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

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)[a]	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
[a]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)[a]	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
[a]CI, confidence interval; UL, upper limit; LL, lower limit.
p value: significant difference of lipid-lowering effect of statins in T521C variant.

($p=0.010$). 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.

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.

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Genetic Predisposition to Neurological Symptoms in Lacunar Infarction

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

Abstract

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

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Introduction

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

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

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

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

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

Subjects and Methods

Subjects

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

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

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

Brain MRI Examination

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

Detection of Gene Polymorphisms

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

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

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

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

Statistical Analysis

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

Results

Demographic Characteristics of Cases and Controls

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

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

Table 1. Clinical profile of cases and controls

	Lacunar infarction patients	
	symptomatic	asymptomatic
Number (male/female)	151 (88/63)	150 (82/68)
Age, years	68 ± 9	69 ± 9
Hypertension, %	118 (78)	120 (80)
Dyslipidemia, %	66 (44)	59 (39)
Diabetes mellitus, %	35 (23)	23 (15)
Current smoker, %	57 (38)	56 (37)
Number of lacunae		
Whole brain	2.9 ± 2.1	2.7 ± 1.8
Basal ganglia	1.9 ± 1.7*	1.1 ± 1.5
Corona radiata	0.9 ± 1.2	1.0 ± 1.4
Brainstem	0.1 ± 0.3	0.06 ± 0.8
Symptoms[†]		
Hemiparesis	115	
Sensory disturbance	19	
Ataxia	12	
Dysarthria	9	

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

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

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

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

Gene Polymorphism of RAS and Symptomatic Lacunar Infarctions

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

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

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

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

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

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

		Patients with a single symptomatic lacuna (n = 54)	Patients with 4 or more multiple asymptomatic lacunar infarctions (n = 42)
ACE	II	18 (0.33)	28 (0.67)
	ID	24 (0.44)	10 (0.24)
	DD	12 (0.22)	4 (0.10)
		$\chi^2 = 10.60$, f = 2, p = 0.005	
AGT	MM	2 (0.04)	2 (0.05)
	MT	16 (0.30)	12 (0.29)
	TT	36 (0.67)	28 (0.67)
		$\chi^2 = 0.07$, f = 2, p = 0.96	
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-

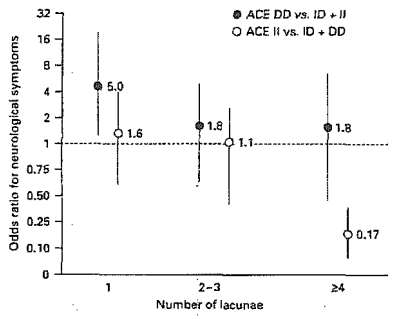

Fig. 1. Odds ratios of ACE genotypes for the manifestation of neurological symptoms of lacunar infarctions according to the number of lacunae. Closed circles indicate the odds ratio for ACE DD genotype compared with ACE ID + II, and open circles indicate the odds ratio for ACE II genotype compared with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

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bjh research paper

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

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Summary

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

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

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

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

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

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

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(Zhao *et al*, 1998) and 325 (Schneider *et al*, 2002). Schneider *et al* (2002) examined the difference in thermal stability and 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

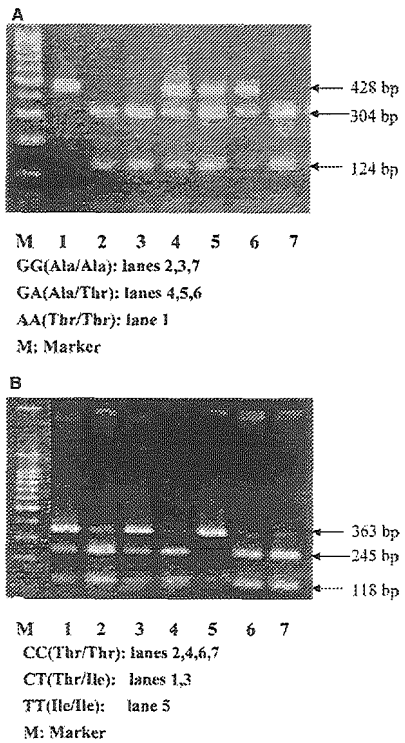

Fig 1. Demonstration of the existence in the human population of Thr/Ala-147 (A) and Thr/Ile-325 (B) polymorphisms in seven typical samples. Each genomic DNA extracted from brain tissues was subjected to polymerase chain reaction (PCR) to amplify a fragment encompassing the codon for the amino acids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

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

Male/female	No sclerosis	Slight sclerosis	Mild sclerosis	Severe sclerosis	FBB total (%)	PBC (%)
Thr/Ala-147						
Thr/Thr	2	8	2	0	12 (7)	3 (3)
Thr/Ala	11	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)

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

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

	No infarction	Small infarction	Large infarction	FBB total (%)	PBC (%)
Thr/Ala-147					
Thr/Thr	6	3	3	12 (6)	3 (3)
Thr/Ala	35	18	22	75 (40)	47 (44)
Ala/Ala	53	23	26	102 (54)	58 (53)
Allele Thr (%)	47 (25)	24 (27)	28 (27)	99 (26)	53 (25)
Allele Ala (%)	141 (75)	64 (63)	74 (73)	279 (74)	163 (75)
Thr/Ile-325					
Thr/Thr	68	30	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)

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).

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Original article

Methyl-CpG binding protein 2 gene (*MECP2*) variations in Japanese patients with Rett syndrome: pathological mutations and polymorphisms

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Abstract

A total of 45 different mutations of methyl-CpG-binding protein 2 gene (*MECP2*) were identified in 145 of 219 Japanese patients with typical or atypical Rett syndrome (RTT) (66.2%). A missense mutation, T158M was the most common mutation of MECP2, identified in 22 (19.1%) patients, followed by four nonsense mutations, R168X (14.8%), R270X (13.0%), R255X (9.6%), and R294X (6.1%) in 115 patients with classical RTT. Two missense mutations, R133C (33.3%) and R306C (23.3%), and a nonsense mutation, R294X (13.3%), were common in 30 patients with atypical RTT, including the preserved speech variant (PSV). Frameshift mutations due to nucleotide deletion or insertion were identified in 22 patients with *MECP2* mutations, and one of them had a 3.6 kb deletion encompassing exons 3 and 4. Three patients with classical RTT had a splicing anomaly. The wide spectrum of phenotypic variability in patients with RTT has been considered to be correlated with the mutation type and location in MECP2, and X-inactivation. However, most patients showed a random X-inactivation pattern evaluated by an androgen receptor gene polymorphism in this study, suggesting that a skewed X-inactivation might not be a main modification factor on clinical phenotypes of RTT. In addition, three new missense mutations, P176R, A378V and T479M, were identified in patients with RTT, but also in healthy Japanese, indicating that these mutations are non-pathogenic in Japanese. Information about rare polymorphic variations is very important for the molecular diagnosis of RTT, although rare polymorphic variants might differ among ethnic groups.

Keywords: Rett syndrome (RTT); Methyl-CpG-binding protein 2 gene (*MECP2*); Single nucleotide polymorphism (SNP); X-inactivation

1. Introduction

Rett syndrome (RTT) (OMIM 312750) is a common cause of mental retardation in females, with a prevalence of 1 in 10,000~15,000 worldwide [1,2]. The clinical manifestations in the classical form of RTT are characterized by cognitive deterioration with autistic features, loss of acquired skills such as language and hand usage, stereotypical hand wringing movements, and gait ataxia, appearing after a period of apparently normal development (until 6–18 months) [3]. However, atypical variants of RTT are also commonly observed, and five distinct categories of atypical forms have been delineated on the bases of clinical criteria: infantile seizure onset type, congenital forth, 'forme fruste', PVS, and late childhood regression form [4].

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Table 1
Mutation analysis in 219 sporadic female patients with RTT

	Mutations in the *MECP2*		
Clinical form	Positive	Negative	Total
Classical RTT	115 (87.8%)	16 (12.1%)	131 (59.8%)
Atypical RTT	30 (34.1%)	58 (65.9%)	88 (40.2%)
Total	145 (66.2%)	74 (33.8%)	219 (100%)

Since the first report on mutations in the methyl-CpG binding protein 2 gene (*MECP2*) in patients with RTT [5], over 200 different mutations of *MECP2* have been identified in patients with classical RTT and atypical RTT [6–26]. Furthermore, patients with X-linked mental retardation syndrome (XLMR) have different mutations in *MECP2 from those in patients with* RTT [27–37]. The wide spectrum of phenotypic variability in patients with *MECP2* mutations has extensively been discussed and considered with respect to mutation type and location in the gene, in addition to the pattern of X-inactivation [6,9,11]. However, some missense mutations reported in patients with RTT and XLMR might be non-pathogenic DNA substitutions such as single nucleotide polymorphisms (SNPs) [16,17,38,39].

To provide further delineation of *MECP2* mutations in RTT patients, we have analyzed *MECP2* in 219 Japanese patients with classical or atypical RTT. We found 45 pathogenic mutations in 145 patients with RTT (66.2%) and three new SNPs with amino acid substitutions in exon 4 of *MECP2*. We suggest that rare missese mutations of *MECP2* should be carefully distinguished from rare non-pathological variations in order to determine the cause of clinical conditions in patients with RTT or XLMR (Table 1).

2. Subjects and methods

2.1. Study patients

A total of 219 unrelated Japanese female patients, ranging in age from 2 to 41 years, were evaluated according to the international diagnostic criteria for RTT [3] by Japanese child neurologists and were referred for molecular analysis of *MECP2*. Classical RTT was diagnosed in 131 patients who had psychomotor regression after a period of normal development (6–12 months), severe mental retardation, deceleration of head growth and loss of language and purposeful hand skills with stereotypical hand movements. Seizures started at 2–3 years of age, but attacks had been well controlled by medication in most cases. Respiratory dysfunction was observed in 60% of patients with classical RTT. Eighty-eight patients had atypical RTT. They had stereotypical hand movements, but had either more severe phenotypes or a mild form of RTT [4]. 'Forme fruste' was prevalent in atypical RTT. Preserved speech variant (PSV) was diagnosed in 12 cases. Congenital onset phenotypes, with severe early motor disability, were diagnosed in five cases. Early seizure onset form was diagnosed in 10 cases.

To determine the frequencies of rare alleles of SNPs, 200 healthy Japanese (100 males and 100 females) were recruited as controls, after obtaining informed consent for DNA analysis.

2.2. Mutation analysis

Using a standard protocol, genomic DNA was extracted from peripheral blood leukocytes from patients with RTT and their parents, after obtaining informed consent from the parents of patients, and controls. DNA amplification of the *MECP2* coding exons was performed by polymerase chain reaction (PCR) method using appropriate primers and conditions as previously reported by Obata et al. [8]. In addition, to amplify exon 1 and the entire regions of exons 3 and 4, primers were designed based on the reported genomic sequence of *MECP2* as follows: Ex1-F: 5′-TAGAGAG-GAGGGACGCCATC-3′, Ex1-R: 5′-CTCGCCAATTAC-GGCATCG-3′, Int 2-1F: 5′-AGTTCCTGGTGTGTCCT-TCC-3′, Int 2-2F: 5′-ACTCGACGAGTAAGAGC-3′, Ex 4-LTD1; 5′-AATGGACCATGCCACCTCAC-3′; Ex4-LTD2: 5′-GCAGCTGATAAGGACTTCC-3′. For amplification of the entire exons 3 and 4, PCR was performed in a final volume of 25 μl of solution including 2 units Taq polymerase (Takara, long PCR kit) and primers, In2-1F, Int2-2F, Ex4-LTD1, and Ex4-LTD2. PCR conditions for long PCR were initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 min, and annealing and extension at 60 °C for 4 min, and by final extension at 72 °C for 10 min. PCR products were sequenced directly using an ABI310 sequencer.

2.3. X-inactivation analysis

The X inactivation pattern was determined by PCR analysis of a polymorphic CAG repeat in the androgen receptor gene (AR) [40]. After digestion with a methylation-sensitive enzyme, *Hpa*II, PCR products from undigested and digested DNAs were separated on an ABI373 automated sequencer and analyzed by Gene Scan software. Methylation of the *Hpa*II site close to this repeats correlates with X inactivation and the PCR product is obtained from only the inactive X chromosome.

3. Results

3.1. Mutation spectrum of MECP2

Forty-five different mutations in *MECP2* were identified in 145 of 219 (66.2%) sporadic female patients with classical or atypical RTT (Tables 1 and 2); missense mutations in 63 (43.4%), nonsense mutations in 57 (39.3%), frameshift mutations due to nucleotide deletion or insertion in 22 (15.2%) and splicing anomalies in three (2.1%). All mutations were not observed in their parents, indicating de novo mutation.

Table 2
List of *MECP2* mutations found in 145 patients in this study

Genotypes	Nucleotide change	Involved functional domain	Phenotype	References (The first report)
Missense mutations (63)				
A131D (1)	**C392A**	**MBD**	**Classical RTT**	**This report**
S134F (1)	**C401T**	**MBD**	**Classical RTT**	**This report**
D156A (1)	**A467C**	**MBD**	**Classical RTT**	**This report**
P302T (1)	**C904A**	**TRD**	**Classical RTT**	**This report**
K305R (1)	**A914G**	**TRD**	**Classical RTT**	***This report***
R106W (3)	C316T	MBD	Classical RTT	5
R106Q (2)	G317A	MBD	Classical RTT	13
L124F (1)	G372T	MBD	Classical RTT	8
R133C (11)	C397T	*MBD*	Atypical RTT (4), PVS (7)	5
S134C (1)	C401G	MBD	Classical RTT	14
P152R (6)	C455G	MBD	Classical RTT	14
D156E (2)	C468G	MBD	Classical RTT	19
T158M (22)	C473T	MBD	Classical RTT	5
P302H (2)	C905A	TRD	Classical RTT	10
P302R (1)	C905G	TRD	Classical RTT	13
R306C (7)	C916T	TRD	Atypical RTT (6) Classical RTT (1)	6
Nonsense mutations (57)				
R68X (1)	**C203G**	**MBD, TRD, NLS, WDR**	**Classical RTT**	**This report**
Y141X (1)	**C423G**	**MBD, TRD, NLS, WDR**	**Classical RTT**	**This report**
R168X (17)	C502T	TRD, NLS, WDR	Classical RTT	6
Q170X (1)	C508T	TRD, NLS, WDR	Classical RTT	13
R255X (11)	C763T	TRD, NLS, WDR	Classical RTT	5
R270X (15)	C808T	TRD, NLS, WDR	Classical RTT	9
R294X (11)	C880T	TRD, WDR	Classical RTT (7) *Atypical RTT (4)*	8
Deletion and insertion mutations (22)				
1 bp del (1)	**806 (G)**	**TRD, NLS, WDR**	**Classical RTT**	**This report**
2 bp del (1)	**107–108(AA)**	**MBD, TRD, NLS, WDR**	**Classical RTT**	**This report**
2 bp del (1)	**543–544(TC)**	**TRD, NLS, WDR**	**Classical RTT**	**This report**
4 bp del (1)	**1448–51(AGAG)**	**WDR**	**Classical RTT**	**This report**
35 bp del (1)	**1159–1193**	**WDR**	**Atypical RTT**	**This report**
212 bp del (1)	**1110–1321**	**WDR**	**Atypical RTT**	**This report**
507 bp del (1)	**616–1121**	**MBD, TRD, WDR**	**Classical RTT**	**This report**
3.6 kb del (1)	**Exs 3 and 4**	**MBD, TRD, WDR**	**Classical RTT**	**This report**
1 bp Ins (1)	**243 (C)**	**MBD, TRD, NLS, WDR**	**Classical RTT**	**This report**
1 bp Ins (1)	**1194 (C)**	**WDR**	**Atypical RTT**	**This report**
1 bp Ins (1)	**1320 (T)**	**WDR**	**Atypical RTT**	**This report**
1 bp del (1)	233 (C)	MBD, TRD, NLS, WDR	Classical RTT	8
1 bp del (1)	375 (C)	MBD, TRD, NLS, WDR	Classical RTT	8
1 bp del (1)	695 (G)	TRD, NLS, WDR	Classical RTT	8
1 bp del (1)	696 (C)	TRD, NLS, WDR	Classical RTT	8
1 bp del (2)	803 (C)	TRD, NLS, WDR	Classical RTT	8
4 bp del (1)	754–8(GGCA)	TRD, NLS, WDR	Classical RTT	8
44 bp del (3)	1155–1198	WDR		
71 bp del (1)	1132–1202	WDR	Atypical RTT	24
Splicing anomalies (3)				
Ex1 donor site (1)		**GT/AT**	**Classical RTT**	**This report**
Ex3 donor site (1)		**GT/TT**	**Classical RTT**	**This report**
Ex4 acceptor site (1)	**CAG/GAG**		**Classical RTT**	**This report**

Novel mutations are denoted by bold letters. Numbers in parentheses are observed subjects in this study. PVS: preserved speech variant, MBD: methyl-CpG-binding domain, TRD: translation repression domain, NLS: nuclear localization signal, WDR: group II WW domain binding region.

3.1.1. Missense mutations

Sixteen different missense mutations including five new ones (A131D, S134F, D156A, P302T, and K305R) were identified in 63 patients and the phenotypes in these patients are summarized in Table 2. The most common mutation was c473t resulting in T158M, which was

identified in 22 of 145 (15.2%) unrelated patients with *MECP2* mutations. All patients with T158M showed the most typical clinical features of classical RTT. The second most common missense mutation was R133C, which was identified in 11 patients with atypical RTT including eight with PSV. They started to walk at 18 months without support and to walk in adulthood. In addition, R306C was identified in eight patients with a mild phenotype of RTT who could walk at 15–20 months and maintain walking at 17 years of age. Missense mutations, R106W/Q, R152R, D156E, and P302H were identified in a few patients with classical RTT. Six patients with P152R could sit with support, but could not walk throughout their life and became bedridden by 10 years of age. Eight other missense mutations, L124F, A131D, S134C/F, D156A, P302R/T and K305R were each identified in only one patient with classical RTT. The patients with these rare mutations could sit but not walk.

3.1.2. Nonsense mutations

Seven nonsense mutations including two new ones (S68X, Y141X) were observed in 57 cases (Table 2). Four nonsense mutations, R168X, R255X, R270X and R294X, were common and were detected in 17 (11.7%), 11 (7.6%), 15 (10.3%) and 11 (7.6%) out of 145 patients with mutations, respectively. Three other nonsense mutations, S68X, Y141X and Q170X were each detected in one patient with classical RTT. The clinical characteristics of patients with R168X, R255X and R270X were very similar to those of patients with T158M, suggesting that 44.8% of patients with classical RTT have T158M, R168X, R255X or R270X in Japan. Many patients with these mutations were not able to walk throughout their life and some patients who started to walk by six years of age became bedridden by 20 years of age. On the other hand, four patients with R294X showed slightly milder form of RTT, and were able to maintain walking in adulthood.

3.1.3. Deletion and insertion mutations

Eighteen different frameshift mutations were detected in 22 patients (Table 2). Most mutations were newly detected in this study. A single base pair deletion was observed in seven patients with classical RTT. The deletion resulted in loss of a methyl-CpG-binding domain (MBD) and a translation repression domain (TRD) of MeCP2 in two patients and loss of TRD in five patients. All patients with 1 bp deletion had classical RTT. A 2 base pair deletion was detected in two patients; one in exon 3 in a patient with classical RTT, whereas the clinical features in the patient with a 543–544(TC) deletion in exon 4 showed PSV of RTT. A 4 base pair deletion was identified in two patients with classical RTT.

Large deletions, 35 bp, 71 bp and 212 bp in the C-terminus of *MECP2* were each found in one patient with atypical RTT, and the three patients with a 44 bp (1155–1198) deletion in the C-terminus of *MECP2* had atypical RTT. Only one patient with 4 bp deletion in the C-terminus had classical RTT. In addition, we identified two patients with a 507 bp deletion (616–122) in exon 4 and a deletion and insertion of 3.6 kb (Ex3-1894-Ex4+65 deletion and Ex4+1-+919 del) with an inverted insertion of 119 bp (Ex4+712-+831 and an unknown sequence region 40 bp). Both had classical RTT.

Compared to deletion mutations, insertion mutations were rare and newly detected in this study, one base pair insertion, in different region of *MECP2* (243insC, 1194insC and 1320insT) was each found in one patient with classical RTT.

3.1.4. Splicing anomalies

Three new splicing anomalies were each detected in one patient with classical RTT. Mutations in the donor site of exon 1 (GT/AT) and exon 3 (GT/TT) were detected in two patients, and in the acceptor site of exon 4 (CAG/GAG) in one patient.

3.1.5. Non-pathogenic variations

Five non-pathogenic variations were detected in patients with RTT and their parents (Fig. 1, Table 3). One variation, A201V, was detected in three patients with RTT, one of their parents and healthy controls. The allele frequency of a rare allele, 201V, was 0.0147 in this Japanese population. G232A was also identified in patients with RTT and healthy controls. Allele frequency of the rare form of G232A was 0.071 in females and 0.048 in males (Table 3). In addition, three new missense mutations in exon 4 of *MECP2*, P176R (C527G), A378V (C1133T) and T479M (T1436T), were detected in patients with RTT, but these mutations were also detected in one of their parents and in unrelated healthy Japanese (Fig. 1). Then, these variations are not pathogenic, but polymorphic, although allele frequencies of rear alleles were less than 0.01 in the Japanese population (Table 3).

3.2. X-inactivation pattern

X-inactivation studies were performed in 123 (84.8%) in 145 patients with a *MECP2* mutation. There were 39 patients (31.7%) who were homozygous for the AR locus who were not informative to detect a skewed X-inactivation pattern. Skewed X-inactivation (greater than 75% expression of one AR allele) was found in 21 (25.0%) of 84 informative patients; four patients showed dominant inactivation by the maternal allele and 17 cases by the paternal allele of the AR allele. However, greater than 90% expression of one AR allele was identified in five patients (5.9%) in whom the *MECP2* mutations were T158M, R168X and 44 bp deletion in the C-terminus. Patients with skewed X-inactivation showed classical RTT and mild clinical features, as did the other patients with the same mutations. The clinical features were not modified by skewed X-inactivation in our study.

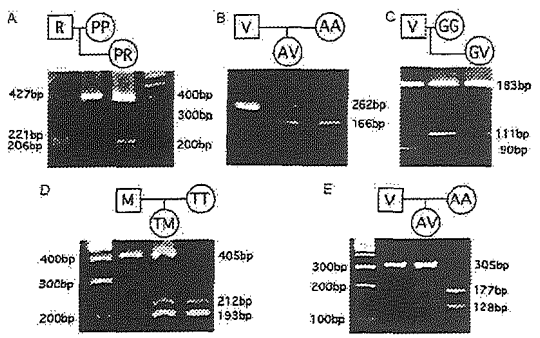

Fig. 1. Missense variations of *MECP2* in a Japanese population. A: three R176R genotypes (*Hha*I polymorphism) (father: hemizygous for a rare allele (R), child: heterozygous for two alleles (PR), mother: homozygous for a common allele (PP) of P176R), B: three A201V genotypes (*Fnu*4HI polymorphism) (father: hemizygous for a rare allele (V) child: heterozygous for two alleles (AV), mother: homozygous for a common allele (AA) of A20 of A201V), C: three G232A genotypes (*Hae*III polymorphism) (father: hemizygous for a rare allele (A), child: heterozygous for two alleles (GA), mother: homozygous for a common allele (GG) of P176R), D: three T479M genotypes (*Hga*I polymorphism) ((father: hemizygous for a rare allele (M), child: heterozygous for two alleles (TM), mother: homozygous for a common allele (TT) of T479M), and E: three A378V genotypes (*Hae*III polymorphism) (father: hemizygous for a rare allele (V), child: heterozygous for two alleles (AV), mother: homozygous for a common allele (AA) of A378V)).

4. Discussion

To date, 218 different mutations have been reported in a total of more than 2100 patients [26]. In our study, 45 different mutations of *MECP2* were identified in 145 of 219 (66.2%) sporadic female patients with classical or atypical RTT, but 74 patients (33.6%) including 16 with classical RTT did not have a *MECP2* mutation in the entire coding and flanking regions. *MECP2* mutations were found in 115 of 131 patients with classical RTT (87.8%), but in 30 of 88 patients with atypical RTT (34.1%) in this study. These results were compatible with many other studies, suggesting that *MECP2* mutations are present in 85–90% of patients with classical RTT and in 30–40% of patients with atypical RTT [9–14]. A number of authors have reported a relationship between type of *MECP2* mutations and the severity of clinical features in patients with RTT [8–16]. However, it has been difficult to evaluate distinct relationship in patients with RTT, because of small number of patients with the same mutations and the broad spectrum of phenotypes [11,16].

The most common missese mutation is T158M in patients with classical RTT in many studies including ours [25,26], suggesting that T158M has crucial function for binding CpG island in genomic DNA. Two missense mutations, R133C and R306C were identified in patients with a mild form of RTT including PSV, as previously reported [5,15–20]. Patients with other missense mutations had classical RTT and four new missense mutations, A131D, S134F, D156A, P302T, and K305R, were identified in patients with classical RTT. Then, more patients with the same missese mutations need to decide the distinct phenotypes of RTT in these missense mutations.

On the other hand, phenotypes in patients with nonsense mutations might be able to establish, because for mutations, R168X, R255X, R270X and R294X have been commonly observed in patients with classical in many studies [5–14]. All patients with three nonsense mutations, R168X, R255X and R270X, showed classical RTT in our study. However, four of 11 patients with R294X had atypical RTT in our study. R168X, R255X, R270X and R294X were not found in PSV of RTT [13]. Locations of many nonsense

Table 3
Non-pathogenic variations of *MECP2* in this study

Phenotype	Nucleotide change	Location	Recognition enzyme	Allele frequencies	
				Common type	Rare type
P176R	C527G	Between MBD/TRD	*Hha*I	0.993 (292)	0.007 (2)
A378V	C1133T	WDR	*Hae*III	0.993 (278)	0.007 (2)
T479M	T1436T	WDR	*Hga*I	0.996 (272)	0.004 (1)
A201V	C602T	TRD	*Fnu*4HI	0.993 (292)	0.007 (2)
G232A	G69SC	TRD	*Hae*III	0.936 (276)	0.064 (19)

Novel SNPs are denoted by bold letters. Numbers in parentheses are observed subjects in this study. MBD: methyl-CpG-binding domain, TRD: translation repression domain, WDR: group II WW domain binding region.

mutations are before nuclear localization signal (NLS) and truncated protein can not migrate in uncles. However, R294X retains NLS domain in truncated protein. Then, phenotypes in patients with mutations causing impairment of NLS are more severe compared to those with other mutations [14].

Frameshift mutations due to nucleotide deletion or insertion were identified in 23 of 145 (15.9%) patients. All these mutations introduced a premature terminal codon in *MECP2*. Large deletions in the C-terminus, 35 bp, 41 bp, 44 bp, 71 bp, 212 bp and 507 bp, were identified in hot spots (1152–1200) of deletions previously reported [9] and in group II WW domain binding region (WDR) of *MECP2* [41]. WDR which is a newly detected functional domain located in 325 aa to the C-terminus, with the interacting *prolin-rich sequence at its center* [41]. Then, dysfunction of WDR might introduce atypical RTT in females. The same deletion, 1155del 44, was identified in three patients who had atypical RTT. A large deletion, 3.6 kb, resulting in loss of three functional domains, MBD, TRD and NLS, of *MECP2* was found in one patient with classical RTT. Her clinical features were not different from those of other patients with classical RTT. Recently, gross rearrangements in *MECP2* were reported in several patients with RTT [18,23,24]. They recommended Southern blot analysis for screening for mutations in patients with RTT. However, the sizes of deletions in these patients except one were 5–9 kb, and break points were in intron 2 and exon 4 in these reports [23,24]. Long PCR is able to amplify around 10 kb, and might be able to define these gross deletions. Then, if long PCR amplification is not able to reveal any mutations, Southern blot might be useful for detection of gross rearrangements of *MECP2* in patients with classical RTT.

At least eight missense mutations were identified in screening of *MECP2* mutations in patients with XLMR in Caucasians [27–37]. A140V was identified in several families with XLMR, indicating that A140V causes nonspecific mental retardation in humans [27–30]. However, the remaining seven missense mutations were each identified in only one patient with autism or mental retardation; five were in functional domains–one in MBD and one in TRD, and three variations were located in WDR. Loss of WDR function causes XLMR in males. However, further study is needed to determine whether these mutations are pathogenic mutations in these patients and family studies are important to distinguish between a rare variation and a disease-causing mutation [36].

In addition, we detected five missense variations in exon 4 of *MECP2* in patients with RTT. Two of these mutations, A201V and G232A, were polymorphisms reported by Amano et al. (2000), and the frequency of a rare allele of G232A was 0.071 in females and 0.048 in males in this study, and 0.054 in a Japanese population studied by Amano et al. [10]. Three other missense mutations, P176R, A378V and T479M, were identified in patients with RTT and healthy Japanese. Allele frequencies of rare types of these variations were less than 0.01 in a Japanese population. Then, seven missense genetic variations, P176R, A201V, G232A, P251L, P376S, A378V and T479M, were non-pathogenic in the Japanese population. To date, 20 missense mutations were found in healthy individuals, indicating that these missense variations are non-pathogenic in Caucasians [6]. Five variations were between MBD and TDR, seven variations were in TRD and 14 variations were in the C-terminus, although missense mutation in the C-terminus of *MECP2* was detected in three patients with RTT; one missense mutation, P322A [13] and two in a terminal codon, X478C [13] and X478T [24]. We found only one of 27 variations, A201V, in our study. Two variations, A201V and P376S, were observed in Caucasians and Japanese, but other variations were specific to each population. These data suggest that rare variations in *MECP2* might differ among different ethic groups and that DNA substitutions in *MECP2* might occur frequently.

In conclusion, we detected 45 different mutations in the 145 patients with classical or atypical RTT. Five mutations, T158M, R168X, R255X, R270X and R294X, were common in patients with classical RTT and two mutations, R133C and R306C were common in patients with atypical RTT. New mutations, three splicing anomalies and a 3.6 kb deletion in exons 3 and 4 were identified in our study, in addition to three new rare SNPs, which might differ among ethnic groups. Non-pathogenic missense variations are very important for the molecular diagnosis of *MECP2* mutations.

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

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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 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).

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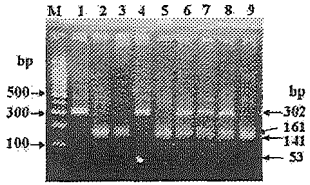
Subjects and methods

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

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hha*I, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-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), 200μM each dNTP, 5μM each primer, 0.5U *Taq* DNA polymerase and 50ng genomic DNA as a template with 35 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 1 min. PCR product size was 355bp, and the G allele was digested by *Hha*I to 53 + 141 + 161 bp, and the A allele to 53 + 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.



M: Size Marker
GG: 2, 3, 5, 9
GA: 6, 7, 8
AA: 1, 4

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

Results

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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Letter to the Editor

COH1 analysis and linkage study in two Japanese families with Cohen syndrome

To the Editor:

Cohen syndrome (MIM 216550) is an autosomal recessive disorder associated with mental retardation, characteristic facial appearance, hypotonia, retinochoroidal dystrophy and neutropenia (1). We previously reported a Japanese family with Cohen syndrome and pointed out the presence of two clinical phenotypes of Cohen syndrome: Finnish type and Jewish type (2), now called non-Finnish type or Cohen-like syndrome (3). Differential clinical findings in two types were retinochoroidal dystrophy and neutropenia, which were only observed in patients with Finnish type of Cohen syndrome (2). Over 100 cases of both types have been reported worldwide, presenting a wide spectrum of clinical features (4–10). However, clinical phenotypes were homogeneous in Finnish patients and distinct diagnostic criteria for Cohen syndrome have been proposed (3, 4).

The locus for Cohen syndrome was assigned to a 10-cM region in 8q22-q23 by linkage analysis in Finnish families (5). Refined linkage studies have suggested the localization of Cohen syndrome in DNA marker loci D8S1808 and D8S546 (6). Recently, a novel gene, *COH1*, was reported as a candidate gene for Cohen syndrome by Kolehmainen et al. (7). They screened *COH1* mutations in 27 Finnish patients and five non-Finnish patients with Cohen syndrome (7). Nine different mutations were identified in 31 patients, but 26 of 27 Finnish patients reported to have the same mutation, 2-bp deletion (c.3348-3349delCT) in exon 23 of *COH1*, which in 15 patients occurred in homozygous and in 11 in heterozygous form (7). Since then, over 50 different mutations in *COH1* have been reported in patients with Cohen syndrome (8–10), and allelic heterogeneity in *COH1* suggested clinical variability in Cohen syndrome (7–10). However, another genetic heterogeneity might exist in patients with Cohen syndrome, because one Finnish patient with Cohen syndrome did not have any mutations in *COH1* (7).

We carried out *COH1* analysis and genetic mapping in two Japanese families with Cohen syndrome whose clinical features were summarized in Table 1. Two affected brothers in family 1 were born from consanguineous healthy parents and had typical clinical features of Cohen syndrome including retinopathy and neutropenia (Table 1) (3). Using direct sequencing from lymphoblastoid cell RNA after reverse transcription polymerase chain reaction and genomic DNA, we identified a novel mutation, 2-bp deletion affecting codons 1936 and 1937 (c.5808-5809delTA) in exon 34 of *COH1* that leads to a frameshift and premature stop codon 11 amino acids downstream in family 1 (Fig. 1, a1). Furthermore, two affected brothers in family 1 were homozygous for the same haplotype of five polymorphic DNA markers, D8S506, D8S1789, D8D559, D8S546 and D8S1762, in 8q22-q23 (Fig. 1, b1).

On the other hand, two affected siblings in family 2 were born from non-consanguineous healthy parents and had clinical features of Cohen syndrome, but they did not have microcephaly, retinopathy and neutropenia (Table 1). No mutations in *COH1* were identified and haplotypes of five DNA markers were different in two siblings in family 2 (Fig. 1, b2). These data suggest that clinical features are not associated with *COH1* in family 2, because D8S1789 is located in intron 33 of *COH1*.

Kolehmainen et al. (9) reported that DNA markers flanking *COH1* were not linked with clinical features in four of 12 families with Cohen-like syndrome. Most patients with Cohen-like syndrome did not have retinopathy and neutropenia (9). Hennies et al. (8) also suggest that early-onset myopia, retinopathy and neutropenia are essential clinical features in patients with *COH1* mutations. These data suggest that Cohen-like syndrome is caused by different gene(s) from *COH1*. The D8S1789 is the most useful DNA marker for linkage study of Cohen syndrome prior DNA analysis of *COH1* in families with Cohen syndrome and Cohen-like syndrome.

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Table 1. Major clinical features of Japanese patients with Cohen syndrome in two families

Clinical characteristics	Family 1		Family 2	
	Sibling 1	Sibling 2	Sibling 1	Sibling 2
Age examined (years)	21	15	15	13
Gender	Male	Male	Male	Female
Growth and development				
Psychomotor retardation	+	+	+	+
Short stature (SD)	−1.2	−1.4	−5.3	−3.1
Truncal obesity	+	+	+	+
Mild hypotonia	+	+	+	+
Cheerful disposition	+	+	+	+
Craniofacial manifestations				
Microcephaly (SD)	**−2.0**	**−2.0**	−1.0	+1.7
Thick eyebrows	+	+	+	+
Prominent root of nose	+	+	+	+
Down-slanted eyes	+	+	+	+
High nasal bridge	+	+	+	+
Short philtrum	+	+	+	+
Prominent upper central incisors	+	+	+	+
Open mouth	+	+	+	+
Limbs				
Long/narrow hands and feet	+	+	+	+
Hyperextensible joints	+	+	+	+
Ophthalmologic findings				
Progressive hyper myopia	+	+	−	−
Strabismus	+	+	+	+
Retinochoroidal dystrophy	+	+	−	−
Peripheral blood counts				
Granulocytopenia	+	+	−	−
White blood count (/mm^3)	**3000–3200**	**3300**	5280–7140	3900–5980

Bolded entities have the frequency of over 80% in Cohen patients reported by Kivitie-Kallio and Norio (4).

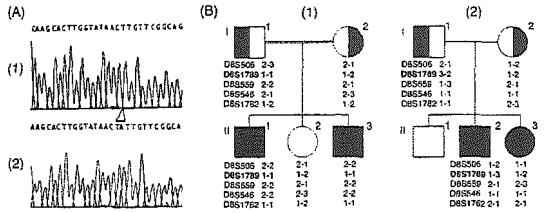

Fig. 1. (a) Sequence analysis of a portion of exon 34 of *COH1* in family 1. (1) c.5808-5809delTA in exon 34 of *COH1* in two affected siblings. (2) Normal sequence. (b) Pedigrees of families 1 (1) and 2 (2) with haplotypes at marker loci mapped to Cohen syndrome on chromosome 8. Solid squares and circle show individuals with Cohen syndrome, open circle shows healthy sister and half-solid squares and circles show obligate carriers for Cohen syndrome. Bold genotypes indicate the DNA marker, D8S1789, in *COH1*.

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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.

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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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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) APOEA(−) 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.

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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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Abstract

Background: The apolipoprotein ε 4 (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.[13,25–27] For VD, we used criteria presented at the NINDA-AIREN International Workshop.[28] Mixed dementia (MD) can be independently diagnosed as either AD alone or VD alone based on clinicopathological findings, and is considered a combination of the two. In the present study, we placed it in a separate category. Diagnosis of Parkinson's disease (PD) was carried out according to criteria proposed by Gibb and Lees[29] and Calne et al.[30]

Clinical neuropathological diagnoses of DLB were made based on the DLB guidelines[31] and Kosaka's classification system.[32] We classified DLB into three groups according to Lewy bodies distribution as Table 1. In the brain stem type, Lewy bodies are located only in the brain stem, that is identical PD. In the limbic type, there are many Lewy bodies in the brain stem and diencephlon, but fewer in the cerebral cortex. In the neocortical type, numerous Lewy bodies are distributed both in the brain stem and diencepahlon 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.