GP: The renin-angiotensin-aldosterone system and carotid artery disease in mild-to-moderate primary hypertension. *J Hypertens* 2000; **18**: 1401–1409.
38. Kawamoto R, Kohara K, Tabara Y, Miki T: An interaction between systolic blood pressure and angiotensin-converting enzyme gene polymorphism on carotid atherosclerosis. *Hypertens Res* 2002; **25**: 875–880.
39. Gerli R, Monti D, Bistoni O, *et al*: Chemokines, sTNF-Rs and sCD30 serum levels in healthy aged people and centenarians. *Mech Ageing Dev* 2000; **121**: 37–46.
40. Inadera H, Egashira K, Takemoto M, Ouchi Y, Matsushima K: Increase in circulating levels of monocyte chemoattractant protein-1 with aging. *J Interferon Cytokine Res* 1999; **19**: 1179–1182.
41. Hwang SY, Cho ML, Park B, *et al*: Allelic frequency of the MCP-1 promoter −2518 polymorphism in the Korean population and in Korean patients with rheumatoid arthritis, systemic lupus erythematosus and adult-onset Still's disease. *Exp J Immunogenet* 2002; **29**: 413–416.

Original Article

Association of Angiotensin II Type 2 Receptor Gene Variant with Hypertension

Jing-Ji JIN, Jun NAKURA, Zhihong WU, Miyuki YAMAMOTO, Michiko ABE, Yusen CHEN, Yasuharu TABARA, Yoshikuni YAMAMOTO, Michiya IGASE, Xiao BO*, Katsuhiko KOHARA, and Tetsuro MIKI

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.

From the Department of Genomic Medicine, School of Medicine, Ehime University, Ehime, Japan, and *Department of Neurology, Xiang Ya School of Medicine, Central South University, Changsha, P.R. China.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Area (C), "Medical Genome Science", from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health, Labour, and Welfare of Japan.

Address for Reprints: Jun Nakura, M.D., Department of Genomic Medicine, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan. E-mail: nakura@gen.ehime-u.ac.jp

Received December 27, 2002; Accepted in revised form March 12, 2003.

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 (8.6)	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 (31.7)	200.7 (30.7)	195.2 (28.6)	204.0 (35.5)	195.7 (31.0)	200.8 (32.0)
HDL-cholesterol (mg/dl)	59.5 (12.7)	60.0 (13.4)	51.4 (12.8)	51.3 (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)	86.7 (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).

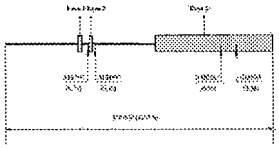

Fig. 1. *Schematic representation of the human AT$_2$-R gene and position of identified SNPs. The minor allele frequency for each SNP is shown in parentheses. * Newly identified SNPs.*

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′-CTTCTGTTTTTCCTGTGTTTT-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′-TCTATATTCCTAGCCCTATTCAATATGTAGTT-3′, the C-allele specific probe was 5′-Fam-TCTTTAAAAACGCTATAAAT-MGB-3′, and the A-allele specific probe was 5′-Vic-TCTTTAAAAAAGCTATAAATTMGB-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, 0.36 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'=0.92, 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	38 (13%)	17 (22%)	0.091			11 (9%)	7 (23%)	0.044			41 (12%)	24 (22%)	0.0058	0.46	0.26–0.81
AC	188 (47%)	37 (48%)				56 (41%)	7 (23%)				158 (45%)	44 (41%)			
CC	93 (40%)	23 (30%)				60 (50%)	17 (54%)				153 (43%)	40 (37%)			
AA	38 (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%)	0.0085	0.46	0.26–0.81
AC+CC	281 (87%)	60 (78%)				116 (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%)				170 (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=0.037; 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 allele 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.

References

1. Kato N: Genetic analysis in human hypertension. *Hypertens Res* 2002; 25: 319–327.
2. Parfrey PS, Markandu ND, Roulston JE, Jones BE, Jones JC, MacGregor GA: Relation between arterial pressure, dietary sodium intake, and renin system in essential hypertension. *BMJ* 1981; 283: 94–97.
3. Munzenmaier DH, Greene AS: Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension* 1996; 27: 760–765.
4. van Kesteren CA, van Heugten HA, Lamers JM, Saxena PR, Schalekamp MA, Danser AH: Angiotensin II-mediated growth and antigrowth effects in cultured neonatal rat cardiac myocytes and fibroblasts. *J Mol Cell Cardiol* 1997; 29: 2147–2157.
5. Carey RM, Wang ZQ, Siragy HM: Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension* 2000; 35: 155–163.
6. Ito M, Oliverio MI, Mannon PJ, *et al*: Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci USA* 1995; 92: 3521–3525.
7. Tsuchida S, Matsusaka T, Chen X, *et al*: Murine double nullizygotes of the angiotensin type 1A and 1B receptor genes duplicate severe abnormal phenotypes of angiotensinogen nullizygotes. *J Clin Invest* 1998; 101: 755–760.
8. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK: Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* 1995; 377: 744–747.
9. Ichiki T, Labosky PA, Shiota C, *et al*: Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 1995; 377: 748–750.
10. Lazard D, Briend-Sutren MM, Villageois P, Mattei MG, Strosberg AD, Nahmias C: Molecular characterization and chromosome localization of a human angiotensin II AT2 receptor gene highly expressed in fetal tissues. *Receptors Channels* 1994; 2: 271–280.
11. Inagami T: Molecular biology and signaling of angiotensin receptors: an overview. *J Am Soc Nephrol* 1999; 11 (Suppl): S2–S7.
12. Martin MM, Elton TS: The sequence and genomic organization of the human type 2 angiotensin II receptor. *Biochem Biophys Res Commun* 1995; 209: 554–562.
13. Ichiki T, Inagami T: Expression, genomic organization, and transcription of the mouse angiotensin II type 2 receptor gene. *Circ Res* 1995; 76: 693–700.
14. Warnecke C, Willich T, Holzmeister J, Bottari SP, Fleck E, Regitz-Zagrosek V: Efficient transcription of the human angiotensin II type 2 receptor gene requires intronic sequence elements. *Biochem J* 1999; 340: 17–24.
15. Takami S, Katsuya T, Rakugi H, *et al*: Angiotensin II type 1 receptor gene polymorphism is associated with increase of left ventricular mass but not with hypertension. *Am J Hypertens* 1998; 11: 316–321.
16. Devlin B, Risch N: A comparison of linkage disequilibrium measures for fine-scale mapping. *Genomics* 1995; 29: 311–322.
17. Schmieder RE, Erdmann J, Delles C, *et al*: Effect of the angiotensin II type 2-receptor gene (+1675 G/A) on left ventricular structure in humans. *J Am Coll Cardiol* 2001; 37: 175–182.
18. Katsuya T, Horiuchi M, Minami S, *et al*: Genomic organization and polymorphism of human angiotensin II type 2 receptor: no evidence for its gene mutation in two families of human premature ovarian failure syndrome. *Mol Cell Endocrinol* 1997; 127: 221–228.
19. Keavney B, McKenzie CA, Connell JM, *et al*: Measured haplotype analysis of the angiotensin-I converting enzyme gene. *Hum Mol Genet* 1998; 7: 1745–1751.
20. Jeunemaitre X, Inoue I, Williams C, *et al*: Haplotypes of angiotensinogen in essential hypertension. *Am J Hum Genet* 1997; 60: 1448–1460.
21. Nakajima T, Jorde LB, Ishigami T, *et al*: Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. *Am J Hum Genet* 2002; 70: 108–123.
22. Horikawa Y, Oda N, Cox NJ, *et al*: Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet* 2000; 26: 163–175.
23. Jin JJ, Nakura J, Wu Z, *et al*: Association of endothelin-1 gene variant with hypertension. *Hypertension* 2003; 41: 163–167.
24. Deinum J, van Gool JM, Kofflard MJ, ten Cate FJ, Danser AH: Angiotensin II type 2 receptors and cardiac hypertrophy in women with hypertrophic cardiomyopathy. *Hypertension* 2001; 38: 1278–1281.
25. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG: Replication validity of genetic association studies. *Nat Genet* 2001; 29: 306–309.
26. Editorial: Freely associating. *Nat Genet* 1999; 22: 1–2 (Editorial).
27. Hilgers KF, Schmieder RE: Association studies in cardiovascular medicine. *J Hypertens* 2002; 20: 173–176.
28. Mancina R, Susini T, Renzetti A, *et al*: Sex steroid modulation of AT2 receptors in human myometrium. *J Clin Endocrinol Metab* 1996; 81: 1753–1757.
29. Armando I, Jezova M, Juorio AV, *et al*: Estrogen upregulates renal angiotensin II AT(2) receptors. *Am J Physiol Renal Physiol* 2002; 283: F934–F943.

Original Article

Genome-Wide Linkage Disequilibrium Mapping of Hypertension in Japan

Zhihong WU, Jun NAKURA, Michiko ABE, Jing-Ji JIN, Miyuki YAMAMOTO, Yusen CHEN, Yasuharu TABARA, Yoshikuni YAMAMOTO, Michiya IGASE, Xiao BO*, Katsuhiko KOHARA, and Tetsuro MIKI

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).

(*Hypertens Res* 2003; 26: 533–540)

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 associated 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 iden-

From the Department of Geriatric Medicine, School of Medicine, Ehime University, Ehime, Japan, and * Department of Neurology, Xiang Ya School of Medicine, Central South University, Changsha, P.R. China.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health Labour and Welfare of Japan.

Address for Reprints: Jun Nakura, M.D., Department of Geriatric Medicine, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791–0295, Japan. E-mail: nakura@m.ehime-u.ac.jp

Received December 19, 2002; Accepted in revised form February 17, 2003.

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.

tification 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 contribute to the development of hypertension, then the relatively 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–environmental 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.

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 further 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

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		OR	95% CI	p value
				HT	NT			
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	1.11–2.28	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	0.135	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		p value	OR	95% CI
	Normotensive (n=1,687)	Hypertensive (n=739)			
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.

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 $10^{-5}\times5.3$. 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.

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		p value	OR	95% CI
	Normotensive (n=1,687)	Hypertensive (n=739)			
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 (*30, 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 (*30*). 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.

References

1. Corvol P, Persu A, Gimenez-Roqueplo AP, Jeunemaitre X: Seven lessons from two candidate genes in human essential hypertension: angiotensinogen and epithelial sodium channel. *Hypertension* 1999; 33: 1324–1331.
2. Kunz R, Kreutz R, Beige J, Distler A, Sharma AM: Association between the angiotensinogen 235T-variant and essential hypertension in whites: a systematic review and methodological appraisal. *Hypertension* 1997; 30: 1331–1337.
3. Staessen JA, Kuznetsova T, Wang JG, Emelianov D, Vlietinck R, Fagard R: M235T angiotensinogen gene polymorphism and cardiovascular renal risk. *Hypertension* 1997; 17: 9–17.
4. Kato N, Sugiyama T, Morita H, Kurihara H, Yamori Y, Yazaki Y: Angiotensinogen gene and essential hypertension in the Japanese: extensive association study and meta-analysis on six reported studies. *J Hypertens* 1999; 17: 757–763.

5. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG: Replication validity of genetic association studies. *Nat Genet* 2001; 29: 306–309.
6. Tamaki S, Iwai N, Tsujita Y, Nakamura Y, Kinoshita M: Polymorphism of alpha-adducin in Japanese patients with essential hypertension. *Hypertens Res* 1998; 21: 29–32.
7. Sugiyama T, Morita H, Kato N, Kurihara H, Yamori Y, Yazaki Y: Lack of sex-specific effects on the association between angiotensin-converting enzyme gene polymorphism and hypertension in Japanese. *Hypertens Res* 1999; 22: 55–59.
8. Matsubara M, Ohkubo T, Michimata M, *et al*: Japanese individuals do not harbor the T594M mutation but do have the P592S mutation in the C-terminus of the beta-subunit of the epithelial sodium channel: the Ohasama study. *J Hypertens* 2000; 18: 861–866.
9. Kajiyama N, Saito Y, Miyamoto Y, *et al*: Lack of association between T-786→C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene and essential hypertension. *Hypertens Res* 2000; 23: 561–565.
10. Matsubara M, Kikuya M, Ohkubo T, *et al*: Aldosterone synthase gene (CYP11B2) C-334T polymorphism, ambulatory blood pressure and nocturnal decline in blood pressure in the general Japanese population: the Ohasama Study. *J Hypertens* 2001; 19: 2179–2184.
11. Tsujita Y, Iwai N, Katsuya T, *et al*: Lack of association between genetic polymorphism of CYP11B2 and hypertension in Japanese: the Suita Study. *Hypertens Res* 2001; 24: 105–109.
12. Rahmutula D, Nakayama T, Soma M, *et al*: Association study between the variants of the human ANP gene and essential hypertension. *Hypertens Res* 2001; 24: 291–294.
13. Fu Y, Katsuya T, Asai T, *et al*: Lack of correlation between Mbo I restriction fragment length polymorphism of renin gene and essential hypertension in Japanese. *Hypertens Res* 2001; 24: 295–298.
14. Matsubara M, Suzuki M, Fujiwara T, *et al*: Angiotensin-converting enzyme I/D polymorphism and hypertension: the Ohasama study. *J Hypertens* 2002; 20: 1121–1126.
15. Nakayama T, Soma M, Rahmutula D, *et al*: Association study between a novel single nucleotide polymorphism of the promoter region of the prostacyclin synthase gene and essential hypertension. *Hypertens Res* 2002; 25: 65–68.
16. Nakayama T, Soma M, Mizutani Y, *et al*: A novel missense mutation of exon 3 in the type A human natriuretic peptide receptor gene: possible association with essential hypertension. *Hypertens Res* 2002; 25: 395–401.
17. Ono K, Mannami T, Baba S, Tomoike H, Suga S, Iwai N: A single-nucleotide polymorphism in C-type natriuretic peptide gene may be associated with hypertension. *Hypertens Res* 2002; 25: 727–730.
18. Xu X, Rogus JJ, Terwedow HA, *et al*: An extreme-sib-pair genome scan for genes regulating blood pressure. *Am J Hum Genet* 1999; 64: 1694–1701.
19. Krushkal J, Ferrell R, Mockrin SC, Turner ST, Sing CF, Boerwinkle E: Genome-wide linkage analyses of systolic blood pressure using highly discordant siblings. *Circulation* 1999; 99: 1407–1410.
20. Sharma P, Fatibene J, Ferraro F, *et al*: A genome-wide search for susceptibility loci to human essential hypertension. *Hypertension* 2000; 35: 1291–1296.
21. Hsueh WC, Mitchell BD, Schneider JL, *et al*: QTL influencing blood pressure maps to the region of PPH1 on chromosome 2q31–34 in Old Order Amish. *Circulation* 2000; 101: 2810–2816.
22. Pankow JS, Rose KM, Oberman A, *et al*: Possible locus on chromosome 18q influencing postural systolic blood pressure changes. *Hypertension* 2000; 36: 471–476.
23. Levy D, DeStefano AL, Larson MG, *et al*: Evidence for a gene influencing blood pressure on chromosome 17: genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the Framingham heart study. *Hypertension* 2000; 36: 477–483.
24. Rice T, Rankinen T, Province MA, *et al*: Genome-wide linkage analysis of systolic and diastolic blood pressure: the Quebec Family Study. *Circulation* 2000; 102: 1956–1963.
25. Perola M, Kainulainen K, Pajukanta P, *et al*: Genome-wide scan of predisposing loci for increased diastolic blood pressure in Finnish siblings. *J Hypertens* 2000; 18: 1579–1585.
26. Atwood LD, Samollow PB, Hixson JE, Stern MP, MacCluer JW: Genome-wide linkage analysis of blood pressure in Mexican Americans. *Genet Epidemiol* 2001; 20: 373–382.
27. Zhu DL, Wang HY, Xiong MM, *et al*: Linkage of hypertension to chromosome 2q14–q23 in Chinese families. *J Hypertens* 2001; 19: 55–61.
28. Kristjansson K, Manolescu A, Kristinsson A, *et al*: Linkage of essential hypertension to chromosome 18q. *Hypertension* 2002; 39: 1044–1049.
29. Angius A, Petretto E, Maestrale GB, *et al*: A new essential hypertension susceptibility locus on chromosome 2p24–p25, detected by genomewide search. *Am J Hum Genet* 2002; 71: 893–905.
30. Lander E, Kruglyak K: Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995; 11: 241–247.
31. Green SA, Turki J, Innis M, Liggett SB: Amino-terminal polymorphisms of the human beta 2-adrenergic receptor impart distinct agonist-promoted regulatory properties. *Biochemistry* 1994; 33: 9414–9419.
32. McGraw DW, Forbes SL, Kramer LA, Liggett SB: Polymorphisms of the 5′ leader cistron of the human beta2-adrenergic receptor regulate receptor expression. *J Clin Invest* 1998; 102: 1927–1932.
33. Ahe M, Nakura J, Yamamoto M, *et al*: Association of GNAS1 gene variant with hypertension depending on smoking status. *Hypertension* 2002; 40: 261–265.
34. Zubenko GS, Hughes HB, Stiffler JS, Hurtt MR, Kaplan BB: A genome survey for novel Alzheimer disease risk loci: results at 10-cM resolution. *Genomics* 1998; 50: 121–128.
35. Hiltunen M, Mannermaa A, Thompson D, *et al*: Genome-wide linkage disequilibrium mapping of late-onset Alzheimer's disease in Finland. *Neurology* 2001; 57: 1663–1668.
36. Majores M, Bagli M, Papassotiropoulos A, *et al*: Allelic association between the D10S1423 marker and Alzheimer's disease in a German population. *Neurosci Lett* 2000; 289: 224–226.
37. Spielman RS, McGinnis RE, Ewens WJ: Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; 52: 506–516.
38. Bennett ST, Lucassen AM, Gough SC, *et al*: Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat Genet* 1995; 9: 284–292.
39. Kaloh T, Mano S, Ikuta T, *et al*: Genetic isolates in East Asia: a study of linkage disequilibrium in the X chromosome. *Am J Hum Genet* 2002; 71: 395–400.
40. Reich DE, Cargill M, Bolk S, *et al*: Linkage disequilibrium in the human genome. *Nature* 2001; 411: 199–204.
41. Zubenko GS, Stiffler JS, Hughes HB, Hurtt MR, Kaplan BB: Initial results of a genome survey for novel Alzheimer's disease risk genes: association with a locus on the X chromosome. *Am J Med Genet* 1998; 81: 98–107.
42. Hiltunen M, Mannermaa A, Kuivisto AM, *et al*: Linkage disequilibrium in the 13q12 region in Finnish late onset Alzheimer's disease patients. *Eur J Hum Genet* 1999; 7: 652–658.

Original Article

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

Yusen CHEN, Jun NAKURA, Jing-ji JIN, Zhihong WU, Miyuki YAMAMOTO, Michiko ABE, Yasuharu TABARA, Yoshikuni YAMAMOTO, Michiya IGASE, Xiao BO*, Katsuhiko KOHARA, and Tetsuro MIKI

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 ($p=0.026$), 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) (*1–5*). 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.

From the Departments of Genomic Medicine, School of Medicine, Ehime University, Ehime, Japan, and *Department of Neurology, Xiang Ya School of Medicine, Central South University, Changsha, China.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health, Labour, and Welfare of Japan.

Address for Reprints: Jun Nakura, M.D., Department of Genomic Medicine, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan. E-mail: nakura@m.ehime-u.ac.jp

Received December 9, 2002; Accepted in revised form January 23, 2003

Table 1. Characteristics of Participants According to Hypertensive Status

Variable	Normotensive ($n=1,609$)	Hypertensive ($n=699$)	p value
Sex (male %)	86.1	89.0	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 (*13*). The sense oligonucleotide primer was 5′-CTCCTAACTGACATGGFGCAA-3′ and the antisense primer was 5′-TAAGGCCACACAAGTCGGGGTT-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.

References

1. Conklin BR, Bourne HR: Structural elements of G alpha subunits that interact with G beta gamma, receptors, and effectors. *Cell* 1993; 73: 631–641.
2. Neer EJ: Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 1995; 80: 249–257.
3. Rens-Domiano S, Hamm HE: Structural and functional relationships of heterotrimeric G-proteins. *FASEB J* 1995; 9: 1059–1066.
4. Dixon RA, Kobilka BK, Strader DJ, *et al*: Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* 1986; 321: 75–79.
5. Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK: Cloning of the cDNA for the human beta 1-adrenergic receptor. *Proc Natl Acad Sci USA* 1987; 84: 7920–7924.
6. Emorine LJ, Marullo S, Briend-Sutren MM, *et al*: Molecular characterization of the human beta 3-adrenergic receptor. *Science* 1989; 245: 1118–1121.
7. Hamm HE: The many faces of G protein signaling. *J Biol Chem* 1998; 273: 669–672.
8. Levine MA, Modi WS, O'Brien SJ: Mapping of the gene encoding the alpha subunit of the stimulatory G protein of adenylyl cyclase (*GNAS1*) to 20q13.2–q13.3 in human by *in situ* hybridization. *Genomics* 1991; 11: 478–479.
9. Feldman RD, Lawton WJ, McArdle WL: Low sodium diet corrects the defect in lymphocyte beta-adrenergic responsiveness in hypertensive subjects. *J Clin Invest* 1987; 79: 290–294.
10. Yoshikawa H, Fukuda K, Wanaka Y, *et al*: Deficient activity of stimulatory nucleotide-binding regulatory protein in lymphocytes from patients with essential hypertension. *Am J Hypertens* 1994; 7: 713–716.
11. Feldman RD, Tan CM, Chorazyczewski J: G protein alterations in hypertension and aging. *Hypertension* 1995; 26: 725–732.
12. Jia H, Hingorani AD, Sharma P, *et al*: Association of the G(s)α gene with essential hypertension and response to β-blockade. *Hypertension* 1999; 34: 8–14.
13. Abe M, Nakura J, Yamamoto M, *et al*: Association of *GNAS1* gene variant with hypertension depending on smoking status. *Hypertension* 2002; 40: 8–14.
14. Eisenhofer G, Lambie DG, Johnson RH: Effects of ethanol on plasma catecholamines and norepinephrine clearance. *Clin Pharmacol Ther* 1983; 34: 143–147.
15. Ireland MA, Vandongen R, Davidson L, Beilin LJ, Rouse

IL: Acute effects of moderate alcohol consumption on blood pressure and plasma catecholamines. *Clin Sci* 1984; 66: 643–648.
16. Puddey IB, Beilin LJ, Vandongen R, Rouse IL, Rogers P: Evidence for a direct effect of alcohol consumption on blood pressure in normotensive men: a randomized controlled trial. *Hypertension* 1985; 7: 707–713.
17. Laurent S, Boutouyrie P, Benetos A: Pathophysiology of hypertension in the elderly. *Am J Geriatr Cardiol* 2002; 11: 34–39.
18. Kannel WB: Some lessons in cardiovascular epidemiology from Framingham. *Am J Cardiol* 1976; 37: 269–282.
19. Kannel WB, Dawber TR, Sorlie P, Wolf PA: Components of blood pressure and risk of atherothrombotic brain infarction: the Framingham study. *Stroke* 1976; 7: 327–331.
20. Dart AM, Kingwell BA: Pulse pressure a review of mechanisms and clinical relevance. *J Am Coll Cardiol* 2001; 37: 975–984.
21. Wakabayashi I, Hatake K: Effects of ethanol on the nervous and vascular systems: the mechanisms of alcohol-induced hypertension. *Nippon Eiseigaku Zasshi* 2001; 55: 607–617 (in Japanese).
22. Yu S, Castle A, Chen M, Lee R, Takeda K, Weinstein LS: Increased insulin sensitivity in Gsα knockout mice. *J Biol Chem* 2001; 276: 19994–19998.
23. Takagi S, Baba S, Iwai N, *et al*: The aldehyde dehydrogenase 2 gene is a risk factor for hypertension in Japanese but does not alter the sensitivity to pressor effects of alcohol: the Suita study. *Hypertens Res* 2001; 24: 365–370.

Hypertension
American Heart Association®
Learn and Live℠

Association of Endothelin-1 Gene Variant With Hypertension
Jing Ji Jin, Jun Nakura, Zhihong Wu, Miyuki Yamamoto, Michiko Abe, Yasuharu Tabara, Yoshikuni Yamamoto, Michiya Igase, Katsuhiko Kohara and Tetsuro Miki

Hypertension 2003;41;163-167; originally published online Nov 25, 2002;
DOI: 10.1161/01.HYP.0000043680.75107.CF
Hypertension is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514

Association of Endothelin-1 Gene Variant With Hypertension

Jing Ji Jin, Jun Nakura, Zhihong Wu, Miyuki Yamamoto, Michiko Abe, Yasuharu Tabara, Yoshikuni Yamamoto, Michiya Igase, Katsuhiko Kohara, Tetsuro Miki

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=2466) 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 90% 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 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 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).

Received June 27, 2002; first decision September 11, 2002; revision accepted October 16, 2002.
From the Department of Geriatric Medicine, School of Medicine, Ehime University, Ehime, Japan.
Correspondence to Dr Jun Nakura, Department of Geriatric Medicine, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan. E-mail nakura@m.ehime-u.ac.jp

Hypertension is available at http://www.hypertensionaha.org
DOI: 10.1161/01.HYP.0000043680.75107.CF

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=206)	P	GG (n=421)	GT (n=322)	TT (n=63)	P	GG (n=1704)	GT (n=1299)	TT (n=269)	P
Men, %	87.1	86.8	83.5	0.50	81.9	78.9	84.1	0.42	85.9	84.8	83.6	0.37
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)	52.5 (0.3)	51.8 (0.5)	0.055
BMI, kg/m²	23.3 (0.1)	23.2 (0.1)	23.2 (0.2)	0.28	23.0 (0.1)	22.9 (0.2)	22.7 (0.3)	0.54	23.2 (0.1)	23.1 (0.1)	23.1 (0.2)	0.21
SBP, mm Hg	131.4 (0.4)	132.3 (0.5)	129.3 (1.0)	0.55	125.1 (0.9)	124.8 (1.1)	121.3 (2.2)	0.55	129.5 (0.4)	130.4 (0.5)	127.4 (1.0)	0.87
DBP, mm Hg	76.5 (0.3)	76.8 (0.3)	75.0 (0.6)	0.51	77.8 (0.6)	77.8 (0.7)	77.9 (1.5)	0.99	76.5 (0.3)	77.1 (0.3)	75.7 (0.6)	0.93
T-Cho, mg/dL	196.5 (0.5)	198.1 (1.0)	197.5 (2.2)	0.68	200.5 (1.6)	196.5 (1.9)	198.3 (4.1)	0.30	199.0 (0.5)	190.2 (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 (0.6)	55.0 (2.0)	0.59	58.8 (0.3)	58.7 (0.4)	59.9 (0.9)	0.78
TG, mg/dL	139.9 (2.4)	137.9 (2.4)	128.8 (4.6)	0.27	128.5 (4.9)	131.9 (6.0)	115.6 (10.7)	0.70	136.5 (2.2)	136.4 (2.3)	125.5 (4.3)	0.52

Data are mean (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.

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[8] 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 ($P=0.044$).

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)	1.96	86.6	<0.0001	0.121	0.17
	Population 2	GG (421)	1.52	90.1	<0.0001	0.045	
		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 (306)	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	0.125	0.44
	Population 2	GG (421)	1.16	51.1	<0.0001	0.067	
		GT+TT (385)	1.18	50.6	<0.0001	0.065	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	0.50

*See Tiret et al.[8]

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.[8] 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]

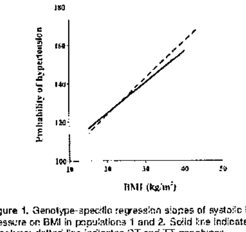

Figure 1. Genotype-specific regression slopes of systolic blood pressure on BMI in populations 1 and 2. Solid line indicates GG genotype; dotted line indicates GT and TT genotypes.

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

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	49	
SBP, mm Hg	129.2 (0.5)	130.1 (0.6)	127.2 (1.2)	0.58	137.3 (0.8)	139.2 (1.0)	136.1 (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)	0.70
Population 2, n	328	256	54		93	66	9	
SBP, mm Hg	123.1 (1.0)	122.6 (1.1)	118.6 (2.2)	0.42	131.9 (2.1)	133.2 (2.3)	137.2 (5.8)	0.56
DBP, mm Hg	76.5 (0.6)	76.3 (0.7)	76.5 (1.7)	0.85	82.5 (1.4)	83.6 (1.5)	85.9 (3.1)	0.43
Populations 1 and 2, n	1265	1002	211		439	297	58	
SBP, mm Hg	127.6 (0.5)	128.2 (0.5)	125.0 (1.1)	0.97	136.1 (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)	81.6 (0.6)	80.2 (1.2)	0.48

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

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.

Acknowledgments

This work was supported by a grant-in-aid for Scientific Research on Priority Areas (C) "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant-in-aid for Research on the Human Genome, Tissue Engineering Food Biotechnology from the Ministry of Health Labor and Welfare.

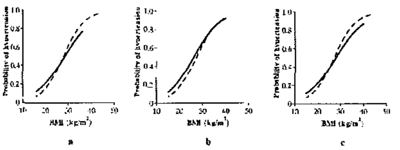

Figure 2. Genotypic variations in relationship between BMI and hypertension. a, In population 1, the regression between BMI and the probability of having hypertension in subjects with GG genotype was represented by the equation y=exp(0.16X−4.53)/[1+exp(0.16X−4.53)]. The equation was y=exp(0.22X−6.10)/[1+exp(0.22X−6.10)] 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(0.18X−4.70)/[1+exp(0.18X−4.70)]. The equation was y=exp(0.21X−5.73)/[1+exp(0.21X−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(0.16X−4.47)/[1+exp(0.16X−4.47)]. The equation was y=exp(0.22X−5.97)/[1+exp(0.22X−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).

References

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;332:411–415.
2. Schiffrin EL, Deng LY, Sventek P, Day R. Enhanced expression of endothelin-1 gene in resistance arteries in severe human essential hypertension. *J Hypertens*. 1997;15:57–63.
3. Shichiri M, Hirata Y, Ando K, Emori T, Ohta K, Kimoto S, Ogura M, Inoue A, Marumo F. Plasma endothelin levels in hypertension and chronic renal failure. *Hypertension*. 1990;15:493–496.
4. Schiffrin EL, Thibault G. Plasma endothelin in human essential hypertension. *Am J Hypertens*. 1991;4(pt 1):303–308.
5. Lenne Ch, Lundeberg T, Theodorsson E, de Faire U. Increased basal concentrations of plasma endothelin in borderline hypertension. *J Hypertens*. 1994;12:1069–1074.
6. Ergul S, Parish DC, Puett D, Ergul A. Racial differences in plasma endothelin-1 concentrations in individuals with essential hypertension. *Hypertension*. 1996;28:652–655.
7. Krum H, Viskoper RJ, Lacourciere Y, Budde M, Charlon V. The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. *N Engl J Med*. 1998;338:784–790.
8. Tiret L, Poirier O, Hallet V, McDonagh TA, Morrison C, McMurray JJ, Dargie HJ, Arveiler D, Ruidavets JB, Luc G, Evans A, Cambien F. The Lys198Asn polymorphism in the endothelin-1 gene is associated with blood pressure in overweight people. *Hypertension*. 1999;33:1169–1174.
9. Asai T, Ohkubo T, Katsuya T, Higaki J, Fu Y, Fukuda M, Hozawa A, Matsubara M, Kitaoka H, Tsuji I, Araki T, Satoh H, Hisamichi S, Imai Y, Ogihara T. Endothelin-1 gene variant associates with blood pressure in obese Japanese subjects: the Ohasama Study. *Hypertension*. 2001;38:1321–1324.
10. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet*. 2001;29:306–309.
11. Freely associating. *Nat Genet*. 1999;22:1–2. Editorial.
12. Ferri C, Bellini C, Desideri G, Di Francesco L, Baldoncini R, Santucci A, De Mattia G. Plasma endothelin-1 levels in obese hypertensive and normotensive men. *Diabetes*. 1995;44:431–436.
13. Parrinello G, Scaglione R, Pinto A, Corrao S, Cecala M, Di Silvestre G, Amato P, Licata A, Licata G. Central obesity and hypertension: the role of plasma endothelin. *Am J Hypertens*. 1996;9(pt 1):1186–1191.
14. Wolpert HA, Steen SN, Istfan NW, Simonson DC. Insulin modulates circulating endothelin-1 levels in humans. *Metabolism*. 1993;42:1027–1030.

Original Article

Association of a GNAS1 Gene Variant with Hypertension and Diabetes Mellitus

Miyuki YAMAMOTO, Michiko ABE, Jing Ji JIN, Zhihong WU, Yasuharu TABARA, Masaki MOGI, Katsuhiko KOHARA, Tetsuro MIKI, and Jun NAKURA

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.

From the Department of Geriatric Medicine, School of Medicine, Ehime University, Ehime, Japan.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Area C, "Medical Genome Science," from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health, Labour, and Welfare.

Address for Reprints: Jun Nakura, M.D., Department of Geriatric Medicine, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791–0295, Japan. E-mail: nakura@m.ehime-u.ac.jp

Received May 11, 2004; Accepted in revised form August 18, 2004.

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±32.8	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′-TAAGGCCACACAAGTCGGGGT-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. GNAS1 Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype	Genotype frequency		p value*	OR*	95% CI*
	Normotensive (n=554)	Hypertensive (n=267)			
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.

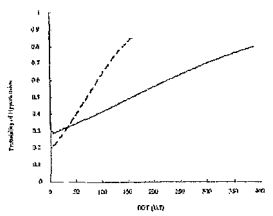

Fig. 1. *Genotypic variations in the positive relationship between GGT and the probability of having hypertension. The solid line indicates the TT and TC genotypes; the dotted line indicates the CC genotype. The regression between GGT and the probability of having hypertension in T carriers was represented 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.

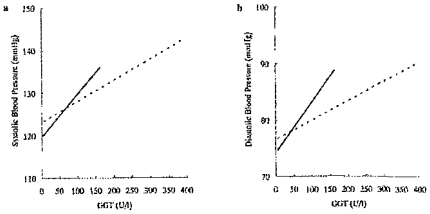

Fig. 2. *Genotype-specific regression slopes of blood pressure on GGT. The solid line indicates the TT and TC genotypes; the dotted line indicates the CC genotype. a: The regression between GGT and SBP in T carriers was represented by the equation,* $Y=0.101X+119.6$. *The equation was* $Y=0.049X+123.0$ *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,* $Y=0.090X+74.1$. *The equation was* $Y=0.034X+76.5$ *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. However, 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.

References

1. Kato N: Genetic analysis in human hypertension. *Hypertens Res* 2002; 25: 319–327.
2. Feldman RD, Lawton WJ, McArdle WL: Low sodium diet corrects the defect in lymphocyte beta-adrenergic responsiveness in hypertensive subjects. *J Clin Invest* 1987; 79: 290–294.
3. Yoshikawa H, Fukuda K, Watanka Y, *et al*: Deficient activity of stimulatory nucleotide-binding regulatory protein in lymphocytes from patients with essential hypertension. *Am J Hypertens* 1994; 7: 713–716.
4. Feldman RD, Tan CM, Chorazyczewski J: G protein alterations in hypertension and aging. *Hypertension* 1995; 26: 725–732.
5. Jia H, Hingorani AD, Sharma P, *et al*: Association of the G(s)alpha gene with essential hypertension and response to beta-blockade. *Hypertension* 1999; 34: 8–14.
6. Abe M, Nakura J, Yamamoto M, *et al*: Association of GNAS1 gene variant with hypertension depending on smoking status. *Hypertension* 2002; 40: 8–14.
7. Chen Y, Nakura J, Xu D, *et al*: Association of GNAS1 gene variant with hypertension dependent on alcohol drinking status. *Hypertens Res* 2003; 26: 439–444.
8. Chick J, Kreitman N, Plant M: Mean cell volume and gamma-glutamyl-transpeptidase as markers of drinking in working men. *Lancet* 1981; 1: 1249–1251.
9. Papoz L, Warnet JM, Pequignot G, Eschwege E, Claude JR, Schwartz D: Alcohol consumption in a healthy population: relationship to gamma-glutamyl transferase activity and mean corpuscular volume. *JAMA* 1981; 245: 1748–1751.
10. Burke V, Puddey IB, Beilin LJ, Vandongen R, Masarei JR: Changes in markers of alcohol intake in men below 'safe' drinking levels. *Alcohol Alcohol* 1992; 27: 677–683.
11. Yersin B, Nicolet JF, Dererey H, Burnier M, van Melle G, Pecoud A: Screening for excessive alcohol drinking: comparative value of carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean corpuscular volume. *Arch Intern Med* 1995; 155: 1907–1911.
12. Alberti KG, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications: part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15: 539–553.
13. Cryer PE, Haymond MW, Santiago JV, Shah SD: Norepinephrine and epinephrine release and adrenergic mediation of smoking-associated hemodynamic and metabolic events. *N Engl J Med* 1976; 295: 573–577.
14. Eisenhofer G, Lambie DG, Johnson RH: Effects of ethanol on plasma catecholamines and norepinephrine clearance. *Clin Pharmacol Ther* 1983; 34: 143–147.
15. Ireland MA, Vandongen R, Davidson L, Beilin LJ, Rouse IL: Acute effects of moderate alcohol consumption on blood pressure and plasma catecholamines. *Clin Sci* 1984; 66: 643–648.
16. Puddey IB, Beilin LJ, Vandongen R, Rouse IL, Rogers P: Evidence for a direct effect of alcohol consumption on blood pressure in normotensive men: a randomized controlled trial. *Hypertension* 1985; 7: 707–713.

Original Paper

Cerebrovascular Diseases

Cerebrovasc Dis 2004;17:273–279
DOI: 10.1159/000077337

Received: December 3, 2002
Accepted: October 2, 2003
Published online: March 16, 2004

Genetic Predisposition to Neurological Symptoms in Lacunar Infarction

Jun-hui Zhang[a] Katsuhiko Kohara[a] Yasumasa Yamamoto[c] Jun Nakura[a]
Yasuharu Tabara[a] Mutsuo Fujisawa[b] Ryosuke Katagi[b] Tetsuro Miki[a]

[a]Department of Geriatric Medicine, Ehime University School of Medicine, and [b]Katagi Neurosurgical Clinic, Ehime, and [c]Kyoto Second Red-Cross Hospital, Kyoto, Japan

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.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2004 S. Karger AG, Basel
0251–5350/04/0174–0273$21.00/0

Accessible online at:
www.karger.com/cvd

Katsuhiko Kohara, MD
Department of Geriatric Medicine, Ehime University School of Medicine
Shigenobu-cho, Onsen-gun
Ehime 791-0295 (Japan)
Tel. +81 89 960 5850, Fax +81 89 960 5852, E-Mail kohara@m.ehime-u.ac.jp

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′-AGCAGAGMGGTTTGCCTTACCTTG-3′ were used [26]. The PCR product (5 μl) was digested with 5 units *Mspi* 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, %	35 (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[†]		
Hemiparesis	115	
Sensory disturbance	19	
Ataxia	12	
Dysarthria	9	

* $p < 0.05$ versus asymptomatic lacunar infarction patients.
† 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.

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 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.

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 = 0.98	
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 lacu-

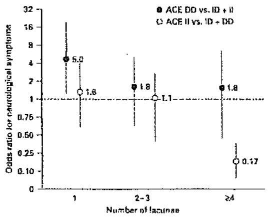

Fig. 1. Odds ratios of ACE genotypes for the manifestation of neurological symptoms of lacunar infarctions according to the number of lacunae. Closed circles indicate the odds ratio for ACE DD genotype compared with ACE ID + II, and open circles indicate the odds ratio for ACE II genotype compared with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

nae 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.

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 lacune [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.

Acknowledgments

This study was supported by research grants from Sasagawa Medical Research Foundation, a Grant-In-Aid for Scientific Research on Priority Area (C) 'Medical Genome Science' from the Ministry of Education, Culture, Sports and Technology of Japan, and a grant for Comprehensive Research on Aging and Health.

References

1 Hassan A, Markus HS: Genetics and ischaemic stroke. Brain 2000;123:1784–1812.
2 Markus HS, Barley J, Lunt R, et al: Angiotensin-converting enzyme gene deletion polymorphism: A new risk factor for lacunar stroke but not carotid atheroma. Stroke 1995;26:1329–1333.
3 Kario K, Kanai N, Saito K, Nago N, Matsuo T, Shimada K: Ischemic stroke and the gene for angiotensin-converting enzyme in Japanese hypertensives. Circulation 1996;93:1630–1633.
4 Takami S, Imai Y, Katsuya T, et al: Gene polymorphism of the renin-angiotensin system associates with risk for lacunar infarction: The Ohasama study. Am J Hypertens 2000;13:121–127.
5 Catto A, Carter AM, Barrett JH, et al: Angiotensin-converting enzyme insertion/deletion polymorphism and cerebrovascular disease. Stroke 1996;27:435–440.
6 Doi Y, Yoshinari M, Yoshizumi H, Ibayashi S, Wakisaka M, Fujishima M: Polymorphism of the angiotensin-converting enzyme (ACE) gene in patients with thrombotic brain infarction. Atherosclerosis 1997;132:145–150.
7 Pfohl M, Fetter M, Koch M, Barth CM, Weiss R, Haring HU: Association between angiotensin I-converting enzyme genotypes, extracranial artery stenosis, and stroke. Atherosclerosis 1998;140:161–166.
8 Notsu Y, Nabika T, Park H-Y, Masuda J, Kobayashi S: Evaluation of genetic risk factors for silent brain infarction. Stroke 1999;30:1881–1886.
9 You R, McNeil JJ, O'Malley HM, Davis SM, Donnan GA: Risk factors for lacunar infarction syndromes. Neurology 1995;45:1483–1487.
10 Gan R, Sacco RL, Kargman DE, Roberts JK, Boden-Albala B, Gu Q: Testing the validity of the lacunar hypothesis: The northern Manhattan stroke study experience. Neurology 1997;48:1204–1211.
11 Lee BI, Nam HS, Heo JH, Kim DI: Yonsei Stroke Registry: Analysis of 1,000 patients with acute cerebral infarctions. Cerebrovasc Dis 2001;12:145–151.
12 Eriksson SE, Olsson JE: Survival and recurrent strokes in patients with different subtypes of stroke: A fourteen-year follow-up study. Cerebrovasc Dis 2001;12:171–180.
13 Moulin T, Tatu L, Vuillier F, Berger E, Chavot D, Rumbach L: Role of a stroke data bank in evaluating cerebral infarction subtypes: Patterns and outcome of 1,776 consecutive patients from the Besançon stroke registry. Cerebrovasc Dis 2000;10:261–271.
14 Besson G, Hommel M, Perret J: Risk factors for lacunar infarcts. Cerebrovasc Dis 2000;10:387–390.
15 Longstreth WT Jr, Bernick C, Manolio TA, Bryan N, Jungreis CA, Price TR, for the Cardiovascular Health Study Collaborative Research Group: Lacunar infarcts defined by magnetic resonance imaging of 3,660 elderly people: The Cardiovascular Health Study. Arch Neurol 1998;55:1217–1225.
16 Lee S-C, Park S-J, Ki H-K, et al: Prevalence and risk factors of silent cerebral infarction in apparently normal adults. Hypertension 2000;36:73–77.
17 Kobayashi S, Okada K, Koide H, Bokura H, Yamaguchi S: Incidence of silent lacunar lesion in normal adults and its relation to cerebral blood flow and risk factors. Stroke 1991;22:1379–1383.
18 Boiten J, Lodder J: Lacunar infarcts: Pathogenesis and validity of the clinical syndromes. Stroke 1991;22:1374–1378.
19 Mast H, Thompson JL, Lee SH, Mohr JP, Sacco RL: Hypertension and diabetes mellitus as determinants of multiple lacunar infarcts. Stroke 1995;26:30–33.
20 Spolveri S, Baruffi MC, Cappelletti C, Semerano F, Rossi S, Pracucci G, Inzitari D: Vascular risk factors linked to multiple lacunar infarcts. Cerebrovasc Dis 1998;8:152–157.
21 Yamamoto Y, Akiguchi I, Oiwa K, Hayashi M, Kimura J: Adverse effect of nighttime blood pressure on the outcome of lacunar infarct patients. Stroke 1998;29:570–576.
22 Kohara K, Jiang Y, Igase M, et al: Postprandial hypotension is associated with asymptomatic cerebrovascular damage in essential hypertensive patients. Hypertension 1999;33:565–568.
23 Kohara K, Zhao B, Jiang Y, et al: Relation of left ventricular hypertrophy and geometry to asymptomatic cerebrovascular damage in essential hypertension. Am J Cardiol 1999;83:367–370.
24 Rigat B, Hubert C, Corvol P, Soubrier F: PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). Nucleic Acids Res 1992;20:1433.
25 Shanmugam V, Sell KW, Saha BK: Mistyping ACE heterozygotes. PCR Methods Appl 1993;3:120–121.
26 Russ AP, Maerz W, Ruzicka V, Stein U, Gross W: Rapid detection of the hypertension-associated Met235→Thr allele of the human angiotensinogen gene. Hum Mol Genet 1993;2:609–610.
27 Nakauchi Y, Suehiro T, Yamamoto M, et al: Significance of angiotensin I-converting enzyme and angiotensin II type 1 receptor gene polymorphisms as risk factors for coronary heart disease. Atherosclerosis 1996;125:161–169.
28 Fisher CM: Lacunes: Small, deep cerebral infarcts. Neurology 1965;15:774–784.
29 Fisher CM: Lacunar stroke and infarcts: A review. Neurology 1982;32:871–876.
30 Fisher CM: The arterial lesions underlying lacunes. Acta Neuropathol 1968;12:1–15.
31 Fisher CM: Capsular infarcts: The underlying vascular lesions. Arch Neurol 1979;36:65–73.
32 Schmal M, Marini C, Carolei A, Di Napoli M, Kessels F, Lodder J: Different vascular risk factor profiles among cortical infarcts, small deep infarcts, and primary intracerebral haemorrhage point to different types of underlying vasculopathy: A study from the L'Aquila Stroke Registry. Cerebrovasc Dis 1998;8:14–19.
33 Boiten J, Lodder J, Kessels F: Two clinically distinct lacunar infarct entities? A hypothesis. Stroke 1993;24:652–656.
34 Jung DK, Devuyst G, Maeder P, Bogousslavsky J: Atrial fibrillation with small subcortical infarcts. J Neurol Neurosurg Psychiatry 2001;70:344–349.
35 Inzitari D, Eliasziw M, Sharpe BL, Fox AJ, Barnett HJ, for the North American Symptomatic Carotid Endarterectomy Trial Group: Risk factors and outcome of patients with carotid artery stenosis presenting with lacunar stroke. Neurology 2000;54:660–666.
36 Wolfe N, Linn R, Babikian VL, Knoefel JE, Albert ML: Frontal systems impairment following multiple lacunar infarcts. Arch Neurol 1990;47:129–132.
37 Matsubayashi K, Okumiya K, Wada T, et al: Postural dysregulation in systolic blood pressure is associated with worsened scoring on neurobehavioral function tests and leukoaraiosis in the older elderly living in a community. Stroke 1997;28:2169–2173.
38 Fujikawa T, Yamawaki S, Touhouda Y: Incidence of silent cerebral infarction in patients with major depression. Stroke 1993;24:1631–1634.
39 Shimada K, Kawamoto A, Matsubayashi K, Nishinaga M, Kimura S, Ozawa T: Diurnal blood pressure variations and silent cerebrovascular damage in elderly patients with hypertension. J Hypertens 1992;10:875–878.

bjh research paper

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

Hiroyasu Akatsu,[1,2] Hidehisa Yamagata,[3] Yusen Chen,[3] Tetsuro Miki,[3] Kouzin Kamino,[4] Masatoshi Takeda,[4] William Campbell,[1] Ikuko Kondo,[3] Kenji Kosaka,[1] Takayuki Yamamoto[1] and Hidechika Okada[1,5]

[1]*Choju Medical Institute, Fukushimura Hospital, Aichi, Toyohashi,* [2]*Department of Geriatric Medicine, Ehime University School of Medicine, Toon-shi, Ehime,* [3]*Division of EcoGenetics, Department of Environmental Health and Social Medicine, Ehime University School of Medicine, Toon-shi, Ehime,* [4]*Division of Psychiatry and Behavioural Proteomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, Suita, Osaka, and* [5]*Institute for Protein Science Co. Ltd, Aichi, Nagoya, Japan*

Received 13 July 2004; accepted for publication 25 August 2004

Correspondence: Dr Hiroyasu Akatsu, Choju Medical Institute, Fukushimura Hospital, 19-14, Azayamanaka, Noyori, Toyohashi, Aichi, 441-8124, Japan. E-mail: akatu@chojuken.net

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

doi:10.1111/j.1365-2141.2004.05219.x

(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.

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 Choja 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 Choja 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

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 ± 8·1/	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	72/41	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	223/115	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.

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-demental 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 Kluver–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 wide-spread 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 AL137143 and AL357758*). 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′-ATC-TTGGGCACCATTTTGAG-3′), TAFI325-F (5′-CACAAAGA-AAAACAGATCACACAG-3′), TAFI325-R (5′-AAAGCCACC-CAATTGTGATT-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 $P < 0{\cdot}05$.

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

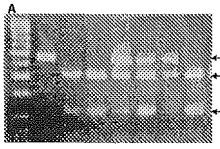

Fig 1. Demonstration of the existence in the human population of Thr/Ala-147 (A) and Thr/Ile-325 (B) polymorphisms in seven typical samples. Each genomic DNA extracted from brain tissues was subjected to polymerase chain reaction (PCR) to amplify a fragment encompassing the codon for the amino acids at positions 147 and 325. PCR products were digested with BbvI (147) and SpeI (325). The TAFI-147 PCR product was 456 bp in size; the G (Ala) allele was digested into 28 + 124 + 304 bp, but the A (Thr) allele gave 28 + 428 bp. The TAFI-325 PCR product size was 363 bp; the C (Thr) allele was digested to 118 + 245 bp, but the T (Ile) allele was not digested by SpeI.

(75%) including those with HT without another risk disease and these were used in our analysis of the relationship between CI and *TAFI* variation.

Details of the patient age and *TAFI* polymorphism distribution in our samples and the population-based controls are described in Table I. Genotyping of the 253 brain bank samples and 108 population-based control individuals was carried out. PCR products were obtained from a number of different human genomic DNAs isolated from patients' brain tissues and all blood samples. The isoform mutated at amino acid 147 (TAFI 147) had a PCR product of 456 bp. After cutting with BbvI, the Thr/Thr-147 homozygote showed two bands (28 + 428 bp), the Thr/Ala-147 heterozygote showed four bands (28 + 124 + 304 + 428 bp) and the Ala/Ala-147 homozygote showed two (28 + 428 bp), as given in Fig 1A. Brouwers *et al* (2001) also reported another *TAFI* polymorphism at amino acid 325. This was a C to T mutation at position 1040 of the *TAFI* gene (GenBank numbers NM-00187 and NM-016413), which would result in the conversion of a Thr codon (ACU) to an Ile codon (AUU) at amino acid position 325. In our study, the *TAFI 325* PCR product size was 363 bp. After SpeI cutting, the Thr/Thr-325 homozygote showed two bands (118 + 245 bp), the Thr/Ile-325 heterozygote showed four bands (118 + 245 + 363 bp) and the homozygote Ile/Ile-325 was not cut (363 bp), as shown in Fig 1B.

The genotype distribution of the Thr/Ala-147 and Thr/Ile-325 polymorphisms was in Hardy–Weinberg equilibrium in the 253 brain bank samples and 108 population-based controls. At the 147 position, frequencies for Thr/Thr, Thr/Ala and Ala/Ala were 6% (15), 40% (102) and 54% (136), respectively, in the brain bank group and 3% (3), 44% (47) and 53% (58), respectively, in the population-based control group (Table I). Among our brain bank samples, the frequency of the Thr allele was 26% (132), and that of the Ala allele was 74% (374); and among the population-based controls, the respective frequencies were 25% (53) and 75% (163) (Table I). In addition, at the 325 position, brain bank frequencies for Thr/Thr, Thr/Ile and *Ile/Ile were 75% (190), 23% (58) and 2% (5) and the respective* population-based frequencies were 75% (81), 20% (21) and 5% (6). The frequency of the Thr allele was 87% (438) in the brain bank group and 85% (183) in the population-based group, and that for the Ile allele was 13% (68) in the brain bank group and 15% (33) in the population-based group (Table I). We could not attach any statistical significance to differences in frequencies between the two groups.

To examine the relationship between cerebral arteriosclerosis and *TAFI* polymorphism at amino acids 147 and 325, we classified the 189 patients with no evidence of disease risk into four groups on the basis of their degree of arteriosclerosis, and found that the disease was absent in 29, slight in 109, moderate in 28 and severe in 14 (Table II). Although no statistical significance was found at the Thr/Ile-325 position, there appeared to be a tendency for patients that were severely affected by arteriosclerosis to have a lower frequency of the Ile (more Thr) allele (Table II). Table III shows the results of our evaluation of the 189 patients in terms of the degree of cerebral arteriosclerosis, as well as clinical history and symptoms, brain imaging (CT scanning), and total macroscopic and microscopic findings. Of these 189 patients, 94 had no infarction, 44 had small infarcts and 51 had large infarcts. Although none of the findings shown in Table III had statistical significance, as in Table II, patients with large infarcts appeared to have a lower frequency of the Ile allele (10%) (Table III).

Table II. Association between Thr/Ala-147 and Thr/Ile-325 polymorphisms of the *TAFI* gene and the arteriasclerosis ratio.

Male/female	No sclerosis	Slight sclerosis	Mild sclerosis	Severe sclerosis	FBB total (%)	PBC (%)
Thr/Ala-147						
Thr/Thr	2	8	2	0	12 (7)	3 (3)
Thr/Ala	11	49	14	6	71 (39)	47 (44)
Ala/Ala	16	61	12	8	97 (54)	58 (53)
Allele Thr (%)	15 (26)	56 (26)	18 (32)	6 (21)	95 (26)	53 (25)
Allele Ala (%)	43 (74)	162 (74)	38 (68)	22 (79)	265 (74)	163 (75)
Thr/Ile-325						
Thr/Thr	18	80	21	12	131 (73)	81 (75)
Thr/Ile	10	27	6	2	45 (25)	21 (20)
Ile/Ile	1	2	1	0	4 (2)	6 (5)
Allele Thr (%)	46 (79)	187 (86)	48 (86)	26 (93)	307 (85)	183 (85)
Allele Ile (%)	12 (21)	31 (14)	8 (14)	2 (7)	53 (15)	33 (15)

FBB, Fukushimura Brain Bank; PBC, population-based controls.
There were not enough samples to provide statistical significance, but for Thr/Ile-325, patients with more severe sclerosis showed a lower frequency of the Ile allele.

Table III. Association between Thr/Ala-147 and Thr/Ile-325 polymorphisms of the *TAFI* gene and the clinicopathological CI grade.

	No infarction	Small infarction	Large infarction	FBB total (%)	PBC (%)
Thr/Ala-147					
Thr/Thr	6	3	3	12 (6)	3 (3)
Thr/Ala	35	18	22	75 (40)	47 (44)
Ala/Ala	53	23	26	102 (54)	58 (53)
Allele Thr (%)	47 (25)	24 (27)	28 (27)	99 (26)	53 (25)
Allele Ala (%)	141 (75)	64 (63)	74 (73)	279 (74)	163 (75)
Thr/Ile-325					
Thr/Thr	68	30	33	131 (73)	81 (75)
Thr/Ile	23	12	10	45 (24)	21 (20)
Ile/Ile	3	2	0	5 (2)	6 (5)
Allele Thr (%)	159 (85)	72 (82)	92 (90)	323 (85)	183 (85)
Allele Ile (%)	29 (15)	16 (18)	10 (10)	55 (15)	33 (15)

FBB, Fukushimura Brain Bank; PBC, population-based controls; CI, cerebral infarction.
On comparing with PBCs, no group showed statistical significance ($P < 0{\cdot}05$).

To analyse the relationship with onset age, we determined the age at the first attack from the pathology of the brain bank samples and the clinical CI history. From the clinical history, the age at first attack was clearly evident in 59 cases. However, we could not detect a correlation between *TAFI* polymorphism at amino acids 147 and 325, and the age at which the first CI event occurred (Table IV).

Discussion

We analysed *TAFI* polymorphisms at amino acids 147 and 325 using samples in which the neuropathology had been confirmed. We designed PCR primers based on the gene sequence encoding human TAFI mapped at 13q14·11 (Vanhoof *et al*, 1996; Boffa *et al*, 1999) and were able to demonstrate the existence in the Japanese population of Thr/Ala-147 and Thr/Ile 325 using the method of Schneider *et al* (2002).

In our analysis, no deviation was noted in relation to sex or age. Among patients at risk of thrombus formation from diseases including DM, VP, AF and HL, as well as neuropathological amyloid angiopathy, the incidence of these polymorphisms was not statistically significant (data not shown). We targeted the remaining 189 neuropathologically diagnosed cases.

We could not detect any correlation between *TAFI* variants and either CI or pathological arteriosclerosis, in terms of genetic polymorphisms. Unfortunately, we did not obtain plasma samples while the patients were alive. At this time, we have no data correlating CI, pathological arteriosclerosis, *TAFI* polymorphism and plasma TAFI antigen (Ag) levels.

The Thr/Ala-147 and Thr/Ile-325 (305A/G and 1040C/T SNP) positions are important determinants of the plasma TAFI Ag levels. Henry *et al* (2001) showed that the TAFI Ag level is strongly affected by associated with the Thr/Ala-147 mutation (Henry *et al*, 2001). In addition, Brouwers *et al*

Table IV. Association between Thr/Ala-147 and Thr/Ile-325 polymorphisms of the *TAFI* gene and patient age at the first CI attack.

	≤60 years	61–70 years	71–80 years	≥81 years	PBC (%)
Thr/Ala-147					
Thr/Thr	0	1	1	0	3 (3)
Thr/Ala	0	8	7	8	47 (44)
Ala/Ala	4	9	10	11	58 (53)
Allele Thr (%)	0 (0)	10 (28)	9 (25)	8 (21)	53 (25)
Allele Ala (%)	8 (100)	26 (72)	27 (75)	30 (79)	163 (75)
Thr/Ile-325					
Thr/Thr	2	13	15	13	81 (75)
Thr/Ile	2	5	3	5	21 (20)
Ile/Ile	0	0	0	1	6 (5)
Allele Thr (%)	6 (75)	31 (86)	33 (92)	31 (82)	183 (85)
Allele Ile (%)	2 (25)	5 (14)	3 (8)	7 (18)	33 (15)

PBC, population-based controls; CI, cerebral infarction.
No statistical significance was noted ($P < 0.05$).

(2001) identified another SNP, Thr/Ile-325 (1040C/T), in the coding region of the *TAFI* gene by comparing published sequences. The C/C genotype (Thr/Thr-325) was associated with the highest levels of TAFI Ag and the T/T genotype (Ile/Ile-325) with the lowest (Brouwers *et al*, 2001). Interestingly, the Thr/Ile-325 polymorphism influences not only the plasma Ag level of TAFI but also TAFIa activity and stability *in vitro*, and can result in increased antifibrinolytic activity; Ile-325 variants exhibited an antifibrinolytic effect that was 60% greater than that of Thr-325 variants (Schneider *et al*, 2002).

In previous reports, it was shown that the plasma TAFI Ag level is important in several vascular diseases and in other conditions (Juhan-Vague *et al*, 2000; Silveira *et al*, 2000; van Tilburg *et al*, 2000; Schroeder *et al*, 2002), and that it is genetically regulated (Brouwers *et al*, 2001; Henry *et al*, 2001).

Accordingly, it is expected that there would be some correlation between TAFI polymorphism and vascular disease. From a query made of the European multicentre database, 533 samples from MI patients and 571 from normal individuals were analysed for Thr/Ala-147, Thr/Ile-325 and plasma TAFI Ag levels (Henry *et al*, 2001; Juhan-Vague *et al*, 2003). A strong correlation was shown between certain polymorphisms and TAFI Ag, however, no statistically significant differences were noted between MI patients and normal individuals (Juhan-Vague *et al*, 2002; Morange *et al*, 2002). In correlating TAFI Thr/Ala-147, Thr/Ile-325 and plasma TAFI Ag levels, a genotype-dependent artefact might develop when levels are measured by an enzyme-linked immunosorbent assay (ELISA) (Guimaraes *et al*, 2004). To address this problem, a genotype 325-specific TAFI ELISA system has been developed (Gils *et al*, 2003). The strong correlation shown between polymorphisms and TAFI Ag was probably because of a genotype-dependent artefact. The relationship between vascular disease and TAFI Ag should therefore be reconsidered using another ELISA system. At the very least, the Thr/Ile-325 polymorphism does not represent a CI risk factor, as was also true of MI (Juhan-Vague *et al*, 2002; Morange *et al*, 2002).

In contrast, previous reports have stated that the Thr/Ala-147 polymorphism is a risk factor for MI (Henry *et al*, 2001; Juhan-Vague *et al*, 2002) and angina pectoris (Morange *et al*, 2003). The distribution of the Thr/Ala-147 polymorphism is similar to that reported by Brouwers *et al* (2001) and Juhan-Vague *et al* (2002). However, in our samples, no Thr/Ala-147 variant put patients at increased risk for cerebral arteriosclerosis or CI (Tables II and III). In addition, with respect to age at the first CI event, no association with either Thr/Ala-147 or Thr/Ile-325 (Table IV) was seen in the younger patients.

However, for Thr/Ile-325, our allele frequencies differed significantly from those of previous reports (Brouwers *et al*, 2001; Morange *et al*, 2002). Compared with the data of Brouwers *et al* (2001) (Thr/Thr 59, Thr/Ile 76, Ile/Ile 17), our χ^2 values were 36·19 ($P < 0·001$) for the Thr/Ile-325 polymorphism and 35·98 ($P < 0·001$) for the total Thr/Ile allele. This tendency was the same as in Morange's multicentre study (Morange *et al*, 2002) in which the χ^2 value for the Thr/Ile polymorphism was 38·86 ($P < 0·001$) (Thr/Thr 267, Thr/Ile 233, Ile/Ile 49) for controls and 48·71 ($P < 0·001$) for MI patients (Thr/Thr 260, Thr/Ile 216, Ile/Ile 49), which corresponded with our data (Thr/Thr 190, Thr/Ile 58, Ile/Ile 5 seen in Table I).

This difference in allele frequencies at position 325 may simply be the result of racial difference between white people and Japanese. However, the average age of the subjects in previous reports was around 50 years (Brouwers *et al*, 2001; Henry *et al*, 2001; Juhan-Vague *et al*, 2002). Our subjects had an average age of over 80 years. It is possible that the deviation observed in our study might reflect the longer life span of our patients, although there was no indication that the extent of deviation corresponded to an increase in range. We intend to examine this difference by analysis of *TAFI* polymorphisms in younger Japanese subjects.

We believe that clinicopathological analyses such as these are important for studying CI, because among the so-called vascular diseases, CI is the end result of a combination of several conditions and risk factors. For this reason, it is not

easy to pick out *TAFI* polymorphisms that represent a CI risk factor. Future studies will focus on CI as an inflammatory disease and TAFI as an acute phase protein of the inflammatory process (Kato *et al*, 2000; Sato *et al*, 2000). In addition, in examining TAFI levels, we will also consider the possibility of artefacts at several time points, and if found, will study their relationship to polymorphism and CI using our unique ELISA system (Tani *et al*, 2003).

Acknowledgements

We received support for this study from the Research Grants for the Future Programme, Japan Society for the Promotion of Science (JSPS). We thank the patients and their guardians for cooperating with our project. We also thank Professor Tetsumei Urano, Department of Physiology, Hamamatsu University School of Medicine for discussions, Mr. Yoshiaki Tani, Mr. Norihiro Ogawa, Mr. Kaoru Taniguwa and Mr. Takeshi Kaneseka for their excellent technical assistance and Ms. Catherine Campbell for her help in editing this manuscript.

References

Akatsu, H., Takahashi, M., Matsukawa, N., Ishikawa, Y., Kondo, N., Sato, T., Nakazawa, H., Yamada, T., Okada, H., Yamamoto, T. & Kosaka, K. (2002) Subtype analysis of neuropathologically diagnosed patients in a Japanese geriatric hospital. *Journal of the Neurological Sciences*, **196**, 65–69.

Bajzar, L., Manuel, R. & Nesheim, M.E. (1995) Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *Journal of Biological Chemistry*, **270**, 14477–14484.

Bajzar, L., Morser, J. & Nesheim, M. (1996) TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *Journal of Biological Chemistry*, **271**, 16603–16608.

Boffa, M.B., Reid, T.S., Joo, E., Nesheim, M.E. & Koschinsky, M.L. (1999) Characterization of the gene encoding human TAFI (thrombin-activable fibrinolysis inhibitor; plasma procarboxypeptidase B). *Biochemistry*, **38**, 6547–6558.

Brouwers, G.J., Vos, H.L., Lechner, K.W., Bulk, S., Schneider, M., Boffa, M., Koschinsky, M., van Tilburg, N.H., Nesheim, M.E., Bertina, R.M. & Gomez Garcia, E.B. (2001) A novel, possibly functional, single nucleotide polymorphism in the coding region of the thrombin-activatable fibrinolysis inhibitor (TAFI) gene is also associated with TAFI levels. *Blood*, **98**, 1992–1993.

Campbell, W. & Okada, H. (1989) An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits. *Biochemical and Biophysical Research Communications*, **162**, 933–939.

Franco, R.F., Fagundes, M.G., Meijers, J.C., Reitsma, P.H., Lourenso, D., Morelli, V., Maffei, F.H., Ferrari, I.G., Piccinato, C.E., Silva, Jr, W.A. & Zago, M.A. (2001) Identification of polymorphisms in the 5′-untranslated region of the TAFI gene: relationship with plasma TAFI levels and risk of venous thrombosis. *Haematologica*, **86**, 510–517.

Gils, A., Alessi, M.C., Brouwers, E., Peeters, M., Marx, P., Ieuts, I., Bouma, B., Hendriks, D., Juhan-Vague, I. & Declerck, P.J. (2003) Development of a genotype 325-specific proCPU/TAFI ELISA. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **23**, 1122–1127.

Guimaraes, A.H.L., Van Tilburg, N.H., Vos, H.L., Bertina, R.M. & Rijken, D.C. (2004) Association between thrombin activatable fibrinolysis inhibitor genotype and levels in plasma: comparison of different assays. *British Journal of Haematology*, **124**, 659–665.

Henry, M., Aubert, H., Morange, P.E., Nanni, I., Alessi, M.C., Tiret, L. & Juhan-Vague, I. (2001) Identification of polymorphisms in the promoter and the 3′ region of the TAFI gene: evidence that plasma TAFI antigen levels are strongly genetically controlled. *Blood*, **97**, 2053–2059.

Hori, Y., Gabazza, E.C., Yano, Y., Katsuki, A., Suzuki, K., Adachi, Y. & Sumida, Y. (2002) Insulin resistance is associated with increased circulating level of thrombin-activable fibrinolysis inhibitor in type 2 diabetic patients. *Journal of Clinical Endocrinology and Metabolism*, **87**, 660–665.

Juhan-Vague, I., Renucci, J.F., Grimaux, M., Morange, P.E., Gouvernet, J., Gourmelin, Y. & Alessi, M.C. (2000) Thrombin-activatable fibrinolysis inhibitor antigen levels and cardiovascular risk factors. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **20**, 2196–2201.

Juhan-Vague, I., Morange, P.E., Aubert, H., Henry, M., Aillaud, M.F., Alessi, M.C., Samnegard, A., Hasse, B., Yudkin, J., Margaglione, M., Di Minno, G., Hamsten, A. & Humphries, S.E. (2002) Plasma thrombin-activatable fibrinolysis inhibitor antigen concentration and genotype in relation to myocardial infarction in the north and south of Europe. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **22**, 867–873.

Kato, T., Akatsu, H., Sato, T., Matsuo, S., Yamamoto, T., Campbell, W., Hotta, N., Okada, N. & Okada, H. (2000) Molecular cloning and partial characterization of rat procarboxypeptidase R and carboxypeptidase N. *Microbiology and Immunology*, **44**, 719–728.

Kostka, H., Kuhlisch, E., Schelling, S. & Siegert, G. (2003) Polymorphisms in the *TAFI* gene and the risk of venous thrombosis. *Clinical Laboratory*, **49**, 645–647.

Morange, P.E., Henry, M., Frere, C. & Juhan-Vague, I. (2002) Thr325Ile polymorphism of the TAFI gene does not influence the risk of myocardial infarction. *Blood*, **99**, 1878–1879.

Morange, P.E., Juhan-Vague, I., Scarabin, P.Y., Alessi, M.C., Luc, G., Arveiler, D., Ferrieres, J., Amouyel, P., Evans, A. & Ducimetiere, P. (2003) Association between TAFI antigen and Ala147Thr polymorphism of the TAFI gene and the angina pectoris incidence. The PRIME Study (Prospective Epidemiological Study of MI). *Thrombosis and Haemostasis*, **89**, 554–560.

Redlitz, A., Tan, A.K., Eaton, D.L. & Plow, E.F. (1995) Plasma carboxypeptidases as regulators of the plasminogen system. *Journal of Clinical Investigation*, **96**, 2534–2538.

Sakharov, D.V., Plow, E.F. & Rijken, D.C. (1997) On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. *Journal of Biological Chemistry*, **272**, 14477–14482.

Sato, T., Miwa, T., Akatsu, H., Matsukawa, N., Obata, K., Okada, N., Campbell, W. & Okada, H. (2000) Pro-carboxypeptidase R is an acute phase protein in the mouse, whereas carboxypeptidase N is not. *Journal of Immunology*, **165**, 1053–1058.

Schneider, M., Boffa, M., Stewart, R., Rahman, M., Koschinsky, M. & Nesheim, M. (2002) Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *Journal of Biological Chemistry*, **277**, 1021–1030.

Schroeder, V., Chatterjee, T., Mehta, H., Windecker, S., Pham, T., Devantay, N., Meier, B. & Kohler, H.P. (2002) Thrombin activatable fibrinolysis inhibitor (TAFI) levels in patients with coronary artery disease investigated by angiography. *Thrombosis and Haemostasis*, **88**, 1020–1023.

Silveira, A., Schattemon, K., Goossens, F., Moor, E., Scharpe, S., Strandqvist, M., Hendriks, D. & Hamsten, A. (2000) Plasma procarboxypeptidase U in men with symptomatic coronary artery disease. *Thrombosis and Haemostasis*, **84**, 364–368.

Tani, S., Akatsu, H., Ishikawa, Y., Okada, N. & Okada, H. (2003) Preferential detection of pro-carboxypeptidase R by enzyme-linked immunosorbent assay. *Microbiology and Immunology*, **47**, 295–300.

van Tilburg, N.H., Rosendaal, F.R. & Bertina, R.M. (2000) Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. *Blood*, **95**, 2855–2859.

Vanhoof, G., Wauters, J., Schatteman, K., Hendriks, D., Goossens, F., Bossuyt, P. & Scharpe, S. (1996) The gene for human carboxypeptidase U (CPU) – a proposed novel regulator of plasminogen activation – maps to 13q14·11. *Genomics*, **38**, 151–155.

Watanabe, R., Wada, H., Watanabe, Y., Sakakura, M., Nakasaki, T., Mori, Y., Nishikawa, M., Gabazza, E.C., Nobori, T. & Shiku, H. (2001) Activity and antigen levels of thrombin-activable fibrinolysis inhibitor in plasma of patients with disseminated intravascular coagulation. *Thrombosis Research*, **104**, 1–6.

Zhao, L., Morser, J., Bajzar, L., Nesheim, M. & Nagashima, M. (1998) Identification and characterization of two thrombin-activatable fibrinolysis inhibitor isoforms. *Thrombosis and Haemostasis*, **80**, 949–955.

Zorio, E., Castello, R., Falco, C., Espana, F., Oso, A., Almenar, L., Aznar, J. & Estelles, A. (2003) Thrombin-activatable fibrinolysis inhibitor in young patients with myocardial infarction and its relationship with the fibrinolytic function and the protein C system. *British Journal of Haematology*, **122**, 958–965.

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Biochemical and Biophysical Research Communications 321 (2004) 329–323

BBRC

www.elsevier.com/locate/ybbrc

Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

Hidehisa Yamagata [a,*], Yusen Chen [b], Hiroyasu Akatsu [c], Kouzin Kamino [d], Jin-ichi Ito [c], Shinji Yokoyama [c], Takayuki Yamamoto [c], Kenji Kosaka [c], Tetsuro Miki [b], Ikuko Kondo [a]

[a] *Department of Medical Genetics, Ehime University School of Medicine, Ehime, Japan*
[b] *Department of Geriatric Medicine, Ehime University School of Medicine, Ehime, Japan*
[c] *Choju Medical Institute, Fukushimura Hospital, Toyohashi, Japan*
[d] *Division of Psychiatry and Behavioral Proteomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, Suita, Japan*
[e] *Department of Biochemistry, Cell Biology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Received 10 June 2004

Abstract

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease (AD) and HIV encephalitis. The FGF1 gene is therefore a strong candidate gene for AD. Using the promoter polymorphism of the FGF1 gene, we examined the relationship between AD and the FGF1 and apolipoprotein E (APOE) genes in 100 Japanese autopsy-confirmed late-onset AD patients and 106 age-matched non-demented controls. The promoter polymorphism (−1385 A/G) was significantly associated with AD risk. The odds ratio for AD associated with the GG vs non-GG genotype was 2.02 (95% CI = 1.16–3.52), while that of ε4 vs non-ε4 in APOE4 gene was 5.19 (95% CI = 2.68–10.1). The odds ratio for APOEε4 and FGF1 GG carriers was 20.5 (95% CI = 6.88–60.9). The results showed that the FGF1 gene is associated with autopsy-confirmed AD.

Keywords: Definite Alzheimer's disease; Fibroblast growth factor 1 gene; Promoter polymorphism; Association study; APOE; Risk factor

Alzheimer's disease (AD; MIM# 104300) is the most common cause of dementia in mid- to late-life. Studying the factors that influence the risk of developing AD may lead to the identification of those at high risk for developing it, strategies for prevention or intervention, and clues to the cause of the disease. Both genetic and environmental factors have been implicated in the development of AD [1], but the cause of AD remains unknown, and no cure or universally effective treatment has yet been developed [2]. Even the diagnosis is difficult. A definitive diagnosis depends on analysis of neuritic plaques and neurofibrillary tangles found in brain tissue [3]. Given the recognition that AD constitutes a heterogeneous disorder, identification of established risk factors would be difficult using conventional methods.

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) is a member of the fibroblast growth factor family that possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes [4]. FGF1 protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease [5,6] and HIV encephalitis [7]. Immunohistochemical examination of postmortem brain tissue of AD revealed that FGF1 was specifically expressed in a subpopulation of reactive astrocytes surrounding senile

* Corresponding author. Fax: +81-89-960-5279.
E-mail address: hideyama@m.ehime-u.ac.jp (H. Yamagata).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.142

plaques. Such upregulation of FGF1 expression might be related to the presence of reactive astrocytes rather than β-amyloid protein deposition [8,9]. Recent studies suggest that FGF1 upregulates APOE synthesis and subsequently HDL production in reactive astrocytes in an autocrine or paracrine manner, and exerts its effect after central nervous system (CNS) damage through APOE secretion [10,11]. Besides, the fact that FGF1 expression is lower in the hippocampal formation than in motoneurons suggests that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [6,12]. The FGF1 gene is therefore a strong candidate gene for AD. However, there are no reports regarding the association of FGF1 gene polymorphism with AD. Therefore, we investigated whether FGF1 gene polymorphism could contribute to risk in a limited subgroup of AD (autopsy-confirmed AD).

Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected based on the NINCDS-ADRDA criteria for definite AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination [3,13]. Brain and blood samples were obtained with informed consent from subjects in the Chubu and Kansai areas of Japan. A total of 100 unrelated late-onset AD (LOAD) patients had been diagnosed previously, and 106 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence of the patients as described elsewhere [14,15]. The mean age ± SD (years) at the time of this study was as follows: 85.3 ± 6.0 for LOAD, 83.0 ± 4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol–chloroform method [16].

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hha*I, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGCAATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATTATGGTG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5mM $MgCl_2$, 50mM KCl, and 10mM Tris–HCl, pH 8.3), 200μM each dNTP, 5μM each primer, 0.5 U *Taq* DNA polymerase and 50ng genomic DNA as a template with 35 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 1min. PCR product size was 355bp, and the G allele was digested by *Hha*I to 53 − 141 − 161 bp, and the A allele to 53 − 302bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.

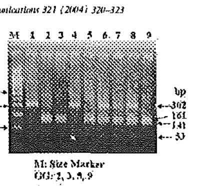

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *Hha*I and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*Hha*I polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

Results

The PCR results were scored by two independent investigators who did not know whether each sample was from a case patient or a control. No intraobserver variability was found on repeated readings of the same gel, and the interobserver variability was less than 1%. All ambiguous samples were analyzed a second time.

The distribution of the three genotypes (GG, GA, and AA) reached Hardy–Weinberg equilibrium. The G allele was found in 75% of the 100 LOAD patients and 63% of the 106 control subjects. A significant association was observed between the −1385 G/A polymorphism and LOAD ($p < 0.03$; Table 1). We then examined the GG genotype as a risk factor for AD, considering the APOE status. As expected, APOEε4 conferred an increased risk for AD [odds ratio (OR) = 5.19]. OR in homozygotes for the G allele was 2.02 [95% confidence interval (CI) = 1.16–3.52]. However, the risk-increasing effect was smaller for −1385 G than for APOEε4 (Table 2). Four categories were defined by the presence (+) or absence (−) of a ε4 or GG genotype. The GG genotype alone showed an increased risk (95% CI: 1.81–7.69), and OR for APOEε4 and the GG genotype was 20.5 (95% CI: 6.88–60.9).

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in promoter of FGF1

Group	Genotype (frequency)				Allele (frequency)	
n	AA	GA	GG	AA + GA	G	A
LOAD (100)	6 (0.06)	38 (0.38)	56 (0.56)*	44 (0.44)**	150 (0.75)	50 (0.25)***
Control (106)	14 (0.13)	51 (0.48)	41 (0.39)	65 (0.61)	133 (0.63)	79 (0.37)

LOAD, late-onset AD.
* $p < 0.03$.
** $p < 0.02$.
*** $p < 0.01$.

Table 2
Relative risk for interaction between APOEε4 and −1385 GG

		LOAD cases	Controls	Odds ratio	95% CI
	−1385 G/A				
	non-GG	44	65	Reference	
	GG	56	41	2.02	1.16–3.52
APOEε4					
−		52	90	Reference	
−		48	16	5.19	2.68–10.1
APOEε4	−1385 GG				
−	−	17	58	Reference	
−	−	35	32	3.73	1.81–7.69
−	−	18	11	5.58	2.21–14.1
−	−	30	5	20.5	6.88–60.9

APOEε4 (−), one or two copies of ε4; APOEε4 (−), no copies of ε4, 95% CI, confidence interval at 95% level.

to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

We have evaluated definite LOAD as a relatively homogeneous case group. Our preliminary data suggest that the FGF1 gene, or a nearby gene, is an additional risk factor, independent of the APOE gene. Association studies often produce conflicting results. There are three possible reasons. First, this might be due to a type I statistical error, where there is a weak association between the polymorphism and the disease. Second, it might arise from the difference in genetic background between the American, French, Asian, and Japanese populations. In some studies, the AD group was made up of a mixture of familial and sporadic patients. We therefore tried to choose homogeneous subjects (autopsy-confirmed and late-onset AD) as much as possible. A third possibility could be linkage disequilibrium with other causative polymorphisms.

Patients with the GG genotype in this study had a higher risk of AD than those with the A allele. This indicates that the GG genotype in the promoter may influence the expression of FGF1 and could be involved in the selective vulnerability of neurons in AD. The results of this study support the hypothesis that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [11,6,12]. This hypothesis should be further examined by functional analysis of FGF1 polymorphisms.

Acknowledgments

We are most grateful to all participants in the study. We thank Drs. Masaki Imagawa, Hideki Yamamoto, Hirotaka Tanabe, Yasuhiro Nonomura, Hiroshi Yoneda, Tsuyoshi Nishimura, Toshiaki Sakai, and Masatoshi Takeda for their help in data collection. We are indebted to Dr. Wendy Gray for revising the manuscript. This work was supported by a grant from the Japanese Millennium Project.

References

[1] C.H. Kawas, R. Katzman, The epidemiology of dementia and Alzheimer disease, in: R.D. Terry, R. Katzman, K.L. Bick, S.S. Sisodia (Eds.), Alzheimer disease, second ed., Lippincott Williams & Wilkins, Philadelphia, 1999, pp. 95–116.
[2] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol. Rev. 81 (2001) 741–766.
[3] G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E.M. Stadlan, Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease, Neurology 34 (1984) 939–944.
[4] F.P. Eckenstein, Fibroblast growth factors in the nervous system, J. Neurobiol. 25 (1994) 1467–1480.
[5] Z. Guo, M. Mattson, Neurotrophic factors protect cortical synaptic terminals against amyloid-and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function, Cereb. Cortex 10 (2000) 50–57.
[6] V. Thomas, E. Masliah, Evidence for neuroprotective effects of acidic fibroblast growth factor in Alzheimer disease, J. Neuropathol. Exp. Neurol. 58 (1999) 296–306.
[7] I.P. Everall, G. Trillo-Pazos, C. Bell, M. Mallory, V. Sanders, E. Masliah, Amelioration of neurotoxic effects of HIV envelope protein gp120 by fibroblast growth factor: a strategy for neuroprotection, J. Neuropathol. Exp. Neurol. 60 (2001) 293–301.

[8] I. Tooyama, H. Akiyama, P.L. McGeer, Y. Hara, O. Yasuhara, H. Kimura, Acidic fibroblast growth factor-like immunoreactivity in brain of Alzheimer patients, Neurosci. Lett. 121 (1991) 155–158.
[9] H. Kimura, I. Tooyama, P.L. McGeer, Acidic FGF expression in the surroundings of senile plaques, Tohoku J. Exp. Med. 174 (1994) 279–293.
[10] S. Ueno, J. Ito, Y. Nagayasu, T. Furukawa, S. Yokoyama, An acidic fibroblast growth factor-like factor secreted into the brain cell culture medium upregulates APOE synthesis, HDL secretion and cholesterol metabolism in rat astrocytes, Biochim. Biophys. Acta 1589 (2002) 261–272.
[11] T. Tada, J. Ito, M. Asai, S. Yokoyama, Fibroblast growth factor 1 is produced prior to apolipoprotein E in the astrocytes after cryo-injury of mouse brain, Neurochem. Int. 45 (2004) 23–30.
[12] V. Thomas, F. Licastro, E. Masliah, Locally reduced levels of acidic FGF lead to decreased expression of 28-kDa calbindin and contribute to the selective vulnerability of the neurons in the entorhinal cortex in Alzheimer's disease, Neuropathology 21 (2001) 203–211.
[13] M.F. Folstein, S.E. Folstein, P.R. McHugh, Mini-mental state, a practical method for grading the cognitive state of patients for the clinician, J. Psychiatr. Res. 12 (1975) 189–198.
[14] H. Akatsu, M. Takahashi, N. Matsukawa, Y. Ishikawa, N. Kondo, T. Sato, H. Nakazawa, T. Yamada, H. Okuda, T. Yamamoto, K. Kosaka, Subtype analysis of neuropathologically diagnosed patients in a Japanese geriatric hospital, J. Neurol. Sci. 196 (2002) 63–69.
[15] M. Matsubara, H. Yamagata, K. Kamino, T. Nomura, K. Kohara, I. Kondo, T. Miki, Genetic association between Alzheimer disease and the alpha-synuclein gene, Dement. Geriatr. Cogn. Disord. 12 (2001) 106–109.
[16] J. Sambrook, E.F. Fritsch, T. Maniatis, in: Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, New York, 1989, pp. 9–14.
[17] T. Yoneyama, H. Kasuya, H. Onda, H. Akagawa, N. Jinnai, T. Nakajima, T. Hori, I. Inoue, Association of positional and functional candidate genes FGF1, FBN2, and LOX on 5q31 with intracranial aneurysm, J. Hum. Genet 48 (2003) 309–314.
[18] R.L. Myers, R.A. Payson, M.A. Chotani, L.L. Deaven, J.M. Chiu, Gene structure and differential expression of acidic fibroblast growth factor mRNA: identification and distribution of four different transcripts, Oncogene 8 (1993) 341–349.
[19] R.A. Payson, M.A. Chotani, I.M. Chiu, Regulation of a promoter of the fibroblast growth factor 1 gene in prostate and breast cancer cells, J. Steroid Biochem. Mol. Biol. 66 (1998) 93–103.
[20] I.M. Chiu, K. Touhalisky, C. Baran, Multiple controlling mechanisms of FGF1 gene expression through multiple tissue-specific promoters, Prog. Nucleic Acid Res. Mol. Biol. 70 (2001) 155–174.

PSYCHOGERIATRICS 2004; 4: 24–32

ORIGINAL ARTICLE

Increased incidence of dementia with Lewy bodies in patients carrying the ε4-allele of apolipoprotein E

Hiroyasu AKATSU,[1] Kouzin KAMINO,[2] Hidehisa YAMAGATA,[3] Daisuke ISOJIMA,[1] Ikuko KONDO,[3] Takayuki YAMAMOTO,[1] Tomoyuki KIDA,[2] Masatoshi TAKEDA,[2] Tetsuro MIKI[4] and Kenji KOSAKA[1]

[1]Choju Medical Institute, Fukushimura Hospital, Toyohashi; [2]Division of Psychiatry and Behavioral Proteomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, Osaka; [3]Department of Medical Genetics, and [4]Geriatric Medicine Ehime University School of Medicine, Ehime, Japan

Correspondence: Dr Hiroyasu Akatsu, Choju Medical Institute, Fukushimura Hospital, 19-14, Aza-Yamanaka, Noyori, Toyohashi 441-8124, Japan. Email: akatu@chojuken.net

Received 7 July 2004; accepted 17 August 2004.

Key words: Alzheimer's disease, apolipoprotein E, cerebral amyloid angiopathy, dementia with Lewy bodies, limbic neurofibrillary tangle dementia, vascular dementia.

Abstract

Background: The apolipoprotein ε4 (APOE4) allele is a risk factor for Alzheimer's disease, but it remains undetermined whether this allele is related to the pathological development of neurofibrillary tangles (NFT) and the formation of Lewy bodies.

Methods: In the present study, we examined the relationship between these changes and the APOE4 allele in 255 consecutive neuropathologically diagnosed cases. APOE genotyping was carried out by the polymerase chain reaction–restriction fragment length polymorphism method.

Results: Nearly all our cases of dementia with Lewy bodies (DLB) showed the common form, having numerous senile plaques in the cerebral cortex and NFT in the parahippocampal and hippocampal regions and were also associated with the APOE4 allele. Limbic neurofibrillary tangle dementia (LNTD), characterized by the presence of NFT in limbic areas as well as the absence of senile plaques, did not appear to be associated with the APOE4 allele.

Conclusions: The APOE4 allele is a risk factor for DLB as well as Alzheimer's disease and cerebral amyloid angiopathy, but not for LNTD.

INTRODUCTION

Apolipoprotein E (APOE) is one of the major components of circulating lipoproteins and participates in the regulation of lipid metabolism. It exists as E2, E3, and E4 isoforms, which are encoded by the APOE2, APOE3 and APOE4 alleles of APOE, respectively.[1] Since it was first noted that the APOE4 allele is a risk factor for Alzheimer's disease (AD) and that APOE4 interacts with β-amyloid (Aβ),[2–4] APOE has been a focus for research on the etiopathology of neurodegenerative diseases, especially AD, from the standpoint of its role in lipid metabolism in the brain, as well as in Aβ metabolism. The APOE genotype has also been analyzed with respect to its association with frontotemporal dementia (FTD),[5–7] dementia with Lewy bodies (DLB),[8–10] the neurofibrillary tangle (NFT) predominant form of senile dementia,[11] and progressive supranuclear palsy (PSP).[12] However, there has been no study which has examined the pathological changes in terms of the relationship between the APOE4 allele and the production of Lewy bodies and tau phosphorylation.

The association between APOE and Aβ deposition in the AD brain remains controversial. Using an animal model, interesting evidence was obtained showing that APOE directly interacts with Aβ.[13] However, from the viewpoint of cholesterol metabolism, it is plausible that a risk posed by one of the APOE genotypes could be balanced by positive effects in normal membrane repair, since human APOE3-expressing astrocytes from human APOE3 knock-in mice can supply cholesterol to neurons to a greater extent than APOE4-expressing astrocytes.[14] As for the morphology of Aβ deposits, there are two forms; senile plaques (SP) and cerebral amyloid angiopathy (CAA). CAA is characterized by the deposition of Aβ

an cortical and leptomeningeal vessel walls, and this Aβ is thought to originate from smooth muscle cells.[15,16] On the other hand, another study showed that in AD, Aβ accumulates in periarterial interstitial fluid drainage pathways of the brain.[17] In addition, APOE2 and APOE3 isoforms prevent blood-to-brain transport of Aβ,[18] suggesting that APOE4 enters brain microvessels and parenchyma as a stable complex with soluble Aβ, reduces peptide degradation and might predispose to cerebrovascular damage, and possibly enhance amyloid formation under pathological conditions.

In the present report, to examine the risk that APOE4 might pose in the development of neuropathological changes, we analyzed APOE genotypes in Fukushimura Brain Bank (FBB) samples examined neuropathologically for evidence of AD, DLB and vascular dementia (VD).[19] Based on statistical analysis, we reported relationships between APOE genotypes and the major forms of dementia.

SUBJECTS AND METHODS

Patients

The 255 cases examined in the present study were composed of patients hospitalized in Fukushimura Hospital, Toyohashi, Japan. All of these patients were cognitively evaluated by neuropsychological testing, using such tests as the mini-mental state examination (MMSE),[20] and Hasegawa's dementia scale (HDS)[21] or the HDS revised version (HDS-R),[22] which is commonly utilized in Japan. We also recorded interviews employing a comprehensive questionnaire covering psychological and medical symptoms, chronic conditions, treatment, and activities of daily living. Autopsies were carried out at Fukushimura Hospital, from October 1990,[19] and APOE genotyping was performed using DNA samples extracted from dissected brain tissues obtained between January 1993 and July 2002, after obtaining the agreement of the patients' guardians for use of these tissues for the purpose of diagnosis, research and genetic analysis. The present study was approved by the ethics committee of Fukushimura Hospital. The patients consisted of 122 men and 133 women, with a mean ± SD age at death of 82.3 years ±8.5, range 44–102 years.

To obtain non-demented controls, elderly individuals were recruited in Suita City, Osaka, Japan, and evaluated by a questionnaire that included an inquiry *into past and present illnesses. Written informed* consent was obtained from each individual, according to a protocol approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine, Osaka, Japan. These population-based non-demented controls (PBC) consisted of 174 men and 213 women, with a mean ± SD age at blood drawing of 75.3 years ±5.0, range 63–92 years.

Autopsy and sampling of brain tissues

The 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 hemisphere showing abnormal findings by computed tomography scanning, or from the left hemisphere when no difference between the left and the right was found, and fixed in 4% paraformaldehyde (PFA) as a hemisphere block. The other hemisphere was divided into several regions. Some samples of lesions were frozen for further analyzes and stored at −80°C, while other areas were removed and 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. The specimens were embedded in paraffin and processed into 5 μm sections for conventional histological and immunohistochemical examination.

Neuropathological diagnostic criteria

Specimens were stained using hematoxylin-eosin and Kluver-Barerra staining methods. Methenamine silver (MS) staining was used to detect SP, CAA and NFT.[23] Ubiquitin, α-synuclein, Aβ and tau-immunostaining methods were also used when necessary. When samples were positively stained by MS staining, sections were also subjected to an immunohistochemical assay for detection of CAA using monoclonal anti-Aβ 1–40 and 1–42 antibodies (:BL, Fujioka, Japan) at a dilution of 1:1000 and a standard ABC method. Using MS and CR staining and we diagnosed diffuse and widespread CAA affecting the entire cerebral area. The pathological diagnosis of AD was carried out in accordance with the Consortium to Establish a Registry for Alzheimer Disease (CERAD) criteria guidelines.

in addition to scoring according to CERAD criteria, SP and NFT as AD pathology were quantified, as described by Molsa *et al.*[24] Sections from the midfrontal, midtemporal, and angular gyri, as well as from the CA1 area of the hippocampus and from the entorhinal cortex were scanned under a light microscope (10× objective), and the numbers of SP and NFT per field (area 0.92 mm^2) were estimated. In the present report, we separated the AD group into two subgroups; early-onset AD (EOAD) with an onset before 65 years, and late-onset AD (LOAD) with onset after 65 years.

In the past, we identified a new group characterized by NFT without SP, and termed this condition limbic neurofibrillary tangle dementia (LNTD), which is identical to senile dementia of the NFT type[25] and a tangle-predominant form of senile dementia.[11] We have previously reported the diagnostic criteria for LNTD.[19,25–27] For VD, we used criteria presented at the NINDA-AIREN International Workshop.[28] Mixed dementia (MD) can be independently diagnosed as either AD alone or VD alone based on clinicopathological findings, and is considered a combination of the two. In the present study, we placed it in a separate category. Diagnosis of Parkinson's disease (PD) was carried out according to criteria proposed by Gibb and Lees[29] and Calne *et al.*[30]

Clinical neuropathological diagnoses of DLB were made based on the DLB guidelines[31] and Kosaka's classification system.[32] We classified DLB into three groups according to Lewy bodies distribution as Table 1. in the brain stem type, Lewy bodies are located only in the brain stem, that is identical PD. In the limbic type, there are many Lewy bodies in the *brain stem and diencephlon, but fewer in the cerebral* cortex. in the neocortical type, numerous Lewy bodies are distributed both in the brain stem and diencephalon as well as in the cerebral cortex and basal ganglia. All of these DLB are divided into two forms: a pure form and a common form. With the common form, numerous SP can be found in the cerebral cortex and, to a greater or lesser extent, also NFT can be found in the parahippocampal and hippocampal legions. But, it is not enough to diagnose as AD. On the other hand, the pure form has only a few senile changes or none at all.

All of our autopsy samples, we classified as control brain that there is no pathological finding only with physiological changes.

Apolipoprotein E subtyping

DNA of autopsied cases was extracted from brain tissues by the phenol-chloroform method. The peripheral blood of the elderly in the PBC group was collected in tubes containing EDTA, and DNA was extracted using a QIAamp DNA Blood Kit (Qiagen, Valencia, CA) and stored at 4°C. APOE genotyping was carried out by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method, according to a procedure reported by Wenham *et al.*[33]

Statistical analysis

Statistical analysis was carried out with both the χ^2-test with Yates's correction and Fisher's exact test using 2 × 2 tables. A difference was considered significant when the *P*-value was less than 0.05.

Table 1 Distribution of apolipoprotein E (APOE) subtypes among dementia with Lewy bodies (DLB) subtypes compared with normal aging and population-based control (PBC) groups

	Brain stem (%)	Limbic (%)	Neocortical* (%)	PBC (%)
APOE genotype				
2/2	0	0	0	1 (0.3)
2/3	0	0	0	32 (8.2)
2/4	0	0	0	3 (0.8)
3/3	6 (67)	7 (64)	7 (64)	293 (75.7)
3/4	3 (33)	4 (36)	3 (27)	55 (14.2)
4/4	0	0	1 (9)	3 (0.8)
Total	9 (100)	11 (100)	11 (100)	387 (100)
APOE allele				
E2	0	0	0	37 (4.8)
E3	15 (83)	18 (82)	17 (77)*	673 (87.0)
E4	3 (17)	4 (18)	5 (23)*	64 (8.2)

*DLB alleles 3 and 4 compared to PBC, $P < 0.05$. Percentages are the frequencies of allele subtypes in each type of DLB.

RESULTS

Frequencies of neuropathological findings

The frequencies and mean ages at death of the neuropathologically diagnosed subgroups are summarized in Table 2. With the FBB samples, the main neuropathological disorders were cerebrovascular (cerebral infarcts and hemorrhages with or without dementia; 44%), AD (35%) and DLB (12%). Two types of diagnostic changes were noted in 38 cases, and three types were observed in one case (AD pathology, amyloid angiopathy and infarction). Twenty-four patients (9%) were diagnosed with disorders such as cerebral arteriosclerosis, NPH or subdural hemorrhage. Female cases of AD were more frequent than male cases, but no gender bias was noted in other disorders. Percentages of the main neuropathological diagnoses were similar to those of our previous report.[19]

Frequencies of apolipoprotein E alleles and genotypes

Since only 20 (8%) of the FBB samples showed signs of physiological aging alone, we used a population-based non-demented group of elderly subjects (PBC) as a reference control in comparing alleles and genotype frequencies of the APOE gene (Table 3). The genotype distribution of the reference control was similar to that in a previous report.[34] As the population advanced in age, the frequency of the APOE2 allele increased and that of the APOE4 decreased, although the difference between the seventh and the ninth decades was not significant. It was noted that the APOE2 allele frequency in FBB control brain was similar to that of the PBC group.

The FBB samples, as a whole, had a higher frequency of the APOE4 allele compared to the PBC samples ($P < 0.01$) (Table 3). The FBB group was *significantly different from the PBC group in both* APOE genotype and allele frequencies ($P < 0.01$), and this difference was evident in individuals over 70 years ($P = 0.002$ for the group aged 70–79 years; $P < 0.001$ for that over 80 years) (Table 3). And, the frequencies of APOE2 alleles were not enough, but the APOE2 frequency of FBB group decreased in older age against in that of PBC group. On the other hand, the frequencies of APOE4 in the FBB group were decreased in the same manner as in the PBC group.

Analysis of apolipoprotein E genotypes in the main neurological groups

Distributions of APOE genotypes within the main pathological disorders are summarized in Table 4. The frequencies of APOE genotypes were significantly different in the AD ($P < 0.0001$) and DLB groups ($P < 0.005$), compared to the PBC group. In addition, frequencies in the AD group were significantly different when compared with the physiological aging patients ($P < 0.02$).

Cerebrovascular disorders without CAA showed no association with the APOE genotype. Of six patients with Binswanger's disease, a subtype of vascular dementia, five had the 3/3 subtype and one had 2/4.

Apolipoprotein E analysis of amyloid-β and tangle diseases

Apolipoprotein E genotypes of AD and LNTD are summarized in Table 5. EOAD and LOAD was linked tightly

Table 2 Summary of the main neuropathological subgroup diagnoses[a]

	Men (%)	Women (%)	Total (%)	Mean ± SD age at death (years)
FBB samples				
AD	36 (30)	54 (41)	90 (35)	83.5 ± 7.62
DLB	14 (11)	17 (13)	31 (12)	80.0 ± 9.46
VD/CI	56 (46)	57 (43)	113 (44)	82.2 ± 7.93
LNTD	2 (2)	2 (2)	4 (2)	86.0 ± 5.72
Control brain	10 (8)	10 (8)	20 (8)	86.8 ± 5.50
Total	122	133	255	82.3 ± 8.49
PBC samples	174	213	387	75.3 ± 5.0[b]

[a]Thirty-eight patients had two diagnoses and one had three. Therefore, the total subgroup percentages were over 100%. Each subgroup percentage was determined from the ratio of the number of patients with a specific diagnosis to the total patient number. Twenty-four patients were diagnosed with other neuropathological diseases (not shown). [b]Age at time blood was drawn. AD, Alzheimer's disease; CI, cerebral infarct; DLB, dementia with Lewy bodies; FBB, Fukushimura Brain Bank; LNTD, limbic neurofibrillary tangle dementia; PBC, population-based control; VD, vascular dementia.

Table 3 Distribution of apolipoprotein E (APOE) genotypes within each Fukushimura Brain Bank (FBB) (upper) and population-based control (PBC) (lower) group[a]

	Age (years)			
	≤69 (%)	70–79 (%)	80≤ (%)	Total (%)
APOE genotype				
2/2	0	0	0	0
	0	1 (0.3)	0	1 (0.2)
2/3	1 (4.8)	4 (6.2)	4 (2.4)	9 (3.5)
	1 (4.8)	23 (7.8)	8 (12.5)	32 (8.2)
2/4	1 (4.8)	2 (3.1)	1 (0.6)	4 (1.6)
	0	3 (1.0)	0	3 (0.8)
3/3	13 (61.9)	40 (61.5)	117 (69.2)	170 (66.7)
	15 (71.4)	230 (7.2)	48 (75.0)	293 (75.7)
3/4	4 (19.0)	16 (24.6)	42 (24.9)	62 (24.3)
	4 (19.0)	43 (14.3)	8 (12.5)	55 (14.2)
4/4	2 (10.5)	3 (4.6)	5 (3.0)	10 (3.9)
	1 (4.8)	2 (0.7)	0	3 (0.8)
Total	21	65	169	255
	21	302	64	387
APOE allele				
E2	2 (4.8)	6 (4.6)	5 (1.5)	13 (2.5)
	1 (2.4)	28 (4.6)	8 (6.3)	37 (4.8)
E3	31 (73.8)	100 (76.9)	280 (82.8)	411 (80.6)
	35 (83.3)	526 (87.1)	112 (87.4)	673 (87.0)
E4	9 (21.4)	24 (18.5)	53 (15.7)	86 (16.9)
	6 (14.3)	50 (8.3)	8 (6.3)	64 (8.2)

[a]Percentages are the frequencies of subtypes in each age group.

Table 4 Distribution of apolipoprotein E (APOE) subtypes according to the main neuropathological Fukushimura Brain Bank (FBB) findings compared with those of the population-based control (PBC) group[a]

	AD	DLB	VD/CI	Control brain	FBB total	PBC
APOE genotype						
2/2	0	0	0	0	0	1 (0.3)
2/3	1 (1)	0	6 (5)	2 (10)	9 (3.5)	32 (8.2)
2/4	1 (1)	0	2 (2)	0	4 (1.6)	3 (0.8)
3/3	44 (49)	20 (65)	85 (75)	14 (70)	170 (66.7)	293 (75.7)
3/4	37 (41)	10 (32)	19 (17)	4 (20)	62 (24.3)	55 (14.2)
4/4	7 (8)	1 (3)	1 (1)	0	10 (3.9)	3 (0.8)
Total	90 (100)	31 (100)	113 (100)	20 (100)	255 (100)	387 (100)
APOE allele						
E2	2 (1)	0	8 (4)	2 (5)	13 (2.5)	37 (4.8)
E3	126 (70)*	50 (81)**	195 (83)	34 (85)	411 (80.6)	673 (87.0)
E4	52 (29)*	12 (19)**	23 (10)	4 (10)	86 (16.9)	64 (8.2)

*AD alleles 3 and 4 compared to aging patients, $P < 0.02$ and PBC, $P < 0.001$. **Dementia with Lewy bodies alleles 3 and 4 compared to PBC, $P < 0.005$. [a]Percentages represent the frequency of each finding. AD, Alzheimer's disease; CI, cerebral infarct; DLB, dementia with Lewy bodies; VD, vascular dementia.

to the APOE4 allele in comparison with the PBC group ($P < 0.0001$). In addition, compared with our physiological aging samples, eight cases with diffuse and widespread CAA affecting the entire cerebral area showed the highest association (data not shown).

On the other hand, though the number was only four, LNTD, a kind of tauopathy, had no association with APOE4 allele (Table 5).

Apolipoprotein E analysis of dementia with Lewy bodies subtypes

The DLB group did not show as strong an association with APOE genotype as the AD group. A significant difference in the APOE allele frequencies in the DLB group was noted, however, when this group was compared with the PBC group ($P = 0.004$) (Table 4). According to the standardized criteria,[31] 31 DLB

Table 5 Distribution of apolipoprotein E (APOE) subtypes of patients with Aβ and/or NFT deposition diseases compared with FBB normals and PBC*

	LOAD (%)	EOAD (%)	LNTD (%)	PBC (%)
APOE genotype				
2/2	0	0	0	1 (0.3)
2/3	1 (1)	0	0	32 (8.2)
2/4	1 (1)	0	0	3 (0.8)
3/3	58 (50)	6 (43)	3 (75)	293 (75.7)
3/4	31 (62)	6 (43)	1 (25)	55 (14.2)
4/4	5 (6)	2 (14)	0	3 (0.8)
Total	76 (100)	14 (100)	4 (100)	387 (100)
APOE allele				
E2	2 (1)	0	0	37 (4.8)
E3	108 (71)	16 (64)	7 (88)	573 (67.0)
E4	42 (28)*	10 (36)**	1 (12)	64 (8.2)

*All APOE alleles in LOAD patients compared to aging patients, $P < 0.05$ and to Saka controls, $P < 0.001$. **All APOE alleles in EOAD patients compared to aging patients, $P < 0.05$ and to Saka controls, $P < 0.001$. †Percentages were calculated from the frequency of each subtype to the total number of samples of each disease. EOAD, early-onset of Alzheimer's disease; LNTD, limbic neurofibrillary tangle dementia; LOAD, late-onset of Alzheimer's disease; PBC, population-based control.

cases were classified into nine cases with the brain stem, 11 with the limbic and 11 with the neocortical types (Table 1). All DLB cases except for two with the brain stem type had the common form of DLB with AD pathology. The frequency of the APOE4 allele in the neocortical type of DLB was significantly higher than that in the PBC group ($P = 0.039$), and the same tendency was seen in both the brain stem (17%) and limbic (18%) types.

DISCUSSION

Since 1993, it has been known that having the APOE4 allele places an individual at increased risk for LOAD.[2,3] However, its frequency varies according to ethnic background,[35] such as among Caucasians and Japanese.[34] Evans *et al.*[36] reported that the frequency of the APOE4 allele is higher in black populations than among Caucasians, but this higher frequency is not associated with an increased risk of AD. Our results showed that the frequencies of the APOE alleles in the PBC group were similar to those of a Japanese population investigated in a previous study.[34] It seems reasonable to consider the samples used in the present study as representative of the Japanese elderly with respect to the frequencies of APOE genotypes.

It has been noted that the APOE4 allele, which promotes premature atherosclerosis, is significantly less frequent in centenarians than in controls.[37] The APOE2 allele, in contrast, has been positively associated with advancing age,[38] in our reference controls (PBC group), the ratio of the APOE2 allele increased with age and that of the APOE4 allele decreased (Table 3). However, an interesting and deceptively conflicting finding with regard to the APOE4 allele was that the ratio of the APOE4 allele at younger ages was higher than that of older people, even in the PBC group (Table 3). This was because the group of younger subjects might have included normal persons who might eventually develop AD at some future time. The APOE2 allele was seldom found in our FBB samples, and we were unable to detect any particular tendency. Although the number of normal aging FBB samples was limited, the APOE2-positive cases included only patients over 80 years of age. This supports the findings of a previous report.[38] The normal FBB samples showed the same tendency as the PBC with respect to the APOE4 allele. Because 35.2% of FBB samples revealed some form of AD pathology, the frequency of the APOE4 allele in the total FBB group was higher (16.9%) than in the normal group (Table 4). But even in our FBB group of which 35.2% showed AD pathology, the presence of the APOE4 allele might not only represent an AD risk factor, but might also influence longevity, as in the PBC and normal FBB groups (Table 3).

On the other hand, one cannot make comparisons related to the age at death of FBB patients and the age at blood drawing of PBC. The mean ± SD age at death of the patient group (82.3 years ±8.5) was obviously higher than that of the PBC group at blood drawing (75.3 years ±5.0). However, the allele and genotype frequencies of the PBC group could be considered as reference data on Japanese elderly since this group was population-based.

Therefore, allele and genotype frequencies of the patient group or subgroups differing by diagnosis could be compared to those of this non-demented control group.

With respect to dementia, the frequencies of APOE alleles in AD and DLB were significantly different from those of the PBC group (Table 4), and analysis of allele subtype frequencies in both the diseases showed interesting results.

Compared with our 20 control brains and PBC, percentages of the various subtypes in EOAD and

LOAD patients were very different. These differences have already been discussed in previous reports from 1993.[2,3] Among the patients who had CAA, the APOE4 allele tended to have a stronger correlation with CAA than with AD (data not shown) but this will be analyzed in detail at a future time.

The phosphorylated form of tau was more prominent in cases of familial and sporadic AD which were positive for the APOE4 allele and its amounts increased with the gene dose.[39] In an *in vitro* study, the authors reported that isoform-specific interactions between APOE and tau might be important in the regulation of intraneuronal tau metabolism in AD and could alter the rate of formation of paired helical filaments (PHF) and NFT.[40] In our study, we did not analyze correlations between the frequencies of APOE alleles and the quantity of PHF/NFT in AD or LNTD, but we did note that the APOE genotype was not a risk factor for LNTD (Table 5), which is a NFT-only dementia without significant numbers of either diffuse amyloid or neuritic plaques. This would be in agreement with Bancher *et al.* who stated that, although the APOE genotype is not a risk factor for LNTD, LNTD patients would have APOE4 alleles,[11] would be AD. We have only a few autopsied cases with common tauopathies such as PiD, PSP and corticobasal degeneration (CBD). Therefore, we could not statistically examine any correlation between tau phosphorylation and the APOE4 allele. But, according to our results on LNTD and PiD, APOE4 might not influence tau formation.

Dementia with Lewy bodies is the second most frequent neurodegenerative dementia, following AD. Among our FBB samples, 12% had changes characteristic of DLB. As a whole, our DLB group had a high frequency of APOE4 (Table 4) and compared with the PBC, the difference was statistically significant ($P < 0.01$). Using the previously established guidelines,[31] DLB samples were classified into a brain stem type (nine cases), a limbic type (11 cases) and a neocortical type (11 cases) (Table 1). Only the neocortical type showed a statistically significant relationship ($P < 0.05$) with the APOE genotype, but it should be recognized that the single 4/4 neocortical DLB sample would have a strong influence on the result. This case also had CAA changes. In a sample comparison, however, the frequencies of allele 4 in our normal aging group was 10% and in the PBC group, 8.2%, compared to 17% in the brain stem, 18% in the limbic and 23% in the neocortical type of DLB. Each group of DLB had a higher APOE4 allele frequency than the normal groups. In our previous examination of Yokohama City University samples,[8] 39% of those with neocortical DLB had the APOE4 allele. Another Japanese group reported that the frequencies of the APOE4 allele in AD and DLB were similar.[9] In addition, Wakabayashi *et al.* analyzed Lewy body pathology with respect to APOE alleles and concluded that when PD occurs in APOE4-positive individuals, these patients concomitantly develop cortical Lewy body pathology which in a proportion of cases results in limbic (transitional) or neocortical-type Lewy body disease.[10] We also found that the frequency of the APOE4 allele increased going from the brain stem type to the neocortical type. However, all of our limbic and neocortical DLB cases were of the common form. Among our six cases having the brain stem type with a 3/3 genotype, two had the pure form of DLB and four had the common form (Table 1). All three with the APOE-3/4 genotype had the common form. This tendency reflected AD pathology. In the report by Wakabayashi *et al.*[10] samples positive for the APOE4 allele had an increased Lewy body density, and the plaque density was also high. Lewy body disease without concomitant AD pathology (pure form) ($n = 12$) has also been analyzed and the APOE4 allele frequency was found not to be significantly increased.[41] In *in vitro* studies investigating α synuclein as a Lewy body constituent, its interaction with lipid vesicles was highly dependent on their phospholipid composition.[42,43] However, the participation of apolipoprotein in Lewy body formation is not yet clear. Further biochemical analyzes and epidemiological investigations of a sufficient number of pure form DLB samples are needed.

In conclusion, while it is known that the frequencies of APOE alleles in Japan are different from those of Western countries, we found that AD and DLB have a positive correlation with the APOE4 allele. From previous reports, APOE interacts with Aβ and plays a role in SP formation and CAA development. In the present study, APOE4 was confirmed to be a risk factor for AD. As for DLB, we mainly analyzed the common form with AD pathology. Therefore, further data are needed in order to determine whether the APOE4 might also be a risk factor for Lewy body development.

ACKNOWLEDGMENTS

We received support for the present study from the Grant of Research for the Future Program, Japan Society for the Promotion of Science (JSPS).

We thank the patients and their guardians for cooperating with our project, as well as the medical staff and attending physicians. We also thank Dr Yoshiyuki Kanai, Dr Kiyohide Kojima and Dr Morito Endoh for scientific discussion and advice.

We are grateful to Mr Yoshiaki Tani, Mr Norihiro Ogawa, Mr Kaoru Tanigawa and Mr Takeshi Kanesaka for their excellent technical assistance, and to Dr William Campbell and Ms Catherine Campbell for their help in editing this manuscript.

REFERENCES

1 Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988; 240: 622–630.
2 Strittmatter WJ, Saunders AM, Schmechel D *et al.* Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 1993; 90: 1977–1981.
3 Corder EH, Saunders AM, Strittmatter WJ *et al.* Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 1993; 261: 921–923.
4 Schmechel DE, Saunders AM, Strittmatter WJ *et al.* Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc Natl Acad Sci USA* 1993; 90: 9649–9653.
5 Ingelsson M, Fabre SF, Ullvs L *et al.* Increased risk for frontotemporal dementia through interaction between tau polymorphisms and apolipoprotein E epsilon4. *Neuroreport* 2001; 12: 905–909.
6 Kowalska A, Asada T, Arima K *et al.* Genetic analysis in patients with familial and sporadic frontotemporal dementia: two tau mutations in only familial cases and no association with apolipoprotein epsilon4. *Dement Geriatr Cogn Disord* 2001; 12: 387–392.
7 Verpillat P, Camuzat A, Hannequin D *et al.* Apolipoprotein E gene in frontotemporal dementia: an association study and meta-analysis. *Eur J Hum Genet* 2002; 10: 399–405.
8 Kawanishi C, Suzuki K, Odawara T *et al.* Neuropathological evaluation and apolipoprotein E gene polymorphism analysis in diffuse Lewy body disease. *J Neurol Sci* 1996; 136: 140–142.
9 Higuchi M, Tashiro M, Arai H *et al.* Glucose hypometabolism and neuropathological correlates in brains of dementia with Lewy bodies. *Exp Neurol* 2000; 162: 247–256.
10 Wakabayashi K, Kakita A, Hayashi S *et al.* Apolipoprotein E epsilon4 allele and progression of cortical Lewy body pathology in Parkinson's disease. *Acta Neuropathol (Berl)* 1998; 95: 450–454.
11 Bancher C, Egensperger R, Kosel S *et al.* Low prevalence of apolipoprotein E epsilon 4 allele in the neurofibrillary tangle predominant form of senile dementia. *Acta Neuropathol (Berl)* 1997; 94: 403–409.
12 Teubel Y, Josephs KA, Cookson N *et al.* APOE E4 is a determinant for Alzheimer type pathology in progressive supranuclear palsy. *Neurology* 2003; 60: 240–245.
13 Bales KR, Verina T, Dodel RC *et al.* Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nat Genet* 1997; 17: 263–264.
14 Gong JS, Kobayashi M, Hayashi H *et al.* Apolipoprotein E (ApoE) isoform-dependent lipid release from astrocytes prepared from human ApoE3 and ApoE4 'knock-in' mice. *J Biol Chem* 2002; 277: 29919–29926.
15 Wisniewski HM, Wegiel J, Kotula L. Review. David Oppenheimer Memorial Lecture 1995: some neuropathological aspects of Alzheimer's disease and its relevance to other disciplines. *Neuropathol Appl Neurobiol* 1996; 22: 3–11.
16 Wisniewski HM, Wegiel J. Beta-amyloid formation by myocytes of leptomeningeal vessels. *Acta Neuropathol (Berl)* 1994; 87: 233–241.
17 Weller RO, Massey A, Newman TA *et al.* Cerebral amyloid angiopathy: amyloid beta accumulates in putative interstitial fluid drainage pathways in Alzheimer's disease. *Am J Pathol* 1998; 153: 725–733.
18 Martel CL, Mackic JB, Matsubara E *et al.* Isoform-specific effects of apolipoproteins E2, E3, and E4 on cerebral capillary sequestration and blood–brain barrier transport of circulating Alzheimer's amyloid beta. *J Neurochem* 1997; 69: 1995–2004.
19 Akatsu H, Takahashi M, Matsukawa N *et al.* Subtype analysis of neuropathologically diagnosed patients in a Japanese geriatric hospital. *J Neurol Sci* 2002; 196: 53–60.
20 Folstein MF, Folstein SE, McHugh PR. 'Mini-mental state'. A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975; 12: 189–198.
21 Hasegawa K, Imoue K, Moriya K. An investigation of dementia rating scale for the elderly. *Seishin Igaku* 1974; 16: 968–969. (in Japanese).
22 Katoh S, Shimogaki H, Onodera A *et al.* Development of the revised version of Hasegawa's dementia scale (HDS-R). *Jpn J Geriat Psychiatry* 1991; 2: 1339–1347. (in Japanese).
23 Haga C, Ikeda K, Iwabuchi K *et al.* Methenamine-silver staining: a simple and sensitive staining method for senile plaques and neurofibrillary tangles. *Biotech Histochem* 1994; 69: 295–300.
24 Molsa PK, Paljarvi L, Rinne JO, Rinne UK, Sako E. Validity of clinical diagnosis in dementia: a prospective clinicopathological study. *J Neurol Neurosurg Psychiatry* 1985; 48: 1085–1090.
25 Kosaka K, Iseki E, Odawara T. Limbic neurofibrillary tangle dementia. *Brain Pathol* 1997; 7: 1114.
26 Kosaka K, Iseki E, Arai H. Recent advances in dementia research in Japan: Alzheimer-type dementia. *Psychiatry Clin Neurosci* 1999; 53: 1–10.
27 Kosaka K, Iseki E. Recent advances in dementia research in Japan: non-Alzheimer-type degenerative dementias. *Psychiatry Clin Neurosci* 1998; 52: 367–373.
28 Roman GC, Tatemichi TK, Erkinjuntti T *et al.* Vascular dementia: diagnostic criteria for research studies. Report of the NINDS-AIREN International Workshop. *Neurology* 1993; 43: 250–260.
29 Gibb WR, Lees AJ. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry* 1988; 51: 745–752.
30 Cairns DB, Snow BJ, Lee C. Criteria for diagnosing Parkinson's disease. *Ann Neurol* 1992; 32: S125–S127.
31 McKeith IG, Galasko D, Kosaka K *et al.* Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. *Neurology* 1996; 47: 1113–1124.

32 Kosaka K. Diffuse Lewy body disease in Japan. *J Neurol* 1990; 237: 197–204.
33 Wenham PR, Price WH, Blandell G. Apolipoprotein E genotyping by one-stage PCR. *Lancet* 1991; 337: 1158–1159.
34 Eto M, Watanabe K, Ishii K. A racial difference in apolipoprotein E allele frequencies between the Japanese and Caucasian populations. *Clin Genet* 1996; 50: 422–427.
35 Mayeux R, Stern Y, Ottman R *et al.* The apolipoprotein epsilon 4 allele in patients with Alzheimer's disease. *Ann Neurol* 1993; 34: 752–754.
36 Evans DA, Bennett DA, Wilson RS *et al.* Incidence of Alzheimer disease in a biracial urban community: relation to apolipoprotein E allele status. *Arch Neurol* 2003; 60: 185–189.
37 Schachter F, Faure-Delanef L, Guenot F *et al.* Genetic associations with human longevity at the APOE and ACE loci. *Nat Genet* 1994; 6: 29–32.
38 Ree IM, McDowell I, McMaster D *et al.* Apolipoprotein E alleles in nonagenarian subjects in the Belfast Elderly Longitudinal Free-living Aging Study (BELFAST). *Mech Ageing Dev* 2001; 122: 1367–1372.
39 Thaker U, McDonagh AM, Iwatsubo T *et al.* Tau load is associated with apolipoprotein E genotype and the amount of amyloid beta protein, Abeta40, in sporadic and familial Alzheimer's disease. *Neuropathol Appl Neurobiol* 2003; 29: 35–44.
40 Strittmatter WJ, Saunders AM, Goedert M *et al.* Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: implications for Alzheimer disease. *Proc Natl Acad Sci USA* 1994; 91: 11183–11186.
41 Nielsen AS, Ravid R, Kamphorst W, Jorgensen OS. Apolipoprotein E epsilon 4 in an autopsy series of various dementing disorders. *J Alzheimers Dis* 2000; 5: 119–125.
42 Perrin RJ, Woods WS, Clayton DF *et al.* Exposure to long chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. *J Biol Chem* 2001; 276: 41958–41962.
43 Zhu M, Fink AL. Lipid binding inhibits alpha-synuclein fibril formation. *J Biol Chem* 2003; 278: 16873–16877.

Identification of Hippocampus-Related Candidate Genes for Alzheimer's Disease

Keiko Taguchi, MD,[1] Hidehisa D. Yamagata, MD, PhD,[2] Wangtao Zhong, MD,[1] Kouzin Kamino, MD, PhD,[3] Hiroyasu Akatsu, MD, PhD,[4] Ryuji Hata, MD, PhD,[5] Takayuki Yamamoto, MD, PhD,[4] Kenji Kosaka, MD, PhD,[4] Masatoshi Takeda, MD, PhD,[3] Ikuko Kondo, MD, PhD,[2] and Tetsuro Miki, MD, PhD[1]

Alzheimer's disease (AD) is a complex multifactorial disease in which many genetic and environmental factors are involved. We performed an association study using 376 AD patients and 376 control subjects. We studied 35 single nucleotide polymorphisms in 35 genes that were significantly downregulated or upregulated only in the AD hippocampus compared with control and found that 9 single nucleotide polymorphisms were associated with AD. Our data indicated that single nucleotide polymorphisms could highly reflect differences in gene expression. Furthermore, an intronic polymorphism (+9943T/C) in *POU2F1* was most significantly associated with AD (p = 0.0007). Our results suggest that *POU2F1* is a candidate gene for AD.

Ann Neurol 2005;57:585–588

Alzheimer's disease (AD; MIM #104300) is a neurodegenerative disorder characterized by progressive memory impairment and multiple cognitive deficits in mid to late life.[1] Its pathological hallmarks consist of neuritic plaques and neurofibrillary tangles in the cerebral cortex, accompanied by neuronal loss.[2–4] These neuropathological findings are prominent in the temporal neocortex and hippocampus. To date, four genes have been established to be associated with AD phenotypes, including the amyloid precursor protein gene, apolipoprotein E (*ApoE*) gene, and presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes.[5] The majority of familial AD cases are associated with *PSEN1* mutations, and the majority of sporadic cases are related to *ApoE-ε4*.[6–8] It has become clear that genetic and environmental factors are involved in the pathophysiology of this disease, but it remains unclear how these factors combine and ultimately lead to the neurodegenerative process.[1,2]

Recent advances in molecular biological technology have demonstrated that single nucleotide polymorphisms (SNPs) are a valuable tool for investigating the genetic basis of disease. SNPs may be used in not only positional cloning studies, but also genome-wide association studies.[9] Previously, we reported significantly upregulated or downregulated gene expression in the AD hippocampus using a complementary DNA microarray.[10] The most upregulated gene proved to be calcineurin Aβ (*PPP3CB*). We made a list of the top 20 named genes upregulated or downregulated (Table 1). Because SNPs may themselves represent genetic variants that affect disease susceptibility or progression, evaluating variants in a disease-associated gene is of great importance to identify alleles responsible for disease susceptibility or progression.

Subjects and Methods

Subjects

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected using National Institute of Neurological and Communication Disorders-Alzheimer's Disease and Related Disorders Association criteria for definite or probable AD, and nondemented control subjects were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination.[3,11] Brain and blood samples were obtained with informed consent from the patients (or their guardians) in the Chubu, Kansai, and Ehime areas of Japan. A total of 376 unrelated AD patients had been diagnosed previously, and 376 control subjects (outpatients or healthy volunteers) were selected and matched for age and place of residence with each patient. The mean age ± SD at the time of this study was 78.2 ± 8.3 years for late-onset AD and 75.5 ± 4.9 years for control subjects. The female proportion was greater in the AD group (70.5%) than in the control group (54.7%). Genomic DNA was purified by standard procedures from lymphocytes, lymphoblastoid cell lines, or brain samples.[12]

We compared allele frequencies between sporadic late-onset AD and healthy control subjects. Because *ApoE-ε4* is a risk factor for AD, we stratified the population by ε4 carrier status. *ApoE* genotyping was performed as described previously. Allelic and genotypic distributions were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. p values less than 0.05 were considered significant. Odds ratios (ORs) were calculated with two-tailed p values and 95% confidence intervals (CIs). Bonferroni correction was applied to reduce type I error. The relation of genotypic factors and the effect of *ApoEε4* to AD was assessed with logistic regression analysis. Statistical analyses were performed with SPSS software version 11.0 (SPSS, Chicago, IL).

From the Departments of [1]Geriatric Medicine and [2]Medical Genetics, Ehime University School of Medicine, National University Corporation, Ehime; [3]Division of Psychiatry and Behavioral Proteomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, Suita; [4]Choju Medical Institute, Fukushimura Hospital, Toyohashi; and [5]Division of Functional Histology, Ehime University School of Medicine, National University Corporation, Ehime, Japan.

Received Nov 22, 2004, and in revised form Jan 13, 2005. Accepted for publication Jan 24, 2005.

Published online Mar 28, 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20433

Address correspondence to Dr Yamagata, Department of Medical Genetics, Ehime University School of Medicine, National University Corporation, Toon-shi, Ehime 791-0295, Japan.
E-mail: hideyama@m.ehime-u.ac.jp

Published by Wiley-Liss, Inc., through Wiley Subscription Services

Table 1. List of 17 (or 18) Genes of Top 20 Genes and SNPs Significantly Up-Regulated or Down-Regulated in Hippocampus from AD Brain but not Control Brain (Chromosomal Location)

Up-Regulated Genes	Down-Regulated Genes
PPP3CB (10q22): calcineurin A beta: rs12644	*HMMR* (5q34): hyaluronan-mediated motility receptor: rs299290
RANBP1 (22q11): RAN binding protein 1: hCV2613312	*LAMB1* (7q31): laminin, beta 1: rs2237685
GNA11 (19p13): guanine nucleotide binding protein 11: rs308064	*POU2F1* (1q24.2): POU domain, class 2, transcription factor 1: rs3407814
CSN1 (4q13): casein, alpha: rs2279526	*MYH8* (17p13): myosin, heavy polypeptide 8, skeletal muscle: rs2024076
FCER1G (1q23): Fc fragment of IgE, high affinity 1: rs11421	*TM4SF5* (17p13): transmembrane 4 superfamily member 1: rs3851
ART3 (4q21): ADP-ribosyltransferase 3: hCV450363	*ADORA2B* (17p12): adenosine A2b receptor: rs1076424
FGL2 (7q11): fibrinogen-like 2: rs2075761	*COL11A1* (1p21.1): collagen, type XI alpha: rs3753841
ZAP128 (14q24.3): peroxisomal long-chain acyl-CoA thioesterase: hCV11164654	*PDCD11* (10q24.33): human mRNA for KIAA0185 gene: rs2986014
CLCNKB (1p36): chloride channel, kidney, B: hCV1814709	*TGM4* (3p21): transglutaminase 4: rs1995641
MCM3AP (21q22): minichromosome maintenance 3-associated protein: rs3788252	*PCK2* (14q11.2): phosphoenolpyruvate carboxykinase 2: rs2071586
FACL4 (Xq23): fatty acid CoA ligase, long chain 4: rs1324805	*HSPC242* (22q12): Homo sapiens PAC clone DJ130H16: rs2072158
RPS15 (19p13.3): ribosomal protein S15: rs1847602	*LCK* (1p35): lymphocyte-specific protein tyrosine kinase: hCV1895446
GBP2 (1p22): guanylate binding protein 2, interferon-inducible: rs4656097 (hCV2431431)	*TNFRSF8* (1p36): tumor necrosis factor receptor superfamily, member 8: hCV9567
PHKG2 (16p11.2): phosphorylase kinase, gamma 2: hCV27530858	*DPYS* (8q22): dihydropyrimidinase: rs2246815
AVPR1A (12q14.2): arginine vasopressin receptor 1A: rs1042615	*EGR2* (10q21.3): early growth response 2: rs2297489
PSMB7 (9q33.3): proteasome subunit, beta-type 7: hCV3112402	*CD36* (7q21): CD36 antigen: rs1358337
EFEMP1 (2p16): EGF-containing fibulin-like extracellular matrix protein 1: rs1344733	*CAV2* (7q31): caveolin 2: rs2270189
	AKAP8 (19p13.12): A kinase anchor protein 95: hCV2596739

Genotyping

We selected 35 among 40 genes due to the available database; 18 genes were significantly downregulated and 17 genes were significantly upregulated in the AD hippocampus compared with control according to our previous report (see Table 1). We performed a genotype of one SNP in each of the 35 candidate genes. The selected SNPs met the following criteria. First, the polymorphism was confirmed in the Japanese population. Second, the minor allele frequency was between 0.1 and 0.5 according to common disease–common variant hypothesis.[13] Third, the variant may potentially influence gene expression (eg, promoter, exon, missense, and so forth). Intronic SNPs in the strong linkage disequilibrium block around the promoter region. Fourth, a TaqMan probe was available. Genotyping of SNPs was performed using the TaqMan-polymerase chain reaction method. Amplification was performed according to the manufacturer's protocol. The fluorescence intensity of the polymerase chain reaction products was measured using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA).

Results

Two (*CAV2, RANBP1*) of 35 SNPs were not polymorphic in our samples (data not shown). We analyzed the data for the remaining 33 SNPs. The distribution obtained for the patients and control subjects almost reached Hardy–Weinberg equilibrium.

Of 17 SNPs with downregulated gene expression tested, 5 (*POU2F1, MYH8, CD36, DPYS, COL11A1*) showed a significant association with AD. Of 16 SNPs with upregulated gene expression, 4 (*GNA11, FCER1G, MCM3AP, GBP2*) showed a significant association with AD. The genotypic and allelic distributions of each SNP in the patients and control subjects are shown in Table 2.

Among them, we found a strong association between the *POU2F1* +9943T/C polymorphism and AD (p = 0.0007; p = 0.023 after Bonferroni correction). The allelic frequency for *POU2F1* +9943T was 0.09 in control subjects and 0.15 in AD patients. After the logistic regression analysis, a recessive model provided the

Table 2. Genotype and Allele Numbers of Nine SNPs Significantly Associated with AD Risk

	Gene Name	rs number	Celera	Location		AD : Control (Genotype)	P value	AD : Control (allele)	P value	OR (95% CI) APOE4(−) Subjects
Down-regulated	*POU2F1*	rs3407814	hCV1622428	intron 2	9943T/C	TT/TC/CC = 11/90/275 : 2/65/309	0.0022**	T/C = 112/640 : 69/683	0.0007**	1.73 (1.11–2.69)
	MYH8	rs2024076	hCV2179107	intron 18	13583T/C	TT/TC/CC = 9/132/235 : 23/110/243	0.047*	T/C = 150/602 : 156/596	0.709	1.98 (0.78–5.00)
	CD36	rs1358337	hCV1803785	intron 3	12329A/G	GG/GA/AA = 41/132/180 : 51/182/143	0.021*	G/A = 240/512 : 284/468	0.017*	1.35 (0.79–2.29)
	DPYS	rs2246815	hCV9595729	intron 5	38687A/G	GG/GA/AA = 175/163/39 : 137/192/47	0.023*	G/A = 511/241 : 466/286	0.015*	1.45 (0.80–2.60)
	COL11A1	rs3753841	hCV2947954	exon 52	193817A/G	AA/AG/GG = 178/160/38 : 158/156/62	0.036*	A/G = 516/236 : 472/280	0.017*	1.62 (0.95–2.75)
Up-regulated	*GNA11*	rs308064	hCV3149365	intron 2	18527T/C	TT/TC/CC = 144/176/56 : 156/186/34	0.046*	T/C = 464/288 : 498/254	0.068	1.02 (0.70–1.46)
	FCER1G	rs11421	hCV1841966	exon 5	3825T/C	TT/TC/CC = 132/159/85 : 140/183/53	0.009**	T/C = 423/329 : 463/289	0.036*	1.05 (0.73–1.52)
	MCM3AP	rs3788252	hCV3270916	intron 25	43966T/C	TT/TC/CC = 18/144/214 : 31/115/230	0.026*	T/C = 180/572 : 177/575	0.856	1.19 (0.83–1.71)
	GBP2	rs4656097	hCV2431431	intron 6	6900T/A	TT/TA/AA = 17/155/204 : 22/122/232	0.041*	T/A = 189/563 : 166/586	0.162	1.14 (0.85–1.54)

*$P < 0.05$, **$P < 0.01$, OR: odds ratio, 95% CI: confidence interval.

best fit (p = 0.020; OR, 6.33; 95% CI, 1.33–30.0), but a dominant model could not be rejected (p = 0.070; OR, 1.42; 95% CI, 1.42–2.08). We then examined the TT genotype as a risk factor for AD, considering the *ApoE* status. To quantify possible interactions between *ApoE-ε4* and *POU2F1*, we analyzed the data with respect to various carrier status combinations, taking subjects who had neither *ApoE-ε4* nor *POU2F1* as a reference (Table 3). Four categories were defined by the presence (+) or absence (−) of an ε4 or TT genotype. As expected, *ApoE-ε4* conferred an increased risk for AD (OR, 5.09; 95% CI, 3.61–7.18). The TT genotype alone showed an increased risk (OR, 1.73; 95% CI, 1.11–2.69), and the OR for *ApoE-ε4* and the TT genotype was 6.08. As for the interaction between the ApoE-ε4 and *POU2F1-T* alleles for the risk for AD, logistic regression analysis did not indicate a significant effect (p = 0.30). The synergetic effect of TT allele in patients having *ApoE-ε4* was weak. The other eight gene SNPs did not show significant associations in the *ApoE-ε4* (−) subjects (see Table 2).

Discussion

In this study, we hypothesized that genes demonstrating significant differences in expression level between AD and control hippocampus might play a potential role as disease modifiers or disease susceptibility genes. To confirm this assumption, we performed an association study using these AD candidate genes. Consequently, we found 9 significant associations in 33 SNPs (genes). Compared with general association studies, the detection rate of positive SNPs (genes) in this study was markedly high. Our data indicated that SNPs could highly reflect difference in gene expression.

Previously, we reported a comparison of the gene expression in the hippocampus containing neurofibrillary tangle–associated lesions from an AD patient with that in the parietal cortex from the same patient, which lacked those lesions. Compared with control brain, the genes significantly upregulated or downregulated only in the AD brain were determined. The most upregulated gene proved to be calcineurin Aβ (*PPP3CB*), although its SNP showed no association (allele: p = 0.51; genotype: p = 0.81). Our analysis showed that 5 of 17 SNPs with downregulated gene expression (*POU2F1, MYH8, CD36, DPYS, COL11A1*) were associated with AD, and 4 of 16 SNPs with upregulated gene expression (*GNA11, FCER1G, MCM3AP, GBP2*) were associated with AD.

Among them, the POU domain, class 2, transcription factor 1 (POU2F1; also called Oct-1) showed the strongest association with AD. *POU2F1*, a member of the POU family transcription factors, is ubiquitously expressed in both the embryo and the adult.[14] *POU2F1* also takes a part in regulation of cell type–specific gene expression. It regulates some genes in the immune system including those encoding light and heavy chains of immunoglobulins, IL-2, IL-3, IL-5, IL-8, granulocyte/macrophage colony stimulating factor, and CD20.[15] Furthermore, an alternatively spliced variant of human *POU2F1* is only expressed in lymphoid tissues and brain.[15]

Our data suggest that *POU2F1* mediates immune and inflammatory responses in the AD brain. In fact, increasing evidence suggests that the immune system may play an important role in the degenerative process of AD.[16] The +9943T allele was significantly associated with AD in individuals lacking an ApoE-ε4 allele. Therefore, *POU2F1* (1q24.2) is an additional risk factor, synergistic with the *ApoE* gene. According to the SNPbrowser Version 2.0 (Applied Biosystems), strong linkage disequilibrium is shown around the *POU2F1* gene. Therefore, it is reasonable to think that +9943T/C polymorphism in intron 2 can contribute

Table 3. Relative Risks for Interaction Between APOEε4 and +9943T in POU2F1

		AD Cases	Controls	Odds Ratio	95%CI		AD Cases	Controls	Odds Ratio	95%CI
	9943T/C					APOEε4				
	non-TT	365	374	Reference	—	−	193	317	Reference	—
	TT	11	2	5.64	1.24–25.6	+	183	59	5.09	3.61–7.18
APOEε4	9943T									
−	−	145	266	Reference	—					
−	+	48	51	1.73	1.11–2.69					
+	−	130	43	5.55	3.72–8.27					
+	+	53	16	6.08	3.35–11.0					

APOEε4 (+), one or two copies of ε4; APOEε4 (−), no copies of ε4, 95% CI: confidence interval.

to promoter activity. +9943T/C may be the representative marker that influences gene expression. Our data suggest that these nine genes are susceptibility genes of sporadic AD. This should be examined further by functional analysis of the nine gene polymorphisms. Also, extensive investigations using different SNPs in the same genes, different populations, and a larger sample size are required to clarify the role of the nine gene polymorphisms.

This work was supported by grants from the Japanese Millennium Project (MIL 01413, M.T.), the Japan Society for the Promotion of Science and the Novartis Foundation for the Gerontological Research (H.D.Y.).

We are grateful to all participants in this study.

References

1. Kawas CH, Katzman R. The epidemiology of dementia and Alzheimer disease. In Terry RD, Katzman R, Bick KL, et al, eds. Alzheimer disease. Philadelphia: Lippincott Williams & Wilkins, 1999:95–116.
2. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001;81:741–766.
3. McKhann G, Drachman D, Folstein M, et al. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 1984;34:939–944.
4. Bird TD, Sumi SM, Nemens EJ, et al. Phenotypic heterogeneity in familial Alzheimer's disease: a study of 24 kindreds. Ann Neurol 1989;25:12–25.
5. Campion D, Flaman JM, Brice A, et al. Mutations of the presenilin gene in families with early-onset Alzheimer's disease. Hum Mol Genet 1995;4:2373–2377.
6. Strittmatter WJ, Saunders AM, Schmechel D, et al. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer's disease. Proc Natl Acad Sci U S A 1993;90:1977–1981.
7. van Duijn C, de Knijff P, Cruts M, et al. Apolipoprotein E4 allele in a population-based study of early-onset Alzheimer's disease. Nat Genet 1994;7:74–78.
8. Houlden H, Crook R, Backhovens H, et al. ApoE genotype is a risk factor in nonpresenilin early-onset Alzheimer's disease families. Am J Med Genet 1998;81:117–121.
9. Erichsen HC, Chanock SJ. SNPs in cancer research and treatment. Br J Cancer 2004;90:747–751.
10. Hata R, Masumura M, Akatsu H, et al. Up-regulation of calcineurin Abeta mRNA in the Alzheimer's disease brain: assessment by cDNA microarray. Biochem Biophys Res Commun 2001;284:310–316.
11. Folstein MF, Folstein SE, McHugh PR. Mini-Mental state: a practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–198.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989:9–14.
13. Doris PA. Hypertension genetics, single nucleotide polymorphisms, and the common disease: common variant hypothesis. Hypertension 2002;39:323–331.
14. Ryan AK, Rosenfeld MG. POU domain family values: flexibility, partnerships, and developmental codes. Genes Dev 1997;11:1207–1225.
15. Lechina NN, Krivega IV, Pankratova EV. Human Oct-1L isoform has tissue-specific expression pattern similar to Oct-2. Immunol Lett 2003;85:237–241.
16. Monsonego A, Weiner HL. Immunotherapeutic approaches to Alzheimer's disease. Science 2003;302:834–838.

Original Article

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

Shinji TAMAKI, Yasuyuki NAKAMURA*[1], Yasuharu TABARA*[2], Tomonori OKAMURA*[3], Yoshikuni KITA*[3], Takashi KADOWAKI*[3], Yasuyuki TSUJITA*[4], Minoru HORIE*[4], Tetsuro MIKI*[5], and Hirotsugu UESHIMA*[3]

We examined the interactions between lifestyle and polymorphisms of salt-sensitive genes and their effects on hypertension in a general Japanese sample (The Shigaraki Study). The study group consisted of 2,902 subjects who underwent a medical examination in 1999 in Shigaraki, a suburban area in Shiga. Among 1,647 subjects not receiving antihypertensive medication, in a combined analysis of angiotensinogen (AGT) and adducin (ADD1) polymorphisms, double homozygosity of 235Thr or 460Trp was not found to be associated with hypertension. A multiple logistic regression analysis showed that age (odds ratio [OR]: 1.07, 95% confidence interval [95% CI]: 1.06–1.08), body mass index (BMI) (OR: 1.18, 95% CI: 1.13–1.23), alcohol consumption (OR: 1.39, 95% CI: 1.16–1.66), family history of hypertension (OR: 1.67, 95% CI: 1.18–2.07), and combined AGT M235T Thr/Thr and ADD1 Trp/Trp polymorphisms (OR: 1.37, 95% CI: 1.03–1.82) were associated with hypertension. However, there was no interaction between eating salty food and combined AGT and ADD1 polymorphisms. Furthermore, eating salty food was not associated with hypertension in a multivariate analysis. Therefore, a combination of the AGT and ADD1 polymorphisms appears to be associated with hypertension. However, a simple questionnaire regarding salt intake was not sufficient to confirm the relationship between salt intake and hypertension and/or salt-sensitive genes. (*Hypertens Res* 2005; 28: 645–650)

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

From the Division of Cardiology, Department of Medicine, Kohka Public Hospital, Kohka, Japan; *[1]Cardiovascular Epidemiology, Kyoto Women's University, Kyoto, Japan; *[2]Division of EcoGenetics, Department of Environmental Health and Social Medicine and *[3]Department of Geriatric Medicine, Ehime University School of Medicine, Ehime, Japan; and *[4]Department of Health Science and *[5]Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu, Japan.

This study represents joint research with Ehime University. The study was supported in part by a contract from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid for Scientific Research on Priority Areas [C]: 12204039, Grant-in-Aid for Scientific Research [B]: 02454211, Grant-in-Aid for Scientific Research [C]: 06670414) and the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research [C]: 16590500).

Address for Reprints: Yasuyuki Nakamura, M.D., Ph.D., Cardiovascular Epidemiology, Department of Living and Welfare, Faculty of Home Economics, Kyoto Women's University, 35 Imakumano Kitahiyoshi-cho, Higashiyama-ku, Kyoto 605-8511, Japan. E-mail: nakamury@kyoto-wu.ac.jp

Received October 25, 2004; Accepted in revised form June 13, 2005.

Introduction

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

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

Methods

Study Population

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

Blood Pressure (BP) and Biochemical Examinations

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

Assessment of Lifestyle Factors

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

Genetic Analysis

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

Statistical Analysis

The Statistical Package for Social Science (SPSS ver. 11.0J; SPSS Japan, Tokyo, Japan) was used for the statistical analysis. Student's or Welch's *t*-test and the Wilcoxon rank sum test (*for alcohol consumption*) were used for comparisons of means between two categories. For comparisons of means among three or more categories, a one-way analysis of variance or the Kruskal-Wallis test (for alcohol consumption) was used according to the distributions. The χ^2 test was used to compare proportions. Age-adjusted prevalence was calculated directly. A multiple logistic regression analysis was used to clarify the contribution of each independent variable to hypertension. In this analysis, hypertension was regarded as a dependent variable, and each genotype and other factors were regarded as independent variables. The significance of the interaction of eating salty food with AGT and ADD1 genotypes was also tested using an interaction term in this model. All confidence intervals were estimated at the 95% level.

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

Risk characteristics	Men (638)				Women (1,009)			
	Met/Met	Met/Thr	Thr/Thr	*p*-value	Met/Met	Met/Thr	Thr/Thr	*p*-value
N (1,647)	29	168	441		40	303	666	
Age (years)	55.9±14.9	56.1±16.3	56.1±15.3	0.997	53.8±16.7	52.7±15.8	53.5±15.2	0.739
BMI (kg/m²)	22.4±3.7	*22.1±3.0*	*22.6±2.8*	*0.208*	*21.9±3.0*	*22.2±3.0*	*22.1±3.0*	*0.890*
sBP (mmHg)	128.0±16.9	130.6±16.4	130.2±17.5	0.760	125.0±17.3	123.2±18.5	125.2±19.9	0.324
dBP (mmHg)	77.0±11.3	78.5±12.2	78.3±11.6	0.819	76.0±10.2	73.3±11.1	74.6±11.8	0.194
Alcohol consumption (gou/day)	1.38	0.80	0.80	0.251	0.06	0.07	0.07	0.145
Eating salty food (%)	31.0	25.6	19.5	0.119	15.0	6.9	7.5	0.191

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

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

Risk characteristics	Men (638)				Women (1,009)			
	Gly/Gly	Gly/Trp	Trp/Trp	*p*-value	Gly/Gly	Gly/Trp	Trp/Trp	*p*-value
N (1,647)	123	305	210		201	497	311	
Age (years)	55.4±15.9	55.3±15.7	57.6±15.1	0.240	54.7±16.6	53.3±15.0	53.2±15.5	0.187
BMI (kg/m²)	22.3±2.5	22.5±3.0	22.5±3.0	0.673	21.6±2.8	22.3±3.0	22.1±3.0	0.031
sBP (mmHg)	*130.6±17.0*	*129.6±17.0*	*130.8±17.6*	*0.726*	*124.7±20.2*	*124.8±19.6*	*124.3±18.6*	*0.951*
dBP (mmHg)	78.3±11.8	78.0±11.6	78.6±12.0	0.836	72.8±12.1	75.0±11.6	74.0±11.1	0.061
Alcohol consumption (gou/day)	0.77	0.87	0.79	0.390	0.06	0.08	0.07	0.352
Eating salty food (%)	24.4	22.3	19.0	0.482	8.0	8.7	5.8	0.322

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

Results

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

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

Table 4 shows the multivariate adjusted odds ratios of combined AGT and ADD1 polymorphisms for hypertension when the data for men and women were combined. Multiple logistic regression analysis adjusting for age, BMI, alcohol consumption, eating salty food, family history of hypertension, number of cigarettes per day and sex showed that age, BMI, alcohol consumption, family history of hypertension, and combined AGT and ADD1 polymorphisms were associated with hypertension. However, there was no correlation between eating salty food and hypertension. In addition, there was no interaction between eating salty food and the AGT M235T Thr/Thr plus ADD1 Trp/Trp polymorphism ($p=0.829$).

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

Risk characteristics	Men (638)			Women (1,009)		
	Others	Thr/Thr and Trp/Trp	*p*-value	Others	Thr/Thr and Trp/Trp	*p*-value
N (1,647)	488	150		800	209	
Age (years)	55.3±15.8	58.6±14.6	0.026	53.4±15.5	52.7±15.4	0.549
BMI (kg/m²)	22.4±2.9	22.8±3.0	0.189	22.1±3.0	22.1±2.9	0.971
sBP (mmHg)	129.6±16.9	132.0±18.0	0.140	124.3±19.3	125.8±19.7	0.335
dBP (mmHg)	77.9±11.7	79.6±11.9	0.125	74.2±11.6	74.7±11.5	0.598
Alcohol consumption (gou/day)	0.83	0.81	0.723	0.07	0.07	0.144
Family history of hypertension (%)	24.4	18.0	0.104	29.9	30.6	0.834
Eating salty food (%)	22.7	18.0	0.217	8.1	5.7	0.248
Hypertension (%)	28.9	38.0	0.035	23.4	27.3	0.241
Hypertension (%)†	28.9	35.9	0.127	23.4	28.1	0.125

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

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

Risk characteristics	Odds ratio (95% CI)	*p*-values
AGT T/T and ADD1 T/T (both TT=1, others=0)	1.37 (1.03–1.82)	0.031
Age (years)	1.07 (1.06–1.08)	<0.001
BMI (kg/m²)	1.18 (1.13–1.23)	<0.001
Alcohol consumption (gou/day)	1.39 (1.16–1.66)	<0.001
Eating salty food (yes=1, no=0)	1.25 (0.88–1.77)	0.218
Family history of hypertension	1.57 (1.18–2.07)	0.002
Smoking (number of cigarettes/day)	1.00 (0.99–1.01)	0.481
Sex (men=0, women=1)	1.09 (0.81–1.47)	0.481

Abbreviations are listed in Tables 1 and 2.

Discussion

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

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

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

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

Acknowledgements

We received excellent technical assistance from the staff at Ehime University for the determination of the ADD1 gene polymorphism.

References

1. Kunz R, Kreutz R, Beige J, Distler A, Sharma AM: Association between the angiotensinogen 235T variant and essential hypertension in whites: a systematic review and methodological appraisal. *Hypertension* 1997; 30: 1331–1337.
2. Katsuya T, Ishikawa K, Sugimoto K, Rakugi H, Ogihara T: Salt sensitivity of Japanese from the viewpoint of gene polymorphism. *Hypertens Res* 2003; 26: 521–525.
3. Hunt SC, Celeijnse JM, Wu LL, Wilteman JC, Williams RR, Grobbee DE: Enhanced blood pressure response to mild sodium reduction in subjects with the 235T variant of the angiotensinogen gene. *Am J Hypertens* 1999; 12: 460–466.
4. Schorr U, Blaschke K, Beige J, Distler A, Sharma AM: Angiotensinogen M235T variant and salt sensitivity in young normotensive Caucasians. *J Hypertens* 1999; 17: 475–479.
5. Beeks E, Janssen RG, Kroon AA, *et al*: Association between the alpha-adducin Gly460Trp polymorphism and systolic blood pressure in familial combined hyperlipidemia. *Am J Hypertens* 2001; 14: 1185–1190.
6. Cusi D, Barlassina C, Azzani T, *et al*: Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. *Lancet* 1997; 18; 349: 1353–1357.
7. Tamaki S, Iwai N, Tsujita Y, Nakamura Y, Kinoshita M: Polymorphism of alpha-adducin in Japanese patients with essential hypertension. *Hypertens Res* 1998; 21: 29–32.
8. Yamagishi K, Iso H, Tanigawa T, Cui R, Kudo M, Shimamoto T: Alpha-adducin G460W polymorphism, urinary sodium excretion, and blood pressure in community-based samples. *Am J Hypertens* 2004; 17: 385–390.
9. Nakamura Y, Amamoto K, Tamaki S, *et al*: Genetic variation in aldehyde dehydrogenase 2 and the effect of alcohol consumption on cholesterol levels. *Atherosclerosis* 2002; 164: 171–177.
10. Amamoto K, Okamura T, Tamaki S, *et al*: Epidemiologic study of the association of low-K_m mitochondrial acetaldehyde dehydrogenase genotypes with blood pressure level and the prevalence of hypertension in a general population *Hypertens Res* 2002; 25: 857–864.
11. Tamaki S, Nakamura Y, Tsujita Y, *et al*: Polymorphism of the angiotensin converting enzyme gene and blood pressure in a Japanese general population (the Shigaraki Study). *Hypertens Res* 2002; 25: 843–848.
12. Bennett CL, Schrader AP, Morris BJ: Cross-sectional analysis of Met235→Thr variant of angiotensinogen gene in severe, Familial Hypertension. *Biochem Biophys Res Commun* 1993; 197: 833–839.
13. Sugimoto K, Hozawa A, Katsuya T, *et al*: alpha-Adducin Gly460Trp polymorphism is associated with low renin hypertension in younger subjects in the Ohsasma study. *J Hypertens* 2002; 20: 1779–1784.
14. Luft FC, Miller JZ, Weinberger MH, Christian JC, Skrabal F: Genetic influences on the response to dietary salt reduction, acute salt loading, or salt depletion in humans. *J Cardiovasc Pharmacol* 1988; 12: S49–S55.
15. Miller JZ, Weinberger MH, Christian JC, Daugherty SA: Familial resemblance in the blood pressure response to sodium restriction. *Am J Epidemiol* 1987; 126: 822–830.
16. Hunt SC, Cook NR, Oberman A, *et al*: Angiotensinogen genotype, sodium reduction, weight loss, and prevention of hypertension. Trials of hypertension prevention, Phase II. *Hypertension* 1998; 32: 393–401.
17. Beeks E, Kessels AG, Kroon AA, Van der Kuuw MM, De Leeuw PW: Genetic predisposition to salt-sensitivity: a systematic review. *J Hypertens* 2004; 22: 1243–1249.
18. Province MA, Arnett DK, Hunt SC, *et al*, for the HyperGEN Group: Association between the alpha-adducin gene and hypertension in the HyperGen Study. *Am J Hypertens* 2000; 13: 710–718.
19. Larson N, Hutchinson R, Boerwinkle E: Lack of association of 3 functional gene variants with hypertension in African Americans. *Hypertension* 2000; 35: 1297–1300.
20. Matsubara M, Sato T, Nishimura T, *et al*: *CYP11B2* polymorphisms and home blood pressure in a population-based cohort in Japanese: the Ohsasma study. *Hypertens Res* 2004; 27: 1–6.
21. Shioji K, Kokubo Y, Mannami T, *et al*: Association between hypertension and the alpha-adducin, beta1-adrenoreceptor, and G-protein beta3 subunit genes in the Japanese population: the Suita study. *Hypertens Res* 2004; 27: 31–37.
22. Tanaka T, Okamura T, Miura K, *et al*: A simple method to estimate populational 24-h urinary sodium and potassium excretion using a casual urine specimen. *J Hum Hypertens* 2002; 16: 97–103.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
BBRC
Biochemical and Biophysical Research Communications 335 (2005) 596–602
www.elsevier.com/locate/ybbrc

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

Haruhiko Osawa [a,*], Hiroshi Onuma [a], Masaaki Ochi [a], Akiko Murakami [a], Junko Yamauchi [a], Tomomi Takasuka [a], Fumiko Tanabe [a], Ikki Shimizu [b], Kenichi Kato [b], Wataru Nishida [a], Kazuya Yamada [c,d], Yasuharu Tabara [c], Masaki Yasukawa [f], Yasuhisa Fujii [b], Jun Ohashi [g], Tetsuro Miki [h], Hideichi Makino [a,*]

[a] Department of Laboratory Medicine, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan
[b] Ehime Prefectural Hospital, Japan
[c] Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Japan
[d] CREST, Japan Science and Technology Agency, Japan
[e] Department of Medical Genomics, Ehime University School of Medicine, Japan
[f] First Department of Internal Medicine, Ehime University School of Medicine, Japan
[g] Department of Human Genetics, School of International Health, Graduate School of Medicine, The University of Tokyo, Japan
[h] Department of Geriatric Medicine, Ehime University School of Medicine, Japan

Received 6 July 2005

Abstract

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

Keywords: Resistin; Insulin resistance; SNP; Polymorphism; Promoter; mRNA; Monocyte; Adipocyte; Type 2 diabetes; Serum level

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

Resistin (resistance to insulin) is secreted from adipocytes and antagonizes insulin both in vitro and in vivo [7–14]. Serum levels of resistin are increased in obese diabetic mice and are reduced by PPARγ ligands, suggesting that levels of this molecule are correlated with whole body insulin resistance [7]. Transgenic mice that overexpress *resistin* in the liver have high serum resistin levels and are insulin-resistant [11]. *Resistin* (−/−) mice show lower fasting blood glucose levels, which are increased by resistin injection [13]. A reduction in resistin gene expression by antisense oligonucleotides improves and enhancement by adenovirus encoding *resistin worsens insulin resistance [15,16]. These findings* suggest that higher serum resistin levels cause insulin resistance and diabetes in rodents whereas some other studies did not agree with this role of resistin [17].

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

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

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

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

Materials and methods

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

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

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

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

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

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

Table 1
Clinical characteristics of control and T2DM subjects

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

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

* Corresponding authors. Fax: +81 89 960 5627.
E-mail addresses: haruosawa@m.ehime-u.ac.jp (H. Osawa), hidemak@m.ehime-u.ac.jp (H. Makino).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.122