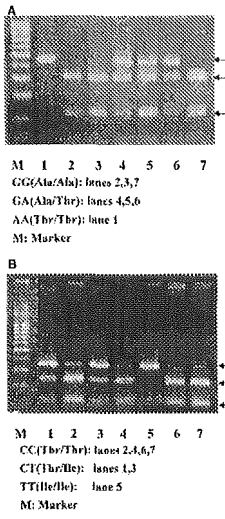

Fig 1. Demonstration of the existence in the human population of Thr/Ala-147 (A) and Thr/Ile-325 (B) polymorphisms in seven typical samples. Each genomic DNA extracted from brain tissues was subjected to polymerase chain reaction (PCR) to amplify a fragment encompassing the codon for the amino acids 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-001187 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 (85)*	*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	1	9	10	11	58 (53)
Allele Thr (%)	0 (0)	10 (28)	9 (23)	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 36·19 ($P < 0.001$) for the Thr/Ile-325 polymorphism and 33·98 ($P < 0.001$) 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 360, 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 (31.3%) 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, P152R, A70V 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

Clinical form	Mutations in the *MECP2*		
	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′-TAGAGAGGAGGGACGCCATC-3′, Ex1-R: 5′-CTCGCCAATTACGGCATCG-3′, Int 2-1F: 5′-AGTTCCTGGTGTGTCCTTCC-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
L134F (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)	6
			Classical RTT (1)	
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)	8
			Atypical RTT (4)	
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)	Ex3 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 (1)	1155–1198	WDR		
71 bp del (1)	1133–1203	WDR	Atypical RTT	24
Splicing anomalies (3)				
Ex1 donor site (1)		GTAT	Classical RTT	This report
Ex3 donor site (1)		GTTT	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.

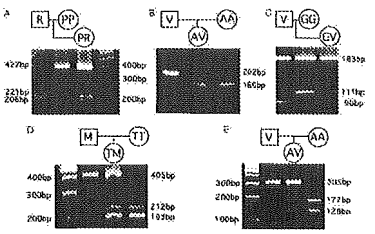

Fig. 1. Missense variations of *MECP2* in a Japanese population. A: three R176R genotypes (*Hha*I polymorphism) (father: hemizygous for a rare allele (R), child: heterozygous for two alleles (PR), mother: homozygous for a common allele (PP) of P176R). B: three A201V genotypes (*Fau*4III polymorphism) (father: hemizygous for a rare allele (V) child: heterozygous for two alleles (AV), mother: homozygous for a common allele (AA) of A201 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	*Fau*4III	0.993 (292)	0.007 (2)
G232A	G695C	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 nucles. 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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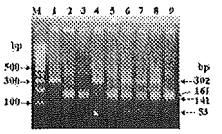
Subjects and methods

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) (rs3401[1]) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hhal*, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGC AATTCTCCTCCTCCTT-3′) and FGF1-R (5′-CCACTTCAACCGCATT ATGGTCG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5mM $MgCl_2$, 50mM KCl, and 10 mM Tris–HCl, pH 8.3), 200μM each dNTP, 5μM each primer, 0.5 U *Taq* DNA polymerase and 50ng genomic DNA as a template with 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. PCR product size was 355bp, and the G allele was digested by *Hhal* to 53 − 141 − 161 bp, and the A allele to 53 − 302bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.



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

Results

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

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

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614 to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

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

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

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

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

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

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

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

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

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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

	Family 1		Family 2	
Clinical characteristics	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).

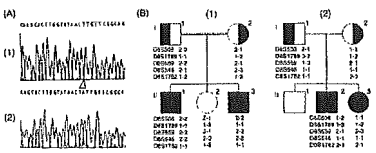

Fig. 1. (a) Sequence analysis of a portion of exon 34 of *COH1* in family 1. (1) c.5808-5809delTA in exon 34 of *COH1* in two affected siblings. (2) Normal sequence. (b) Pedigrees of families 1 (1) and 2 (2) with haplotypes at marker loci mapped to Cohen syndrome on chromosome 8. Solid squares and circle 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.[9,11] 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; hCV2596759

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

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

	Gene Name	rs number	Celera	Location		AD : Control (Genotype)	P value	AD : Control (allele)	P value	OR (95% CI) APOE4(−) Subjects
Down-regulated	*POU2F1*	rs1407814	hCV1622428	intron 2	9943T/C	TT/TC/CC = 11/90/275 : 2/65/309	0.0022**	T/C = 112/640 : 69/683	0.0007**	1.73 (1.11–2.69)
	MYH8	rs2024076	hCV2179407	intron 18	+3583T/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 : 51/183/143	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	hCV2947951	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 (0.76–1.36)
	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	6090T/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.

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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,[16] and APOE genotyping was performed using DNA samples extracted from dissected brain tissues obtained between January 1993 and July 2002, after obtaining the agreement of the patients' guardians for use of these tissues for the purpose of diagnosis, research and genetic analysis. The present study was approved by the ethics committee of Fukushimura Hospital. The patients consisted of 122 men and 133 women, with a mean ± SD age at death of 82.3 years ±8.5, range 44–102 years.

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

Autopsy and sampling of brain tissues

The brain was removed at autopsy, weighed, cut midsagittally and examined for vascular and other macroscopically detectable lesions. Specimens for diagnostic examination were taken from the hemisphere showing abnormal findings by computed tomography scanning, or from the left hemisphere when no difference between the left and the right was found, and fixed in 4% paraformaldehyde (PFA) as a hemisphere block. The other hemisphere was divided into several regions. Some samples of lesions were frozen for further analyzes and stored at −80°C, while other areas were removed and fixed in 4% PFA for immunohistochemical analysis.

Samples for diagnostic purposes were taken from the frontal, temporal, parietal and occipital lobes, hippocampal formation, amygdala, basal ganglia, thalamus, and the midbrain including the substantia nigra, pons, medulla, and cerebellum. The specimens were embedded in paraffin and processed into 5 μm sections for conventional histological and immunohistochemical examination.

Neuropathological diagnostic criteria

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

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

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

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

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

Apolipoprotein E subtyping

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

Statistical analysis

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

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

	Brain stem (%)	Limbic (%)	Neocortical (%)	PBC (%)
APOE genotype				
2/2	0	0	0	1 (0.3)
2/3	0	0	0	32 (8.2)
2/4	0	0	0	3 (0.8)
3/3	6 (67)	7 (64)	7 (64)	293 (75.7)
3/4	3 (33)	4 (36)	3 (27)	55 (14.2)
4/4	0	0	1 (9)	3 (0.8)
Total	9 (100)	11 (100)	11 (100)	387 (100)
APOE allele				
ε2	0	0	0	37 (4.8)
ε3	15 (83)	18 (82)	17 (77)*	673 (87.0)
ε4	3 (17)	4 (18)	5 (23)*	64 (8.2)

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

RESULTS

Frequencies of neuropathological findings

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

Frequencies of apolipoprotein E alleles and genotypes

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

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

Analysis of apolipoprotein E genotypes in the main neurological groups

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

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

Apolipoprotein E analysis of amyloid-β and tangle diseases

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

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

	Men (%)	Women (%)	Total (%)	Mean ± SD age at death (years)
FBB samples				
AD	36 (30)	54 (41)	90 (35)	83.5 ± 7.52
DLB	14 (11)	17 (13)	31 (12)	80.0 ± 9.46
VD/CI	56 (46)	57 (43)	113 (44)	82.2 ± 7.93
LNTD	2 (2)	2 (2)	4 (2)	95.0 ± 5.72
Control brain	10 (8)	10 (8)	20 (8)	86.8 ± 5.50
Total	122	133	255	82.3 ± 8.49
PBC samples	174	213	387	75.3 ± 5.0[b]

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

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

	Age (years)			
	≤69 (%)	70–79 (%)	80≤ (%)	Total (%)
APOE genotype				
2/2	0	0	0	0
	0	1 (0.3)	0	1 (0.2)
2/3	1 (4.8)	4 (6.2)	4 (2.4)	9 (3.5)
	1 (4.8)	23 (7.6)	8 (12.5)	32 (8.2)
2/4	1 (4.8)	2 (3.1)	1 (0.6)	4 (1.6)
	0	3 (1.0)	0	3 (0.8)
3/3	13 (61.9)	40 (61.5)	117 (69.2)	170 (66.7)
	15 (71.4)	230 (77.2)	48 (75.0)	293 (75.7)
3/4	4 (19.0)	16 (24.6)	42 (24.9)	62 (24.3)
	4 (19.0)	43 (14.3)	8 (12.5)	55 (14.2)
4/4	2 (10.5)	3 (4.6)	5 (3.0)	10 (3.9)
	1 (4.8)	2 (0.7)	0	3 (0.8)
Total	21	65	169	255
	21	302	64	387
APOE allele				
E2	2 (4.8)	6 (4.6)	5 (1.5)	13 (2.5)
	1 (2.4)	26 (4.5)	8 (6.3)	37 (4.8)
E3	31 (73.8)	100 (76.9)	280 (82.6)	411 (80.6)
	35 (83.3)	526 (87.1)	112 (87.4)	673 (87.0)
E4	9 (21.4)	24 (18.5)	53 (15.7)	86 (16.9)
	6 (14.3)	50 (8.3)	8 (6.3)	64 (8.2)

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

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

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

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

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

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

Apolipoprotein E analysis of dementia with Lewy bodies subtypes

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

Table 5 Distribution of apolipoprotein E (APOE) subtypes of patients with Aβ and/or NFT deposition diseases compared with FBB normals and PBC[7]

	LOAD (%)	EOAD (%)	LNTD (%)	PBC (%)
APOE genotype				
2/2	0	0	0	1 (0.3)
2/3	1 (1)	0	0	32 (8.2)
3/4	1 (1)	0	0	3 (0.8)
3/3	38 (50)	6 (43)	3 (75)	293 (75.7)
3/4	31 (82)	6 (43)	1 (25)	55 (14.2)
4/4	5 (6)	2 (14)	0	3 (0.8)
Total	76 (100)	14 (100)	4 (100)	387 (100)
APOE allele				
E2	2 (1)	0	0	37 (4.8)
E3	108 (71)	16 (54)	7 (88)	673 (87.0)
E4	42 (28)*	10 (36)**	1 (12)	64 (8.2)

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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Regular Article

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

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

A retrospective study was conducted on 66 patients who underwent treatment of hyperlipidemia with HMG-CoA reductase inhibitors in a municipal hospital in a community-based cohort of Ehime prefecture in the southern part of Japan. Plasma lipid concentrations before and after administration were analyzed in patients in relation to the 521T/C (Val-174→Ala) polymorphism in the OATP-C gene (TT: n = 44 (66.7%), TC: n = 20 (30.3%), CC: n = 0 (0.0%), undetermined: n = 2 (3.0%)). Total cholesterol level was significantly lowered after treatment with HMG-CoA reductase inhibitors in all patients ($p<0.001$); moreover, subjects with the 521C allele showed an attenuated total-cholesterol-lowering effect compared with those homozygous for the 521T allele ($-22.3\pm8.7\%$ vs. $-16.5\pm10.5\%$, $p<0.05$).

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

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

Introduction

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

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

Methods

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

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

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

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

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

Results

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

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

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

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

Table 1. Baseline characteristics ($n=66$)

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

N.D.; not determined

Table 2. Lipid concentrations in patients treated with statins

	n		Pre (mg/dL)	Post (mg/dL)	% Change (95% CI, LL/UL)*	p
Total	66	TC	259.2±33.6	203.7±28.7	−20.9 (−23.3/−18.5)	<0.001
	59	LDL-C	167.0±39.3	119.1±24.5	−28.3 (−32.2/−24.3)	<0.001
	62	TG	176.9±131.7	126.1±63.9	−7.6 (−21.6/6.4)	<0.01
	59	HDL-C	58.7±19.6	59.9±14.8	4.6 (0.1/9.2)	0.275
Pravastatin	22	TC	253.6±33.5	208.3±28.5	−17.5 (−21.3/−13.6)	<0.001
	21	LDL-C	161.2±32.3	122.9±20.1	−23.0 (−29.0/−17.0)	<0.001
	21	TG	159.1±83.8	148.2±86	6.8 (−20.3/33.9)	0.555
	20	HDL-C	59.0±12.8	57.5±12.2	−2.0 (−68.0/2.8)	0.302
Atorvastatin	11	TC	249.5±36.9	198.5±31.9	−20.3 (−24.4/−16.1)	<0.001
	8	LDL-C	139.2±54.2	102.2±19	−34.8 (−41/−28.5)	<0.05
	10	TG	282.9±266.1	139.7±69.8	−7.9 (−58.9/43.1)	0.152
	9	HDL-C	56.2±16.0	64.9±12.5	10.7 (−1.43/22.8)	0.059
Simvastatin	33	TC	266.1±32	202.4±28.2	−23.4 (−27.2/−19.6)	<0.001
	30	LDL-C	180.2±33.0	122.2±21.1	−30.2 (−36.5/−23.9)	<0.001
	31	TG	154.8±69.9	106.8±33.1	−17.2 (−33.8/−0.7)	<0.001
	30	HDL-C	58.8±24.4	60.0±17.3	7.2 (−0.4/14.9)	0.582

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

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

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

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

($p=0.010$). 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.[25] 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 Mwinyi *et al.*.[28] On the other hand, Niemi *et al.* recently reported no gene-dose effect of the 521T>C variant on the systemic exposure to pravastatin.[29] Haplotype analysis revealed that the haplotype containing the −11187G>A, 388A>G and 521T>C SNPs had a particularly pronounced effect on the $AUC_{0\text{-}12h}$ 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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