Drug Metab. Pharmacokin. 19 (5): 375–380 (2004).

Regular Article

Effect of Genetic Polymorphism of OATP-C (SLCO1B1) on Lipid-Lowering Response to HMG-CoA Reductase Inhibitors

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Summary: The effect of genetic polymorphism of human organic anion transporting polypeptide C (OATP-C) on the lipid-lowering response to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors was assessed.

A retrospective study was conducted on 66 patients who underwent treatment of hyperlipidemia with HMG-CoA reductase inhibitors in a municipal hospital in a community-based cohort of Ehime prefecture in the southern part of Japan. Plasma lipid concentrations before and after administration were analyzed in patients in relation to the 521T/C (Val-174→Ala) polymorphism in the OATP-C gene (TT: n = 44 (66.7%), TC: n = 20 (30.3%), CC: n = 0 (0.0%), undetermined: n = 2 (3.0%)). Total cholesterol level was significantly lowered after treatment with HMG-CoA reductase inhibitors in all patients ($p < 0.001$); moreover, subjects with the 521C allele showed an attenuated total-cholesterol-lowering effect compared with those homozygous for the 521T allele ($-22.3 \pm 8.7\%$ vs. $-16.5 \pm 10.5\%$, $p < 0.05$).

These data suggest that the 521T/C polymorphism of the OATP-C gene modulates the lipid-lowering efficacy of HMG-CoA reductase inhibitors.

Key words: HMG-CoA reductase inhibitor; genetic polymorphism; transporter; OATP-C; cholesterol; individualized medicine

Introduction

The treatment of common diseases as typified by hyperlipidemia and hypertension gives first priority to lifestyle regimens such as smoking cessation, dietary therapy, kinesitherapy, and maintenance of optimal body weight. However, pharmacotherapy is combined with these measures in patients showing low effectiveness or compliance. Hydoxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are now the most widely prescribed drugs worldwide and are established as the first-line treatment for hyperlipidemia. Inhibition of HMG-CoA reductase, which catalyzes the rate-limiting step of cholesterol biosynthesis, causes a decrease in intracellular cholesterol levels, resulting in upregulation of low density lipoprotein (LDL) receptors, increasing clearance of LDL-cholesterol, and leading to a further lipid-lowering effect. The statins decrease blood levels of total cholesterol, LDL-cholesterol, very low density lipoprotein (VLDL)-cholesterol and triglyceride. High-density lipoprotein (HDL) level is increased to a moderate degree.[1] The clinical significance of statins has been established as the class of drug that most effectively lowers LDL-cholesterol at present. Recent primary and secondary prevention trials have evidenced that statins also reduce the risk of coronary heart disease (CHD).[2-12]

Received; July 28, 2004, Accepted; October 14, 2004
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Pravastatin, one of the statins, is widely used in the treatment of hyperlipidemia. After oral administration, it is absorbed from the gastrointestinal tract, and then taken up from the circulation by the liver through organic anion transporting polypeptide C (OATP-C).[13,14] OATP-C, encoded by the gene SLCO1B1 and also referred to as liver-specific transporter 1 (LST-1) or OATP2, is a liver-specific multispecific organic anion transporter that plays a major role in the hepatic uptake of a variety of endogenous and foreign chemicals.[15-17] In addition to pravastatin, it also plays a major role in the hepatic uptake of pitavastatin,[18] and an inhibition study suggested that lovastatin, simvastatin and atorvastatin are potential substrates of OATP-C.[15] Recently, a number of single nucleotide polymorphisms (SNPs) have been identified in the human OATP-C gene by different groups, and some nonsynonymous SNPs have been found to alter its transport activities.[19-21] The distribution of OATP-C haplotypes varies among ethnic groups. The T521C polymorphism is strongly associated with the A388G variant in Japanese subjects,[22], while in European Americans, the A388C521 (OATP-C*5) allele occurs at a considerable frequency of 14–15%.[20,23]. An *in vivo* pharmacokinetic study in healthy Japanese subjects showed reduced total and nonrenal clearance of pravastatin in subjects with the G388C521(OATP-C*15) allele as compared with individuals homozygous for the G388T521 (OATP-C*1b) allele.[22] The reduced hepatic uptake due to this gene polymorphism may be associated with a lower hepatic concentration, resulting in attenuation of the lipid-lowering effect of statins, since the liver is the target organ of statins. In this retrospective study performed in Japanese patients with hyperlipidemia in whom a statin was prescribed, the effect of genetic polymorphism of OATP-C (T521C) on the lipid-lowering response to statins was assessed.

Methods

Subjects: This retrospective cohort study included 3071 subjects in a rural district of Ehime prefecture in the southern part of Japan. Of these subjects, 101 were prescribed HMG-CoA reductase inhibitors between July 1, 2003 and August 28, 2003.

Follow-up survey was based on the medical records of the municipal hospital. The date of first administration of an HMG-CoA reductase inhibitor was confirmed, and the data of total cholesterol, HDL-cholesterol and triglyceride before and after the first administration were transcribed. LDL-cholesterol concentration was calculated using Friedewald's formula. Subjects who showed low or no drug compliance in their medical record were excluded from the analysis. Sixty six subjects were finally available for analysis.

All subjects gave informed consent, and the study was approved by the ethics committee of Ehime University.

DNA analysis: Genomic DNA was extracted from blood lymphocytes using an extraction kit (QIAGEN GmbH, Hillden, Germany). DNA was amplified by degenerate oligonucleotide-primed PCR (DOP-PCR). DOP-PCR amplification was performed as previously described,[24] with slight modifications as follows. The PCR reactions contained 4 μM DOP-PCR primer (5′-CCGACTCGAGNNNNNNATGTGG-3′), 400 μM dNTPs, 2×GC buffer I, 2.5 mM $MgCl_2$, and 2.5 U Taq polymerase (TaKaRa LA Taq, TAKARA BIO Inc.) in a final volume of 50 μL. The reaction mixture was subjected to an initial denaturation step of 5 min at 95°C; then 10 cycles of 94°C for 30 sec, 30°C for 2 min, and 68°C for 7 min (a ramping step of 0.08°C/sec to 68°C); and then 25 cycles of 94°C for 30 sec, 60°C for 1 min, and 68°C for 7 min. Amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems Inc.). Then the DOP-PCR-preamplified DNA samples were subjected to ExoSAP-IT (Amersham Biosciences Inc.) according to the manufacturer's protocol to remove unincorporated primers and dNTPs and used to determine the gene polymorphism. The TaqMan chemical method was used to detect the OATP-C T521C (Val174Ala) polymorphism. The forward primer was 5′- AGG TTG TTT AAA GGA ATC TGG GTC ATA C -3′, the reverse primer was 5′- CTC CCC TAT TCC ACG AAG CAT ATT -3′, the T allele-specific probe was 5′- FAM- CCC ATG AAC ACA TAT AT-MGB -3′, and the C allele-specific probe was 5′- VIC-CCATGAACGCATATAT-MGB -3′.

Statistical analysis: All values are expressed as mean ± SD. Statistical comparisons among genotypes were performed by ANOVA. Chi-squared tests were used to compare the prevalence among genotypes and to verify Hardy-Weinberg equilibrium. The effect of statin treatment on lipid values was analyzed by *t* test for dependent samples. Analysis of variance for repeated measurements was used to determine the significance of differences in serum lipid concentrations. Probability values less than 0.05 were considered to be significant. Statistical analysis was performed with SPSS statistical software (SPSS Inc.).

Results

Baseline characteristics of the subjects are shown in Table 1. Out of the 66 subjects, 22 were treated with pravastatin, 11 with atorvastatin and 33 with simvastatin. The allele frequencies of the OATP-C T521C polymorphism were 0.85 and 0.15, respectively, and agreed with the results of previous reports in Japanese.[21,22] Genotype frequencies were: TT, 66.7%; TC, 30.3%; CC, 0%; undetermined, 3.0%.

Lipid concentrations in patients treated with statins are shown in Table 2. The mean serum concentrations of total cholesterol, LDL-cholesterol, and triglyceride

decreased from their mean baseline concentrations of 259 to 203, 167 to 119, and 177 to 126 mg/dL, respectively. The mean serum HDL-cholesterol concentration increased slightly from the baseline of 58.7 mg/dL to 59.9 mg/dL. The mean percent changes in total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol concentrations between pre- and post-treatment were −20.9%, −28.3%, −7.6%, and +4.6%, respectively. There were significant differences in the concentration of total cholesterol ($p<0.001$), LDL-cholesterol ($p<0.001$), and triglyceride ($p<0.01$) between pre- and post-treatment. No statistically significant difference was found in HDL-cholesterol ($p=0.275$).

Then the differences in the effect of three kinds of statins; pravastatin, atorvastatin, and simvastatin, were examined. There was no significant difference in the patterns of change of total cholesterol, LDL-cholesterol, and HDL-cholesterol levels. In contrast, the triglyceride-lowering pattern differed (repeated measures ANOVA; $p=0.040$). Out of the three statins, a significant difference between simvastatin and atorvastatin was found by subsequent Tukey's multiple comparison ($p=0.010$). The percent changes in total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol concentrations between pre- and post-treatment showed no significant difference among the three statins.

Table 1. Baseline characteristics (n=66)

Age (years)			70.4±8.4
Sex (male/female)			17/49
Body mass index:BMI (kg/m^2)			23.7±2.6
Drug (n)	Pravastatin		22
	Atorvastatin		11
	Simvastatin		33
Polymorphism of OATP-C (n)	V174A	VV	44 (66.7%)
		VA	20 (30.3%)
		AA	0 (0%)
		N.D.	2 (3.0%)

N.D.; not determined

Table 2. Lipid concentrations in patients treated with statins

	n		Pre (mg/dL)	Post (mg/dL)	% Change (95% CI, LL/UL)*	p
Total	66	TC	259.2±33.6	203.7±28.7	−20.9 (−23.3/−18.5)	<0.001
	59	LDL-C	167.0±39.3	119.1±24.5	−28.3 (−32.2/−24.3)	<0.001
	62	TG	176.9±131.7	126.1±63.9	−7.6 (−21.6/6.4)	<0.01
	59	HDL-C	58.7±19.6	59.9±14.8	4.6 (0.1/9.2)	0.275
Pravastatin	22	TC	253.6±33.5	208.3±28.5	−17.5 (−21.3/−13.6)	<0.001
	21	LDL-C	161.2±32.3	122.9±29.1	−23.0 (−29.0/−17.0)	<0.001
	21	TG	159.1±83.8	148.2±86	6.8 (−20.3/33.9)	0.555
	20	HDL-C	59.0±12.8	57.5±12.2	−2.0 (−68.0/2.8)	0.302
Atorvastatin	11	TC	249.5±36.9	198.5±31.9	−20.3 (−24.4/−16.1)	<0.001
	8	LDL-C	139.2±54.2	102.2±19	−34.8 (−41/−28.5)	<0.05
	10	TG	282.9±266.1	139.7±69.8	−7.9 (−58.9/43.1)	0.152
	9	HDL-C	56.2±16.0	64.9±12.5	10.7 (−1.43/22.8)	0.059
Simvastatin	33	TC	266.1±32	202.4±28.2	−23.4 (−27.2/−19.6)	<0.001
	30	LDL-C	180.2±33.0	122.2±21.1	−30.2 (−36.5/−23.9)	<0.001
	31	TG	154.8±69.9	106.8±33.1	−17.2 (−33.8/−0.7)	<0.001
	30	HDL-C	58.8±24.4	60.0±17.3	7.2 (−0.4/14.9)	0.582

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol
*CI, confidence interval; UL, upper limit; LL, lower limit.
p value: significant difference between pre- and post-treatment.

Table 3. Association of lipid-lowering effect by statins and OATP-C polymorphism

	T521C	N	Pre (mg/dL)	Post (mg/dL)	% Change (95% CI, LL/UL)*	p
TC	TT	44	259.4±35.4	200.3±28.7	−22.3 (−25.0/−19.7)	<0.05
	TC	20	256.8±31.4	213.1±28.3	−16.5 (−21.4/−11.6)	
LDL-C	TT	39	170.2±36.1	118.6±26.8	−29.0 (−33.6/−24.4)	0.094
	TC	20	158.4±46.3	122.6±20.3	−12.4 (−33.4/8.6)	
HDL-C	TT	38	56.1±15.4	57.0±13.7	1.2 (−6.6/9.0)	0.745
	TC	20	63.0±26.0	64.9±16.7	11.1 (−5.3/27.4)	
TG	TT	40	170.7±89.0	125.8±68.0	−10.8 (−28.0/6.4)	0.492
	TC	19	152.8±97.3	127.6±61.2	3.4 (−24.7/31.5)	

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol
*CI, confidence interval; UL, upper limit; LL, lower limit.
p value: significant difference of lipid-lowering effect of statins in T521C variant.

The effect of the T521C polymorphism of the OATP-C gene on the lipid-lowering response to the statins is shown in Table 3. The serum concentration of total cholesterol significantly decreased in subjects with both 521TC and 521TT genotype, from the baseline concentration of 256.8±31.4 to 213.1±28.3 mg/dL and 259.4±35.4 to 200.3±28.7 mg/dL, respectively. Moreover, 521TC heterozygous subjects showed a smaller decrease than 521TT homozygous subjects. A significant effect of the T521C variant was observed in the total-cholesterol-lowering effect of statins (repeated measures ANOVA; $p=0.041$). No statistically significant effect of the T521C variant was found in the other lipid-lowering responses to the statins (LDL-cholesterol, HDL-cholesterol, and triglyceride).

Discussion

Cholesterol-lowering therapy is the central approach in the primary and secondary prevention of CHD. HMG-CoA reductase inhibitors (statins) are currently the most widely used cholesterol-lowering drugs. Large-scale clinical trials have unequivocally demonstrated the efficacy of statin treatment in reducing the risk of CHD.[2-12] On the other hand, an adequate reduction in CHD events is not necessarily achieved in all patients treated with statins.[27] Pharmacogenomic variability is an important determinant of drug response. Assessment of polymorphic genes involved in the pharmacokinetics and pharmacodynamics of statins prior to initiation of treatment may help to identify patients at risk of a low response. Choosing an appropriate therapeutic approach for individual patients may be of great advantage not only from the therapeutic standpoint, but also in relation to cost effectiveness, since therapeutic drugs for lifestyle-related diseases such as statins are prescribed over the long term. In this study, the association of genetic polymorphism of liver-specific organic anion transporter OATP-C, which is concerned with the pharmacokinetics of statins, with the lipid-lowering effect of statins was examined in a community-based cohort.

Previous large scale clinical trials of statins reported 18–27%, 25–46%, 10–16%, and 5–8% reductions on average in serum concentrations of total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol, respectively.[2-12] Our results essentially agree with these results. Serum concentrations of total cholesterol, LDL-cholesterol, and triglyceride significantly decreased after administration of statins, but HDL-cholesterol did not change significantly. The major effect of statins is considered to be the upregulation of LDL receptors. This effect increases the clearance of LDL-cholesterol and leads to a further lipid-lowering effect. Suppression of the synthesis and secretion of VLDL by a reduction of cholesterol synthesis in the liver also decreases serum triglyceride. In contrast, the increase in HDL-cholesterol by statins is moderate.[1,27]

Statins are well tolerated apart from two uncommon but potentially serious adverse effects: (i) elevation of liver enzymes in less than 2% of patients and (ii) skeletal muscle abnormalities, which range from benign myalgia, which may occur in 0.5 to 2.5% of patients, to myopathy (10-fold elevation of creatine kinase with muscle pain or weakness) in up to 0.3% of patients to life-threatening rhabdomyolysis. These serious adverse effects were not recorded in the medical records of the subjects in this study.

The frequency of the CC genotype of the OATP-C T521C polymorphism is very low in Japanese (previous studies reported 0.8% (ref. 22) and 3% (ref. 21)), although the 521C allele occurs at a considerable frequency (16% (ref. 22), 11% (ref. 21)). In the total 3701 subjects in this cohort study, genotype frequencies were: TT; 2175 (70.8%), TC; 750 (24.4%), CC; 80 (2.6%), and undetermined; 66 (2.1%), consistent with previous reports.[21,22] However, no individuals homozygous for the 521C allele were ultimately included in the subjects for analysis.

The therapeutic efficacy of statins for total-cholesterol lowering was compared in subjects with and without the 521C allele. The therapeutic effect was attenuated in subjects with the 521C allele compared with those homozygous for the 521T allele. Therefore, it is possible that the reduced hepatic uptake due to the gene polymorphism is associated with the therapeutic effect of statins. This tendency is expected to be more profound in patients homozygous for the 521C allele according to the results of Nishizato *et al.*[22] and Mwiniyi *et al.*.[23] On the other hand, Niemi *et al.* recently reported no gene-dose effect of the 521T>C variant on the systemic exposure to pravastatin.[25] Haplotype analysis revealed that the haplotype containing the −11187G>A, 388A>G and 521T>C SNPs had a particularly pronounced effect on the $AUC_{0\text{-}12\,hr}$ of pravastatin. This result suggests that the 521T>C variant is not the only predictable SNP of the OATP-C phenotype, and haplotype analysis is more informative than single SNPs analysis. Further study is required to elucidate the most effective SNP or haplotype for predicting OATP-C phenotype.

Unlike pravastatin, atorvastatin and simvastatin have not been shown to be a substrate of OATP-C. Since atorvastatin is administered to patients as the acid form, it is possible that OATP-C accounts for its hepatic uptake. Simvastatin is administered as the lactone form, and it is generally considered that it crosses the plasma

membrane by passive diffusion. However, simvastatin undergoes conversion to the acid form, which is the active form, in the body. A substantial amount of the active form was detected in the blood circulation. Therefore, the acid form may be taken up by the liver by a transporter, presumably by OATP-C. This may account for the attenuated cholesterol-lowering effect of simvastatin treatment in subjects with the 521C allele.

Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets have been linked to individual differences in the efficacy and toxicity of many drugs. Therapeutic effect is determined by the interplay of several genes encoding proteins involved in multiple pathways of drug metabolism, disposition, and effects.[26] To optimize the benefits of medication for individual patients, it is necessary to accumulate clinical data on the association between genotypes and phenotypes for the target drug. Currently, no genetic polymorphisms that are useful for the prediction of effects and adverse drug reactions to statin therapy are available.[27,28] Our results indicated that the T521C polymorphism in the OATP-C gene, which is one of the transporters related to the pharmacokinetics of statins, affected the therapeutic effects of statins on hyperlipidemia. Assessment of the OATP-C T521C polymorphism could be useful for the prediction of therapeutic efficacy and the risk of statin treatment in individualized medicine.

Acknowledgements: This work was supported by a Grant-in Aid for Research on Cancer Prevention and Health Services (H15- cancer prevention - 040) from the Ministry of Health, Labor and Welfare of Japan (T.M.), 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 (T.M.), and by fellowships and grants from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (R.T.).

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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 15, 2004

Genetic Predisposition to Neurological Symptoms in Lacunar Infarction

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

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

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Introduction

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

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

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

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

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

Subjects and Methods

Subjects

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

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

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

Brain MRI Examination

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

Detection of Gene Polymorphisms

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

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

To identify the AGT M235T polymorphism, sense primer 5′-TGACAGGATGGAAGACTGGCTGCTCCCTGC-3′ and antisense primer 5′-AGCAGAGAGGTTTGCCTTACCTTG-3′ were used [26]. The PCR product (5 µl) was digested with 5 units *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 *HaeIII* 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[1]		
Hemiparesis	115	
Sensory disturbance	19	
Ataxia	12	
Dysarthria	9	

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

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

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

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

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

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

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

Gene Polymorphism of RAS and Symptomatic Lacunar Infarctions

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

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

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

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

Lacunar Subtypes and RAS Genes

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

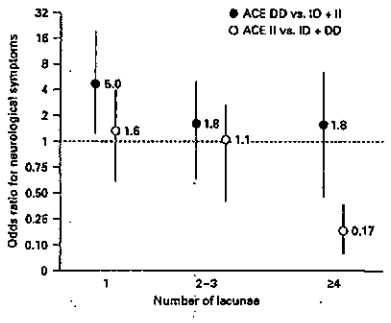

Fig. 1. Odds ratios of ACE genotypes for the manifestation of neurological symptoms of lacunar infarctions according to the number of lacunae. Closed circles indicate the odds ratio for ACE DD genotype compared with ACE ID + II, and open circles indicate the odds ratio for ACE II genotype compared with 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.

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bjh research paper

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

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Received 13 July 2004; accepted for publication 25 August 2004

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Summary

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

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

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

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

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

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

(Zhao *et al*, 1998) and 325 (Schneider *et al*, 2002). Schneider *et al* (2002) examined the difference in thermal stability and antifibrinolyitic 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 Choju Medical Institute (Fukushimura Hospital, Japan) from 1993 to 2002. These were performed after obtaining the agreement of the patients' guardians for diagnosis, and biochemical, molecular biological and genomic research. This study was approved by the Ethics Committee of the Choju Medical Institute on 24 February 2003, and assigned application number 91.

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

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

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

Autopsy and sampling of brain tissues

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

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

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

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

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

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

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

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

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

Chemicals

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

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

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

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

Statistical analysis

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

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

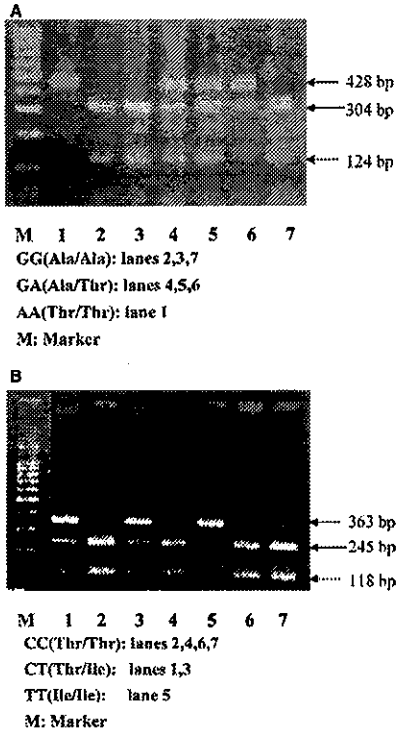

Fig 1. Demonstration of the existence in the human population of Thr/Ala-147 (A) and Thr/Ile-325 (B) polymorphisms in seven typical samples. Each genomic DNA extracted from brain tissues was subjected to polymerase chain reaction (PCR) to amplify a fragment encompassing the codon for the amino acids 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 180 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).

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	40	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)

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

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.

	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	41	139 (74)	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)

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

FBB, Fukushimura Brain Bank, PBC, population-based controls; CI, cerebral infarction.
On comparing with PBCs, no group showed statistical significance ($P < 0.05$).

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 (505A/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, 525 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*, 2002). 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 56·19 ($P < 0.001$) for the Thr/Ile-325 polymorphism and 55·98 ($P < 0.001$) 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 58·86 ($P < 0.001$) (Thr/Thr 267, Thr/Ile 255, 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 Tanigawa and Mr. Takeshi Kanesaka for their excellent technical assistance and Ms. Catherine Campbell for her help in editing this manuscript.

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Biochemical and Biophysical Research Communications 321 (2004) 320–323
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Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

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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

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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 *Hhal*, 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′-TCAAGC AATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATT ATGGTG-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), 200 μM each dNTP, 5 μM each primer, 0.5 U *Taq* DNA polymerase and 50 ng genomic DNA as a template with 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR product size was 355 bp, and the G allele was digested by *Hhal* to 53 + 141 + 161 bp, and the A allele to 53 + 302 bp. 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.

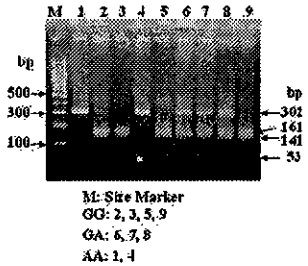

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *Hhal* and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*Hhal* polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

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 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.

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.

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.

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

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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%

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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

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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; rs1407814
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

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).

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 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).

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*	rs1407814	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	0.017*	T/C = 150/602 : 156/596	0.700	1.98 (0.78–5.06)
	CD36	rs1358337	hCV1803785	intron 3	12329A/G	GG/GA/AA = 44/152/180 : 51/182/143	0.024*	G/A = 240/512 : 284/468	0.017*	1.35 (0.79–2.29)
	DPYS	rs2246815	hCV9595720	intron 5	38687A/G	GG/GA/AA = 174/163/39 : 137/192/47	0.023*	G/A = 511/241 : 466/286	0.015*	1.45 (0.80–2.64)
	COL11A1	rs3753841	hCV2947954	exon 52	193817A/G	AA/AG/GG = 178/160/38 : 158/156/62	0.030*	A/G = 516/236 : 472/280	0.017*	1.62 (0.95–2.75)
Up-regulated	*GNA11*	rs308064	hCV3149364	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	6000T/A	TT/TA/AA = 17/155/204 : 22/122/232	0.041*	T/A = 189/563 : 166/586	0.162	1.14 (0.84–1.54)

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

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 (001 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.

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ORIGINAL ARTICLE

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

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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

on 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 (IBL, 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 (10x 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 Caine *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.[18]

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[†]

	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)	95.0 ± 5.72
Control brain	10 (8)	10 (8)	20 (8)	86.8 ± 6.50
Total	122	133	255	82.3 ± 8.49
PBC samples	174	213	387	75.3 ± 5.0[‡]

[†]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). [‡]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[†]

	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 (7.6)	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 (4.6)
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)

[†]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[†]

	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 (86)	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, $P < 0.005$.
[†]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	38 (50)	6 (43)	3 (75)	293 (75.7)
3/4	31 (82)	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)	18 (64)	7 (88)	673 (87.0)
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 Suita controls, $P < 0.001$. **All APOE alleles in EOAD patients compared to aging patients, $P < 0.05$ and to Suita 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.

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