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Dynamin-binding protein gene on chromosome 10q is associated with late-onset Alzheimer's disease

Ryozo Kuwano[1,2,*], Akinori Miyashita[1,2], Hiroyuki Arai[3], Takashi Asada[4], Masaki Imagawa[5], Mikio Shoji[6], Susumu Higuchi[7], Katsuya Urakami[8], Akiyoshi Kakita[9], Hitoshi Takahashi[9], Tamao Tsukie[1], Shinichi Toyabe[10], Kohei Akazawa[10], Ichiro Kanazawa[11] and Yasuo Ihara[12]; The Japanese Genetic Study Consortium for Alzheimer's Disease

[1]Genome Science Branch, Center for Bioresource-Based Researches, Brain Research Institute, Niigata University, Niigata 951-8585, Japan, [2]Center for Transdisciplinary Research, Niigata University, Niigata 951-8585, Japan, [3]Department of Geriatric and Complementary Medicine, Advanced Research Center for Asian Traditional Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan, [4]Department of Psychiatry, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8575, Japan, [5]Department of Neuropsychiatry, Imagawa Clinic, Fukushima-ku, Osaka 553-0003, Japan, [6]Department of Neurology, Neuroscience and Biophysiological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan, [7]Division of Clinical Research, Kurihama Alcoholism Center, National Hospital Organization, Yokosuka 239-0841, Japan, [8]Department of Biological Regulation, Section of Environment and Health Science, Faculty of Medicine, Tottori University, Yonago 683-8503, Japan, [9]Department of Pathology and the Resource Branch for Brain Disease, Brain Research Institute, Niigata University, Niigata 951-8585, Japan, [10]Department of Medical Informatics, Niigata University, Niigata 951-8520, Japan, [11]National Center for Neurology and Psychiatry, Kodaira 187-8502, Japan and [12]Department of Neuropathology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

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The apolipoprotein E (*APOE*) gene has been consistently shown to be a major genetic risk factor; however, all cases of Alzheimer's disease (AD) cannot be attributed to the *ε4* variant of *APOE*, because about half of AD patients have the *APOE-ε3*3* genotype. To identify an additional genetic risk factor(s), we performed large-scale single nucleotide polymorphism (SNP)-based association analysis of 1526 late-onset AD patients and 1666 control subjects in a Japanese population. We prepared two independent sets consisting of exploratory and validation samples, respectively, with only the *APOE-ε3*3* genotype, and first carried out genotyping for the exploratory set with 1206 SNPs in the region between 60 and 107 Mb on chromosome 10q that is implicated by linkage studies as containing an AD susceptibility locus. Thirty-five SNPs that showed significant values ($P < 0.01$) were followed-up to detect any association with the validation samples. Finally, six SNPs exhibited replicated significant associations ($P = 0.000035–0.00048$) on meta-analysis of both sets. These SNPs were clustered in a locus spanning 220 kb at genomic position 101 Mb, and three of the six SNPs were located in the dynamin-binding protein (*DNMBP*) gene. Quantitative real-time RT–PCR analysis demonstrated that neuropathologically confirmed AD brains exhibit a significant reduction of *DNMBP* mRNA compared with age-matched ones ($P < 0.0169$). Thus, we confirmed the association of *DNMBP* with AD individuals with the *APOE-ε3*3* genotype or lacking the *ε4* allele, and *DNMBP* may be one of the susceptibility genes for AD.

*To whom correspondence should be addresssed: Tel: +81 25 227 2343; Fax: +81 25 227 0793; Email: ryosun@gene.med.niigata-u.ac.jp

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INTRODUCTION

Alzheimer's disease (AD) is neuropathologically characterized by a loss of synapses, extracellular deposition of amyloid β-protein (Aβ), intracellular formation of neurofibrillary tangles and neuronal cell death. AD is thought to be a multifactorial disease probably caused by complicated interactions between genetic and environmental factors. The apolipoprotein E (*APOE*) gene located on chromosome 19q13.2 was the first identified genetic risk factor associated with late-onset AD (LOAD) (1,2). To date only the *APOE-ε4* allele has been universally recognized as a major risk factor for LOAD and also as being associated with lowering of the age at onset (AAO) (3). However, about 50% of AD patients do not carry the *APOE-ε4* allele, and only 20% of AD patients and <10% of the variance in AAO appear to be explained by the *APOE-ε4* allele (4,5). The sibling recurrence risk ratio (λ_s) (6) in AD was estimated to be 3.5–5 based on epidemiological data. Assuming that *APOE* was the only genetic risk factor, gene-specific λ_{gs} (*APOE*) was estimated to be 1.7–2.5. Because $\lambda_s > \lambda_{gs}$ (*APOE*), there should be an additional genetic risk factor(s) for AD (7,8). The identification of an additional genetic risk factor(s) would greatly facilitate our understanding of the neuropathological findings, the clinical manifestations and the varying responses to drugs.

Genome-wide linkage studies on LOAD have provided informative data on putative susceptibility genes on several chromosomes (9–15). Chromosomes 1, 9, 10, 12, 21 and X have linkage peaks that have also been observed in *APOE-ε4* negative sib pairs (9,10). There are several candidate genes that are near the chromosome 10 linkage peaks and that are thought to be implicated in LOAD, including the catenin (cadherin-associated protein) alpha 3 (*CTNNA3*) (16,17), the insulin-degrading enzyme (*IDE*) (11,18–21), the urokinase-plasminogen activator (*PLAU*) (22–24) and the glutathione *S*-transferase omega-1 and 2 (*GSTO1* and *GSTO2*) (25–27) genes. However, the association results regarding these candidate genes have not been consistently replicated.

To determine whether or not there are additional genes causing susceptibility to *APOE-ε4* negative LOAD, we screened the region between 60 and 107 Mb on chromosome 10q with 1206 single nucleotide polymorphisms (SNPs). We found that the gene encoding dynamin-binding protein (DNMBP) (28) was significantly associated with LOAD lacking the *APOE-ε4* allele. DNMBP was discovered as a novel scaffold protein that brings the dynamin and actin regulatory proteins together and is concentrated at synapses in the brain. To date expression of its gene in the brain has not been demonstrated. In this study quantitative real-time RT–PCR analysis clearly showed that neuropathologically confirmed LOAD brains contain significantly reduced levels of *DNMBP* mRNA compared with those in age-matched controls. On microarray analysis, the gene expression related to synaptic vesicle trafficking was found to be decrease in the frontal cortex of AD patients (29). In addition, Aβ induces a decrease in the dynamin I level at synaptic sites in rat cultured hippocampal neurons (30). In view of the fact that synaptic dysfunction precedes Aβ deposition in the brains of AD patients (31), our observations raise the possibility that DNMBP, as a risk factor, might play a predominant role in the early stage of LOAD with *APOE-ε3*3* or lacking the *APOE-ε4* allele.

RESULTS

Genetic screening

Although the *APOE-ε4* allele is a strong genetic risk factor for LOAD, about half of the Japanese LOAD patients (46%) have the *APOE-ε3*3* genotype (Table 1). To identify an additional susceptibility gene(s) for LOAD with the *APOE-ε3* allele, we performed SNP-based two-step screening. This two-step procedure was used to examine an exploratory sample set to find significant markers and to confirm their significance with a validation sample set. Both *APOE-ε4* positive and negative subjects show linkage peaks for chromosomes 1, 9, 10, 12, 21 and X (9,10). In this study, we screened a wide region of chromosome 10q showing linkage peaks with a relatively high density of SNP markers. With this strategy, we selected 1322 SNPs (Supplementary Material, Tables S1 and S2) in the region from 60 to 107 Mb on chromosome 10q, of which 1206 were polymorphic, finding no significant deviation ($P > 0.05$) from the Hardy–Weinberg equilibrium (HWE) in a Japanese population.

Using these 1206 SNPs, we first scanned an exploratory sample set. In the first-step screening, 131 of the 1206 SNPs showed allelic *P*-values of <0.05, and 35 of them showed more significant values (allelic $P < 0.01$). To determine whether or not these markers exhibit associations, we genotyped these apparently significant SNPs in another sample set (validation samples), because a large number of SNPs probably exhibit false-positive associations. Replication in both exploratory and validation sample sets strongly suggests a true association of particular SNPs with LOAD. As a result, six of the above 35 SNPs (rs911541, rs3740066, rs11190302, rs11190305, C_11214959_10 and rs3740058) exhibited replicable associations (allelic $P < 0.05$), which remained significant on Mantel–Haenszel meta-analysis of the two sample sets ($P = 0.00003485$–0.0004757) (Table 2). These *P*-values remained at significant levels even after Bonferroni's correction with 35 tests ($P = 0.001220$–0.01665).

The six significant SNPs were located in a region spanning ~220 kb between 101.4 and 101.6 Mb on chromosome 10q (Table 3 and Fig. 1). This associated region contains five genes: the ectonucleoside triphosphate diphosphohydrolase 7 (*ENTPD7*), the cytochrome c oxidase subunit 15 (*COX15*), the cutC copper transporter homolog (*Escherichia coli*) (*CUTC*), the ATP-binding cassette sub-family C member 2 (*ABCC2*) and *DNMBP* genes. SNP rs911541, lying about 171 kb apart from rs3740066, is located in the third intron of *ENTPD7*, which consists of 13 exons. Five SNPs, i.e. rs3740066, rs11190302, rs11190305, C_11214959_10 and rs3740058, are clustered in an ~51.8 kb region including the 3′ flanking regions of both *ABCC2* and *DNMBP* (Fig. 1). SNP rs3740066 is a synonymous SNP (ATC → ATT, Ile1324Ile) as to exon 28 in *ABCC2* that comprises 32 exons. SNP rs11190302 is an intergenic SNP between *ABCC2* and *DNMBP*, lying about 7.0 kb from rs11190305 in *DNMBP*. The other three SNPs, i.e. rs11190305, C_11214959_10 and rs3740058, spanning about 16.1 kb have been mapped to

Table 1. Summary of overall subjects collected by JGSCAD

Overall subjects	LOAD		Control	
	Female	Male	Female	Male
Number of subjects	1103	423	998	668
AAO/AAE (mean ± SD)	73.5 ± 6.6	73.3 ± 6.6	73.0 ± 7.9	73.1 ± 7.7
MMSE (mean ± SD)	15.7 ± 7.0	18.4 ± 6.6	28.0 ± 1.8	28.1 ± 1.8
APOE				
*2*2*	0	1	2	1
*2*3*	31	18	77	55
*2*4*	13	4	9	6
*3*3*	491	208	748	495
*3*4*	465	148	152	104
*4*4*	103	44	10	7
*APOE-ε3*3 sample set*				
Exploratory				
Number of subjects	249	114	223	114
AAO/AAE (mean ± SD)	74.3 ± 6.2	74.6 ± 6.8	80.2 ± 4.1	80.6 ± 4.0
Validation				
Number of subjects	242	94	213	159
AAO/AAE (mean ± SD)	75.0 ± 7.7	72.6 ± 7.6	75.5 ± 4.7	75.7 ± 4.5

AAE, age at examination of control subjects. The *APOE-ε3*3* sample sets (exploratory and validation) used in the first- and second-step screenings are also summarized.

DNMBP, which consists of 17 exons. SNP rs11190305 is a non-synonymous SNP (TGT → TGG, Cys1413Trp) as to exon 16. Both C_11214959_10 and rs3740058 are intronic SNPs, the former being in intron 11 and the latter in intron 10.

Stratified analysis with entire samples

As the next step, the significantly replicated markers were tested by stratified analysis using entire samples with all *APOE*-genotypes, i.e. in 1526 LOAD samples and 1666 control samples (Table 4). It became clear that the six SNPs were strongly associated with *APOE-ε4* negative LOAD (sample set Negative-ε4: range of allelic $P = 0.00005699$–0.001164), whereas none of the six SNPs showed any significant association with *APOE-ε4* positive LOAD at all (sample set Positive-ε4: range of allelic $P = 0.7271$–0.988). We cannot exclude the possibility that the lack of significance may be due to the small sample size, because the *APOE-ε4* allele is fairly rare in controls (frequency 0.0915) in comparison with in LOAD (frequency 0.3027).

Linkage disequilibrium and case–control haplotype analyses

To further characterize these significantly associated SNPs detected on the two-step screening, linkage disequilibrium (LD) and case–control haplotype analyses were performed (Tables 5 and 6). Pair-wise LD measures, $|D'|$, are given in Table 5 for LOAD and control subjects with the *APOE-ε3*3* genotype. We found a strong correlation between the six SNPs and confirmed that the LD block was highly structured in the associated locus identified here. There was no difference in the LD structure between LOAD and control subjects.

In this LD block, five common haplotypes were inferred with the expectation–maximization (EM) algorithm in four sample sets examined: [H1]A-C-C-T-G-G, [H2]G-T-T-G-C-A, [G3]G-T-T-G-C-G, [H4]A-T-T-G-C-A and [H5]A-C-T-G-C-A (Table 6). Haplotype H1, composed of all major alleles of the six SNPs, exhibited the highest frequency (range 0.7118–0.7681) and haplotype H5 the lowest (range 0.0087–0.0188). Haplotype H2 consisted of all minor alleles. For case–control haplotype analysis, the haplotype frequencies in LOAD subjects were compared with those in controls. In the Positive-ε4 sample set, no haplotypes exhibited a significant difference. Global tests also did not give statistically significant results ($P = 0.7872$, permutation $P = 0.8388$). However, regarding All, Negative-ε4 and ε3*3, two haplotypes, H1 and H2, showed significant association. The most significant difference between these two haplotypes was observed for the sample set composed of only *APOE-ε3*3* subjects: H1, $P = 0.0001958$ and permutation $P = 0.0001$; H2, $P = 0.00006021$ and permutation $P < 0.0001$. The frequency of the most common haplotype, H1, was significantly decreased in LOAD, whereas that of the H2 haplotype comprising all minor alleles was significantly increased in LOAD. In contrast, the other three haplotypes, H3, H4 and H5, exhibited no association in any sample set. These findings do not appear to be more significant than the results for the individual SNP, which may be due to the fact that the six SNPs are in one strong LD block and have very similar minor allele frequencies.

Expression of *DNMBP* in the human brain

As *DNMBP* was found to be genetically associated with LOAD, we measured the expression levels of *DNMBP* in autopsy-confirmed LOAD brains using quantitative real-time RT–PCR (Fig. 2). A summary of the brains examined is given in Supplementary Material, Table S3. The mean age at death for LOAD was 76.1 ± 5.5 years, which was not significantly higher ($P = 0.0725$) than that for controls (72.7 ± 6.1 years). To select an appropriate internal standard for the normalization of *DNMBP* mRNA levels, we evaluated, using the TaqMan® Human Endogenous Control Plate

Table 2. Statistics for six replicated SNPs found on the two-step screening involving *APOE-e3*3*

dbSNP/Celera	Exploratory								Validation								Exploratory + validation[a]	
	Genotype	Number of subjects (frequency)		Allele	Number of alleles (frequency)		*P*-value	OR (95% CIs)	Genotype	Number of subjects (frequency)		Allele	Number of alleles (frequency)		*P*-value	OR (95% CIs)	*P*-value	OR (95% CIs)
		LOAD	Control		LOAD	Control				LOAD	Control		LOAD	Control				
rs911541	GG	19 (0.0534)	14 (0.0420)	G	157 (0.2205)	110 (0.1652)	0.009394	1.43 (1.09–1.87)	GG	19 (0.0578)	12 (0.0324)	G	146 (0.2219)	124 (0.1676)	0.01020	1.42 (1.09–1.85)	0.0002612	1.42 (1.18–1.72)
	GA	119 (0.3343)	82 (0.2462)	A	555 (0.7795)	556 (0.8348)			GA	108 (0.3283)	100 (0.2703)	A	512 (0.7781)	616 (0.8324)				
	AA	218 (0.6124)	237 (0.7117)	Sum	712	666			AA	202 (0.6140)	258 (0.6973)	Sum	658	740				
	Sum	356	333						Sum	329	370							
rs3740066	TT	27 (0.0752)	16 (0.0482)	T	182 (0.2535)	127 (0.1913)	0.005546	1.44 (1.11–1.86)	TT	27 (0.0833)	14 (0.0380)	T	169 (0.2608)	141 (0.1916)	0.002054	1.49 (1.16–1.92)	0.00003485	1.46 (1.22–1.75)
	TC	128 (0.3565)	95 (0.2861)	C	536 (0.7465)	537 (0.8087)			TC	115 (0.3549)	113 (0.3071)	C	479 (0.7392)	595 (0.8084)				
	CC	204 (0.5682)	221 (0.6657)	Sum	718	664			CC	182 (0.5617)	241 (0.6549)	Sum	648	736				
	Sum	359	332						Sum	324	368							
rs11190302	TT	29 (0.08010)	15 (0.0457)	T	189 (0.2640)	131 (0.1997)	0.004923	1.44 (1.12–1.85)	TT	27 (0.0811)	14 (0.0381)	T	170 (0.2553)	151 (0.2057)	0.02770	132. (1.03–1.70)	0.0003953	1.38 (1.15–1.65)
	TC	131 (0.3659)	101 (0.3079)	C	527 (0.7360)	525 (0.8003)			TC	116 (0.3483)	123 (0.3351)	C	496 (0.7447)	583 (0.7943)				
	CC	198 (0.5531)	212 (0.6463)	Sum	716	656			CC	190 (0.5706)	230 (0.6267)	Sum	666	734				
	Sum	358	328						Sum	333	367							
rs11190305	GG	27 (0.0763)	14 (0.0420)	G	184 (0.2599)	134 (0.2012)	0.009946	1.39 (1.08–1.80)	GG	27 (0.0821)	14 (0.0385)	G	171 (0.2599)	150 (0.2060)	0.01770	1.35 (1.05–1.74)	0.0004675	1.37 (1.15–1.64)
	GT	130 (0.3672)	106 (0.3183)	T	524 (0.7401)	532 (0.7988)			GT	117 (0.3356)	122 (0.3352)	T	487 (0.7401)	578 (0.7940)				
	TT	197 (0.5565)	213 (0.6396)	Sum	708	666			TT	185 (0.5623)	228 (0.6264)	Sum	658	728				
	Sum	354	333						Sum	329	364							
C_11214959_10	CC	29 (0.0817)	15 (0.0450)	C	188 (0.2648)	135 (0.2027)	0.006617	1.42 (1.10–1.82)	CC	27 (0.0813)	14 (0.0379)	C	172 (0.2590)	154 (0.2087)	0.02580	1.33 (1.03–1.70)	0.0004757	1.37 (1.15–1.63)
	CG	130 (0.3662)	105 (0.3153)	G	522 (0.7352)	531 (0.7973)			CG	118 (0.3554)	126 (0.3415)	G	492 (0.7410)	584 (0.7913)				
	GG	196 (0.5521)	213 (0.6396)	Sum	710	666			GG	187 (0.5633)	229 (0.6206)	Sum	664	738				
	Sum	355	333						Sum	332	369							
rs3740058	AA	24 (0.0680)	13 (0.0388)	A	176 (0.2493)	116 (0.1731)	0.0005534	1.59 (1.22–2.06)	AA	17 (0.0520)	8 (0.0220)	A	135 (0.2064)	117 (0.1612)	0.02980	1.35 (1.03–1.78)	0.00006305	1.47 (1.22–1.78)
	AG	128 (0.3626)	90 (0.2687)	G	530 (0.7507)	554 (0.8269)			AG	101 (0.3089)	101 (0.2782)	G	519 (0.7936)	609 (0.8388)				
	GG	201 (0.5694)	232 (0.6925)	Sum	706	670			GG	209 (0.6391)	254 (0.6997)	Sum	654	726				
	Sum	353	335						Sum	327	363							

Allelic *P*-values are indicated. OR, odds ratio; CIs, confidence intervals.
[a]Computed by the method of Mantel and Haenszel.

Table 3. Summary of associated SNPs

dbSNP/Celera	Genomic position (bp)[a]	Gene (portion)	Function	Alleles[b]	Frequency[c]	HWE[d]
rs911541	101,423,382	*ENTPD7* (intron 3)	—	A/G	0.807/0.193	0.0760
rs3740066	101,594,197	*ABCC2* (exon 28)	Synonymous (Ile1324Ile)	C/T	0.776/0.224	0.2882
rs11190302	101,622,905	— (intergene)	—	C/T	0.767/0.231	0.9244
rs11190305	101,629,867	*DNMBP* (exon 16)	Non-synonymous (Cys1413Trp)	T/G	0.769/0.232	0.8284
C_11214959_10[e]	101,642,926	*DNMBP* (intron 11)	—	G/C	0.766/0.234	0.8625
rs3740058	101,645,972	*DNMBP* (intron 10)	—	G/A	0.803/0.197	0.6722

[a]On the basis of dbSNP build 125 on NCBI build 35.
[b]Nucleotides of the major allele/minor allele.
[c]The major allele/minor allele frequency, calculated using genotype data obtained for the 699 LOAD patients and 709 controls with *APOE-ε3*3* in the exploratory and validation sample sets.
[d]*P*-values were calculated in the HWE test using 709 control subjects with *APOE-ε3*3* in the exploratory and validation sample sets (Table 1).
[e]Assay ID in Celera (CA, USA).

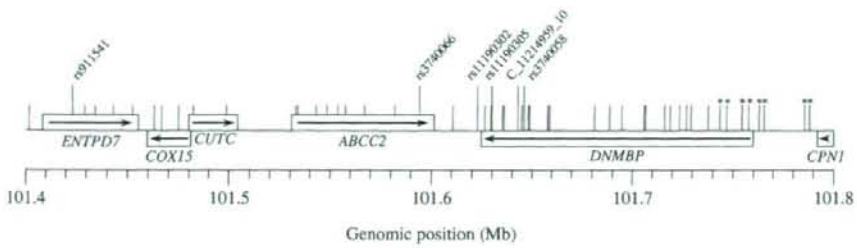

Figure 1. Genomic position of an associated locus. Vertical lines indicate the SNPs used in this study: six significantly associated SNPs, rs911541, rs3740066, rs11190302, rs11190305, C_11214959_10 and rs3740058, are indicated by the long-labeled vertical lines. These SNPs are located in a genomic region in which five genes, *ENTPD7*, *COX15*, *CUTC*, *ABCC2* and *DNMBP*, are clustered, spanning ~350 kb. The asterisked vertical lines in the 5′ region of *DNMBP* show the SNPs additionally designed and analyzed after real-time RT–PCR experiments. Horizontal arrows within open boxes indicate the transcription orientations of individual genes. The mapping position of each SNP is according to dbSNP build 125 on NCBI build 35. *CPN1*, gene encoding carboxypeptidase-*N*-polypeptide 1, 50 kDa.

described under Materials and Methods, the expression levels of endogenous housekeeping genes in eight brains consisting of four LOAD ones and four control ones. The transcripts of β-glucuronidase (*GUSB*) and 18S rRNA genes were found to exhibit little variation within the eight brains (data not shown).

After this preliminary experiment, we increased the samples to 41 and assessed the correlation between the expression of *DNMBP*, *GUSB* and 18S rRNA and age and gender. Neither age- nor gender-dependent significant differences between LOAD patients and controls were observed in the levels of transcripts of *DNMBP*, *GUSB* and 18S rRNA (data not shown). Normalization relative to the quantity of total RNA revealed statistically significant differences between LOAD patients and controls (Fig. 2A, $P = 0.0003$). As can be seen in Figure 2B and C, there was a significant reduction in the *DNMBP* mRNA levels in AD brains compared with that in age-matched controls following normalization as to either *GUSB* ($P = 0.0002$) or 18S rRNA ($P = 0.0169$).

To address whether or not the expression differences between LOAD and control brains are due to genetic variability in *DNMBP*, we first genotyped three associated SNPs (rs11190305, C_11214959_10 and rs3740058) using 41 brain tissue specimens. There were no minor-allele homozygotes in the control brains (Supplementary Material, Table S4), therefore we compared the expression levels of *DNMBP* in total samples with a dominant model (minor-allele homozygotes + heterozygotes versus major-allele homozygotes) (Fig. 2D–F). Two-way ANOVA with the genotype and case–controls as independent variables showed the significant effects of the diagnosis and genotypes examined in this study: rs11190305, $P = 0.0190$; C_11214959_10, $P = 0.0234$ and rs3740058, $P = 0.0205$. However, no significant interactions of *DNMBP* expression, SNPs and diagnosis were observed (Fig. 2D–F).

To determine whether or not the reduced expression of *DNMBP* mRNA was caused by genetic variation including the 5′ upstream region of *DNMBP*, we genotyped the exploratory sample set with eight additional SNPs (indicated by asterisked vertical lines in Figure 1). We did not detect any association of these eight SNPs with LOAD (range of allelic *P*, 0.00526–0.875).

DISCUSSION

This paper describes an attempt to identify an additional susceptibility gene(s) for LOAD with a high density of SNPs in two independent *APOE-ε3*3* sample sets and to verify the finding, if any, in the entire samples. Like many complex diseases, LOAD is caused by interactions between environmental and genetic factors. As genetic risk factors for complex diseases are thought to be of lower penetrance

Table 4. Allelic association of six SNPs in sample sets stratified as to the *APOE* genotype

dbSBP/Celera	Negative-e4[a]								Positive-e4[b]								All[c]							
	Genotype	Number of subjects (frequency)		Allele	Number of alleles (frequency)		*P*-value	OR (95% CIs)	Genotype	Number of subjects (frequency)		Allele	Number of alleles (frequency)		*P*-value	OR (95% CIs)	Genotype	No of subjects (frequency)		Allele	Number of alleles (frequency)		*P*-value	OR (95% CIs)
		LOAD	Control		LOAD	Control				LOAD	Control		LOAD	Control				LOAD	Control		LOAD	Control		
rs911541	GG	42 (0.0566)	43 (0.0314)	G	329 (0.2217)	492 (0.1797)	0.0009932	1.30 (1.11–1.52)	GG	23 (0.0299)	14 (0.0486)	G	289 (0.1877)	110 (0.1978)	0.8625	0.98 (0.77–1.25)	GG	65 (0.0430)	57 (0.0344)	G	618 (0.0244)	602 (0.1817)	0.022	1.16 (1.02–1.31)
	GA	245 (0.3302)	406 (0.2966)	A	1155 (0.7783)	2246 (0.8203)			GA	243 (0.3156)	82 (0.2847)	A	1251 (0.8123)	446 (0.8022)			GA	488 (0.3228)	488 (0.2945)	A	2406 (0.7956)	2712 (0.8183)		
	AA	455 (0.6132)	920 (0.6720)	Sum	1484	2738			AA	504 (0.6545)	192 (0.6667)	Sum	1540	556			AA	9.59 (0.6343)	1112 (0.6711)	Sum	3024	3314		
	Sum	742	1369						Sum	770	288						Sum	1512	1657					
rs3740066	TT	57 (0.0771)	54 (0.0396)	T	378 (0.2558)	559 (0.2049)	0.0001551	1.33 (1.15–1.55)	TT	31 (0.0402)	15 (0.0523)	T	333 (0.2160)	128 (0.2230)	0.7271	0.96 (0.76–1.21)	TT	88 (0.0583)	69 (0.0418)	T	711 (0.2354)	687 (0.2081)	0.0088	1.17 (1.04–1.32)
	TC	264 (0.3572)	451 (0.3306)	C	1100 (0.7442)	2169 (0.7951)			TC	271 (0.3515)	98 (0.3415)	C	1209 (0.7840)	446 (0.7770)			TC	535 (0.3543)	549 (0.3325)	C	2309 (0.7646)	2615 (0.7919)		
	CC	418 (0.5656)	859 (0.6298)	Sum	1478	2728			CC	469 (0.6083)	174 (0.6063)	Sum	1542	547			CC	887 (0.5874)	1033 (0.6257)	Sum	3020	3302		
	Sum	739	1364						Sum	771	287						Sum	1510	1651					
rs11190302	TT	61 (0.0822)	57 (0.0415)	T	395 (0.2662)	598 (0.2178)	0.0003934	1.30 (1.13–1.51)	TT	36 (0.0468)	14 (0.0491)	T	349 (0.2266)	129 (0.2263)	0.988	1.00 (0.80–1.26)	TT	97 (0.0642)	71 (0.0428)	T	744 (0.2460)	727 (0.2192)	0.0116	1.16 (1.03–1.31)
	TC	273 (0.3679)	484 (0.3525)	C	1089 (0.7338)	2148 (0.7822)			TC	277 (0.3597)	101 (0.3544)	C	1191 (0.7734)	441 (0.7737)			TC	550 (0.3638)	585 (0.3528)	C	2280 (0.7540)	2589 (0.7808)		
	CC	408 (0.5499)	832 (0.6060)	Sum	1484	2746			CC	457 (0.5935)	170 (0.5965)	Sum	1540	570			CC	865 (0.5721)	1002 (0.6043)	Sum	3024	3316		
	Sum	742	1373						Sum	770	285						Sum	1512	1658					
rs1119305	GG	59 (0.0806)	55 (0.0407)	G	385 (0.2630)	590 (0.2184)	0.001164	1.28 (1.10–1.48)	GG	34 (0.0447)	14 (0.0498)	G	343 (0.2254)	128 (0.2278)	0.9076	0.99 (0.78–1.24)	GG	93 (0.0623)	69 (0.0423)	G	728 (0.2438)	718 (0.2200)	0.0257	1.14 (1.02–1.29)
	GT	267 (0.3648)	480 (0.3553)	T	1079 (0.7370)	2112 (0.7816)			GT	275 (0.3614)	100 (0.3559)	T	1179 (0.7746)	434 (0.7722)			GT	542 (0.3630)	580 (0.3554)	T	2258 (0.7562)	2546 (0.7800)		
	TT	406 (0.5546)	816 (0.6040)	Sum	1464	2702			TT	452 (0.5940)	167 (0.5943)	Sum	1522	562			TT	858 (0.5747)	983 (0.6023)	Sum	2986	3264		
	Sum	732	1351						Sum	761	281						Sum	1493	1632					
C_11214959_10	CC	60 (0.0810)	56 (0.0410)	C	390 (0.2632)	597 (0.2184)	0.001039	1.28 (1.10–1.48)	CC	35 (0.0453)	14 (0.0491)	C	351 (0.2273)	127 (0.2228)	0.8253	1.03 (0.82–1.29)	CC	95 (0.0628)	70 (0.0424)	C	741 (0.2449)	724 (0.2191)	0.0152	1.16 (1.03–1.30)
	CG	270 (0.3644)	485 (0.3548)	G	1092 (0.7368)	2137 (0.7816)			CG	281 (0.3640)	99 (0.3474)	G	1193 (0.7727)	443 (0.7772)			CG	551 (0.3642)	584 (0.3535)	G	2285 (0.7551)	2580 (0.7809)		
	GG	411 (0.5547)	826 (0.6042)	Sum	1482	2734			GG	456 (0.5907)	172 (0.6035)	Sum	1544	570			GG	867 (0.5730)	998 (0.6041)	Sum	3026	3304		
	Sum	741	1367						Sum	772	285						Sum	1513	1652					
rs3740058	AA	46 (0.0631)	45 (0.0334)	A	337 (0.2311)	482 (0.1790)	0.00005699	1.38 (1.18–1.61)	AA	30 (0.0398)	9 (0.0327)	A	301 (0.1996)	113 (0.2055)	0.7695	0.96 (0.76–1.23)	AA	76 (0.0512)	54 (0.0333)	A	638 (0.2151)	595 (0.1835)	0.00184	1.22 (1.08–1.38)
	AG	245 (0.3361)	392 (0.2912)	G	1121 (0.7689)	2210 (0.8210)			AG	241 (0.3196)	95 (0.3455)	G	1207 (0.8004)	437 (0.7945)			AG	486 (0.3277)	487 (0.3004)	G	2328 (0.7849)	2647 (0.8165)		
	GG	438 (0.6008)	909 (0.6753)	Sum	1458	2692			GG	483 (0.6406)	171 (0.6218)	Sum	1508	550			GG	921 (0.6210)	1080 (0.6663)	Sum	2966	3242		
	Sum	729	1346						Sum	754	275						Sum	1483	1621					

Allelic *P*-values are indicated.
[a]Non-carriers of the *APOE-ε4* allele (*2*2, 2*3* and *3*3*) comprising 749 LOAD patients and 1378 controls.
[b]Carriers of the *APOE-ε4* allele (*2*4, 3*4* and *4*4*) comprising 777 LOAD patients and 288 controls.
[c]All *APOE* genotypes (*APOE-ε2*2, 2*3, 2*4, 3*3, 3*4* and *4*4*) comprising 1526 LOAD patients and 1666 controls.

Table 5. LD measures, $|D'|$, for six SNPs associated with LOAD

dbSNP/Celera	rs911541	rs3740066	rs11190302	4s11190305	C_11214959	rs3740058
rs911541	*	0.9535	0.8958	0.8986	0.8955	0.6865
rs3740066	0.9509	*	0.9403	0.9434	0.9402	0.8609
rs11190302	0.9094	0.9562	*	1.0000	1.0000	0.9537
rs11190305	0.9025	0.9466	0.9954	*	1.0000	0.9533
C_11214959_10	0.9089	0.9565	0.9955	1.0000	*	0.9541
rs3740058	0.5749	0.7795	0.9297	0.9369	0.9376	*

For LD computation, 699 LOAD patients and 709 controls (Table 3) were used. The upper-right half shows LOAD patients and the lower-left half controls.

Table 6. Case–control haplotype analysis

Sample set	Number of subjects		Haplotype[a]	Frequency		Number of estimated alleles		*P*-value	Permutation *P*-value (10,000)	OR (95% CIs)
	LOAD	Control		LOAD	Control	LOAD	Control			
All	1526	1666	**[H1]A-C-C-T-G-G**	0.7367	0.7629	2248	2542	**0.0152**	**0.0204**	087 (0.78–0.97)
			[H2]G-T-T-G-C-A	0.1562	0.1282	477	427	**0.001276**	**0.0017**	1.26 (1.09–1.45)
			[H3]G-T-T-G-C-G	0.0364	0.0430	111	143	0.1812	0.2026	0.84 (0.65–1.08)
			[H4]A-T-T-G-C-A	0.0346	0.0294	106	98	0.2274	0.2458	1.19 (0.90–1.57)
			[H5]A-C-T-G-C-A	0.0168	0.0158	51	53	0.7999	0.8435	1.05 (0.71–1.55)
			Others[c]	0.0194	0.0206	59	69	—	—	—
			Sum	1.000	1.000	3052	3332	—	—	—
			Global	—	—	—	—	0.0695	**0.0401**	
Negative-ε4	749	1378	**[H1] A-C-C-T-G-G**	0.7118	0.7635	1067	2105	**0.0002293**	**0.0001**	0.77 (0.66–0.88)
			[H2] G-T-T-G-C-A	0.1644	0.1236	246	341	**0.0002549**	**0.0003**	1.39 (1.17–1.66)
			[H3] G-T-T-G-C-G	0.0413	0.0453	62	125	0.5466	0.5826	0.91 (0.67–1.24)
			[H4] A-T-T-G-C-A	0.0382	0.0287	57	79	0.0965	0.1011	1.34 (0.95–1.90)
			[H5] A-C-T-G-C-A	0.0188	0.0172	28	47	0.6982	0.7167	1.10 (0.68–1.76)
			Others[c]	0.0255	0.0216	38	59	—	—	—
			Sum	1.000	1.000	1498	2756	—	—	—
			Global	—	—	—	—	**0.0289**	**0.01**	—
ε3*3[b]	699	1243	**[H1]A-C-C-T-G-G**	0.7141	0.7681	998	1909	**0.0001958**	**0.0001**	0.75 (0.65–0.88)
			[H2]G-T-T-G-C-A	0.1645	0.1185	230	295	**0.00006021**	**<0.0001**	1.46 (1.21–1.76)
			[H3] G-T-T-G-C-G	0.0409	0.0457	57	114	0.4585	0.5023	0.88 (0.64–1.22)
			[H4] A-T-T-G-C-A	0.0384	0.0286	54	71	0.088	0.0859	1.37 (0.95–1.96)
			[H5] A-C-T-G-C-A	0.0165	0.0171	23	43	0.845	0.9026	0.95 (0.57–1.58)
			Others[c]	0.0255	0.0218	36	54	—	—	—
			Sum	1.000	1.000	1398	2486	—	—	—
			Global	—	—	—	—	**0.008367**	**0.0026**	—
Positive-ε4	777	288	[H1] A-C-C-T-G-G	0.7608	0.7599	1184	438	0.9429	0.9576	1.01 (0.81–1.26)
			[H2] G-T-T-G-C-A	0.1482	0.1505	230	87	0.8612	0.8891	0.98 (0.75–1.28)
			[H3] G-T-T-G-C-G	0.0316	0.0317	49	18	0.9736	1.0000	1.01 (0.58–1.75)
			[H4] A-T-T-G-C-A	0.0311	0.0331	48	19	0.8054	0.7871	0.93 (0.54–1.60)
			[H5] A-C-T-G-C-A	0.0149	0.0087	23	5	0.2707	0.3934	1.72 (0.65–4.53)
			Others[c]	0.0134	0.0161	20	9	—	—	—
			Sum	1.000	1.000	1554	576	—	—	—
			Global	—	—	—	—	0.7872	0.8388	—

See Table 4 for the information on each sample set, All, Positive-ε4 and Negative-ε4. Statistically significant haplotypes and *P*-values and permutation *P*-values are highlighted in bold.

[a]The SNP order, from left to right, is as follows: rs911541, rs3740066, rs11190302, rs11190305, C_11214959_10 and rs3740058.

[b]A sample set stratified as to the *APOE-ε3*3* genotype, including 699 LOAD patients and 1243 controls.

[c]Haplotypes with frequencies of <0.01 in both LOAD and control subjects.

and heterogeneous, the replication of genetic risk factors in well-characterized case–control samples is critical for validating the markers associated with a complex disease. A strong association between the *APOE-ε4* allele and LOAD has been repeatedly reported by a number of groups. However, as about 50% of AD patients do not carry the *APOE-ε4* allele, the remaining AD cases must be attributed to other risk factors or environmental factors. Thus, to identify a genetic risk factor(s) other than the *APOE-ε4* allele, we prepared two independent sample sets, exploratory and validation ones, from only *APOE-ε3*3* subjects. Our strategy was to scan a broad region on chromosome 10q with a high density of

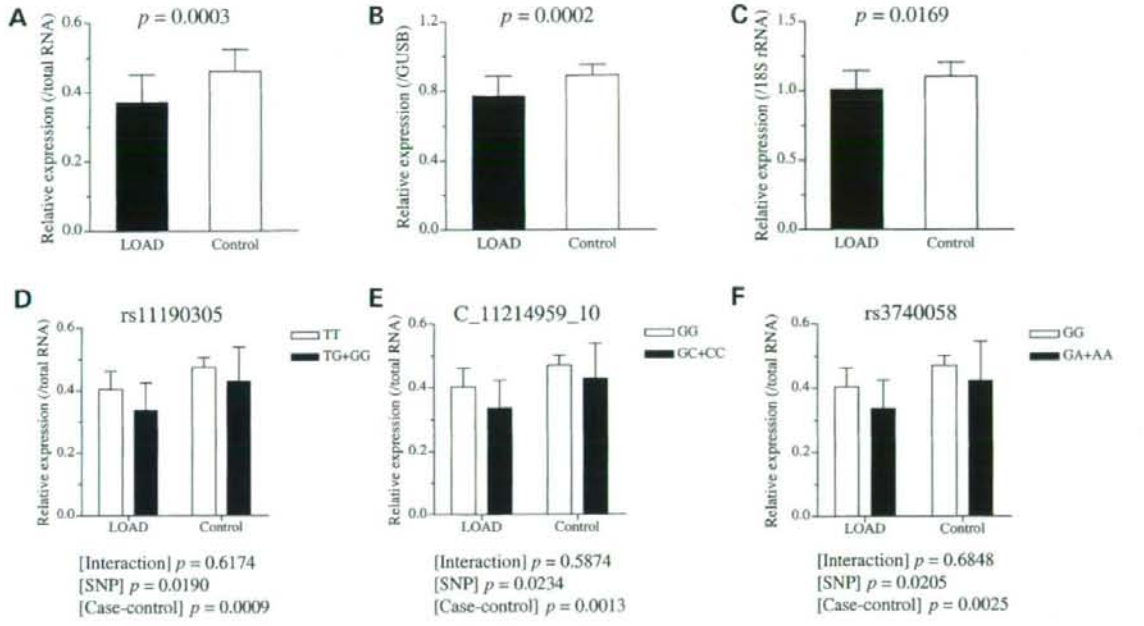

Figure 2. Expression levels of *DNMBP* in post mortem brain tissues. Real-time RT–PCR assaying was carried out to determine the steady-state mRNA level of *DNMBP* by using the standard curve method recommended by the manufacturer. *DNMBP* expression was normalized as to the concentration of total RNA (**A**) used for first-strand cDNA synthesis and the amounts of *GUSB* (**B**) and 18S rRNA (**C**) expression. Values are means ± SD. *P*-values were computed with the two-sided Student's *t*-test. Relative expression of *DNMBP* in LOAD patients and controls was examined with a dominant model (**D**–**F**) by means of two-way ANOVA.

SNPs in the exploratory samples and to follow-up the significant initial markers in the other set (validation samples) and then to examine the significant replicated markers in the entire samples consisting of all *APOE* genotypes. This is similar to the strategy that was adopted for genome-wide (32) or chromosome-wide scanning (33,34). Using this strategy, Grupe *et al.* (34) performed a chromosome 10-specific association study involving 1412 gene-based SNPs. They found one marker, rs498055 (97,344,904 bp), located in a gene homologous to *RPS3A* (*LOC439999*) significantly associated with LOAD.

In a case–control association study involving 1206 SNPs on chromosome 10q, we found a novel locus in which six SNPs, rs911541, rs3740066, rs11190302, rs11190305, C_11214959_10 and rs3740058, were significantly associated with *APOE-ε3*3*-LOAD (Table 2) but not with *APOE-ε4*-LOAD (Table 4). No significant interaction was observed between the *APOE-ε4* allele and the SNPs identified here by logistic regression analysis (data not shown). However, as *APOE-ε4* allele positive samples are fairly rare among controls, we cannot exclude the possibility that the lack of significance in the *APOE-ε4* positive group is due to the small sample size. Further analyses are necessary to determine the interaction of the six SNPs and *APOE* genotypes in a sufficient number of controls.

The six SNPs are located in a locus between 101.4 and 101.7 Mb, which is far from D10S1225 (64.4 Mb) in the strong linkage region described by several groups. Two peaks were found for chromosome 10q when a sample was stratified as to *APOE* genotype on genome-wide linkage analysis (9,10). There was a major peak around 80 cM and another small peak was observed at marker D10S1265 (102.6 Mb) near the six SNPs. The linkage was found in different conditions: in familial AD (9–11,13,15), with plasma Aβ levels as an intermediate quantitative trait (12) and with AAO in AD (14). In this broad region, candidate genes were presumed to be as follows: *CTNNA3* (16,17), *PLAU* (22–24), *IDE* (18–21), *GTSO1* and *GTSO2* (25–27). *CTNNA3* was identified in high plasma Aβ42 pedigrees, and both *IDE* and *PLAU* are suggested to be involved in the degradation of Aβ. We also measured the plasma Aβ40 and Aβ42 levels in the samples to determine whether or not the SNP genotype influences the plasma Aβ level. We did not observe any relationship between the six SNPs identified in this study and the plasma Aβ level (data not shown). Li *et al.* (14) analyzed transcripts in the hippocampus and reported decreased expression of *GSTO1* and *GSTO2* in the region between D10S1239 (98.9 Mb) and D10S1237 (116.1 Mb). We genotyped 167 SNPs in *CTNNA3* (67.35–69.10 Mb), five SNPs in *PLAU* (75.33–75.35 Mb), 19 SNPs in *IDE* (94.20–94.33 Mb), two SNPs in *GSTSO1* (106.00–106.02 Mb) and five SNPs in *GTSO2* (106.01–106.05 Mb) (Supplementary Material, Table S1) in the exploratory samples, but found no association with LOAD. Recently, Grupe *et al.* (34) reported that SNP rs498055 (97,344,904 bp), a locus for the *RPS3A* homolog, is associated with LOAD as described above. The SNPs neighboring the *RPS3A* homolog locus, rs526928

(97,324,281 bp), rs496641 (97,347,389 bp) and rs749049 (97,366,086 bp), were genotyped, but no association was detected for our exploratory sample set. Thus, none of the SNPs in the above-described candidate genes was significantly associated with LOAD with the *APOE-ε3*3* genotype in the Japanese population examined here. However, it is still possible that this finding is due to the ethnic difference.

The novel association locus found in this study contains five genes, *ENTPD7*, *COX15*, *CUTC*, *ABCC2* and *DNMBP* (Fig. 1). SNP rs911541 occurs in intron 3 of *ENTPD7*, also known as *LAPL1*, which encodes apylase with an intracellular catalytic domain (35). ENTPD7 exhibits 71% similarity to LALP70 (36), a lysosomal/autophagolysosomal membrane protein, suggesting that ENTPD7 is also located in a lysosomal/autophagic compartment, but its physiological function is unclear. The SNP rs3740066 (ATC → ATT, Ile1324Ile) is in exon 28 of *ABCC2*, which is a member of the ATP-binding cassette transporter superfamily that transports various molecules across extra- and intracellular membranes. *ABCC2* is expressed predominantly in the liver but was undetectable in human brain on immunocytochemistry (37). The SNP rs11190302 is located in the intergenic region between *ABCC2* and *DNMBP*. Three SNPs, rs11190305, C_11214959_10 and rs3740058, are present at a high density in the 3′ region of *DNMBP*: rs11190305 causes a non-synonymous exchange (TGT → TGG, Cys1413Trp). Taking these data together, we focussed on *DNMBP*, although *ENTPD7* and *ABCC2* may also influence the pathogenesis of AD. This is the first description of a significant association between a *DNMBP* polymorphism and the risk of LOAD with the *APOE-ε3*3* genotype or lacking the *APOE-ε4* allele.

DNMBP binds to dynamin selectively through four N-terminal Src homology-3 (SH3) domains. GTPase dynamin is an essential component for vesicle formation in receptor-mediated endocytosis, synaptic vesicle recycling, caveolae internalization and possibly vesicle trafficking in and out of the Golgi complex (38,39). DNMBP also binds to several actin regulatory proteins including direct binding partners, i.e. N-WASP (neuronal Wiskott–Aldrich syndrome protein) and Ena (Enabled)/VASP (vasodilator-stimulated phosphoprotein), via two SH3 domains at the C-terminus. The DH domain in the middle of DNMBP is involved in the activation of Cdc42. The molecule promotes F-actin nucleation and/or recruitment within cells (28). N-WASP, which acts as a key molecule for fillopodium formation through Cdc42 activation (40), is increased in the AD brain and may be involved in aberrant neuronal sprouting (41). DNMBP is co-localized with synapse-enriched proteins, amphiphysin-1 and dynamin-1 (28). The BAR domain of amphiphysin-1 is required for the triggering of dynamin GTPase activity and fission of the endocytic pit (42). Amphiphysin-knockout mice have defects in synaptic vesicle recycling and major learning deficits (43). The polymorphism of T/G in rs11190305 corresponds to an amino acid change of cysteine to tryptophan (Cys1413Trp). The Cys1413Trp mutation occurs at a position between the two SH3 domains at the C-terminus of DNMBP. It is possible that this amino acid change leads to a conformational alteration, and subsequently affects the interactions with binding partners, although further experiments are necessary to confirm this. To find a new variation, we sequenced all exons, exon–intron boundaries and an about 200 bp 5′ upstream region of *DNMBP* in 92 LOAD patients, but found no polymorphism in these sequenced regions (data not shown).

Thus far, there has been no information about the expression level of *DNMBP* in the AD brain. In this study, we demonstrated a significant reduction of *DNMBP* transcripts in the cerebral cortex of autopsy-confirmed AD patients using quantitative real-time RT–PCR (Fig. 2A–C). Although risk alleles of three SNPs, rs11190305 (allele G), C_11214959_10 (allele C) and rs3740058 (allele A), obviously decreased the *DNMBP* expression level in a dominant model, there were no significant interactions between *DNMBP* gene expression, genotype variation and diagnosis (Fig. 2D—F). *APOE-ε4*-carrying AD subjects also tended to exhibit decreased levels of *DNMBP* expression (data not shown). An alternative and attractive interpretation would be that reduced *DNMBP* expression is caused not only by the SNPs identified here but also by altered expression of other genes. Thus, it is possible that several pathways lead to the reduced *DNMBP* expression that acts as a risk factor for LOAD.

Recently, Yao *et al.* (29) described the reduced expression of a group of genes including those of dynamin I and amphiphysin-1, all of which are involved in synaptic vesicle trafficking, in the frontal cortex of AD brains. AD begins with subtle alterations of hippocampal synaptic function prior to Aβ deposition followed by frank neuronal degeneration (31). Dynamin I is an important mediator of clathrin-dependent endocytosis and synaptic vesicle recycling. These facts may be consistent with our observation that DNMBP is a genetic risk factor for LOAD. The decrease in the level of *DNMBP* mRNA might be related to the pathogenesis traced to synapses in the brain of LOAD patients and is probably caused by multiple environmental and genetic factors and their combination.

MATERIALS AND METHODS

Subjects

To search for susceptibility genes for LOAD by means of genome-wide screening, the Japanese Genetic Study Consortium for AD (JGSCAD) was organized in 2000, and blood samples were collected. The subject information is summarized in Table 1. Expectedly, the *APOE-ε4* allele was found to be a highly significant risk factor for LOAD (OR 4.96, 95% CIs 4.22–5.84; p of chi-square test <0.0001). All individuals included in this study were Japanese. Probable AD cases met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders. Controls who had no signs of dementia and lived in an unassisted manner in the local community were also recruited. AAO is here defined as the age at which the family and/or individuals first noted cognitive problems while working or in daily activities. For evaluation of cognitive impairment, the mini-mental state examination (MMSE) was used.

A total of 41 post mortem brains from LOAD patients and control subjects were obtained from the Bioresource Center, Brain Research Institute, Niigata University (Supplementary Material, Table S3). The distribution of the *APOE-ε4* allele was significantly different between LOAD and control

subjects, as expected (OR 5.70, 95% CIs 1.25–25.93, Fisher's exact $P = 0.0367$). Autopsies were performed after a mean post mortem interval of 4.2 h (range 1–22 h). LOAD patients with dementia were neuropathologically characterized based on consensus criteria that included physiologically age-matched densities of senile plaques and neurofibrillary tangles as distinguished from other neurodegenerative disorders, i.e. dementia with Lewy body disease, frontotemporal dementia and Parkinson's disease according to published criteria. Autopsied controls were confirmed to have no diagnosable brain disease.

The present study was approved by the Institutional Review Board of the University of Niigata and by all participating institutes. Informed consent was obtained from all controls and appropriate proxies for patients, and all samples were anonymously analyzed for genotyping.

Marker selection and genotyping

SNP information was obtained from four open databases: NCBI dbSNP (Build 125, http://www.ncbi.nlm.nih.gov/SNP/), International HapMap Project (Rel#20/phaseII on NCBI Build 35 assembly and dbSNP Build 125, http://www.hapmap.org/index.html), Ensemble Human (Version 37 on NCBI Build 35, http://www.ensembl.org/Homo_sapiens/) and Celera myScience (Version R27 g on NCBI Build 35, http://myscience.appliedbiosystems.com/). We selected 1322 SNPs in the region from 60 to 107 Mb on chromosome 10q (Supplementary Material, Tables S1 and S2); mean intermarker distance ± SD, 34.9 ± 87.4 kb; 95% CIs, 30.2–39.6 kb. To examine the genotyping quality of the 1322 SNPs, the HWE test was performed with 337 control subjects (carrying *APOE-ε3*3* in the exploratory sample set, as shown in Table 1). These SNPs consisted of 29 missense mutations, 27 silent mutations, six SNPs in the 5′-UTR, 29 SNPs in the 3′-UTR, 921 SNPs in introns, 282 SNPs in intergenic regions and 28 SNPs in four loci shared by two different genes (*CTNNA3/LRRTM3*, *CDH23/C10orf54*, *C10orf55/PLAU* and *PGAM1/EXOSC1*). We used 1206 SNPs that were shown to be actually polymorphic in the Japanese population and showed $P > 0.05$ in the HWE test; mean intermarker distance ± SD, 38.3 ± 93.3 kb; 95% CIs, 33.0–43.6 kb.

Genomic DNA was extracted from peripheral blood with a QIAamp® DNA Blood Maxi Kit (Qiagen, Duseldorf, Germany) and examined fluorometrically with a PicoGreen® dsDNA quantification kit (Molecular Probes, CA, USA). SNP genotyping for individual samples was performed with an ABI PRISM® 7900HT instrument using TaqMan technology, and TaqMan SNP Genotyping Assays were purchased from Applied Biosystems (CA, USA).

Sequencing

APOE genotyping of all samples was performed by direct cycle sequencing with an ABI 3100 sequencer and a BigDye® Terminator v3.1 kit (Applied Biosystems) using the following primers: C19APOE001-F (sense 5′-GCCTACAAATCGGAACTGGA-3′) and C19APOE001-R (antisense 5′-ACCTGCTCCTTCACCTCGT-3′). All exons and their exon–intron boundaries and the 5′ upstream region of *DNMBP* were sequenced with 20 primer pairs (Supplementary Material, Table S5).

Case–control study

To identify candidate loci in a broad region of chromosome 10q (60–107 Mb on NCBI build 35), two independent sample sets comprising case–control subjects with *APOE-ε3*3* were constructed (Table 1). The exploratory sample set comprising 363 LOAD patients and 337 control subjects was genotyped, and SNPs showing significant association (allelic $P < 0.01$) were used for further examination using the validation sample set comprising 336 LOAD patients and 372 control subjects. Subsequently, we increased the samples and stratified them as the *APOE-ε4* carrier status: Negative-ε4, *APOE-ε 2*2*, *2*3* and *3*3*; Positive-ε4, *APOE-ε 2*4*, *3*4* and *4*4*; All, all genotypes of *APOE*. The sample numbers for LOAD patients and controls in All, Negative-ε4, ε3*3 and Positive-ε4 were 1526 and 1666, 749 and 1378, 699 and 1243, and 777 and 288, respectively. To examine the genetic association of multiple SNP combinations, case–control haplotype analysis with significant SNPs was performed using the following sample sets: All, Negative–ε4, ε3*3 and Positive-ε4.

Statistical analysis

Using SNPAlyze ver. 3.2.3 software (DYNACOM, Chiba, Japan; http://www.dynacom.co.jp/index.html.en), we performed the HWE test, single SNP case–control analysis, haplotype estimation based on the EM algorithm, case–control haplotype analysis with 10 000 iterated permutations and calculation of LD measures ($|D'|$) to elucidate the LD block structure. The Mantel–Haenszel test was performed using Statcel 2 software (OMS, Tokyo, Japan). Evidence of replication, rather than multiple testing corrections, was used to evaluate the significance of associated SNPs (32–34). We carried out the two-sided Student's *t*-test for comparison of the mean *DNMBP* expression levels between the AD and control brain tissues, using Prism 4.0 b (GraphPad Software, CA, USA). The effects of variation on gene expression were examined using the two-way ANOVA (Prism 4.0 b) with the genotype and case–controls as independent variables.

Quantitative real-time PCR

Frozen materials (Supplementary Material, Table S3) were prepared on dry ice blocks from 1 cm thick slices of the cerebral cortices. RNA was extracted directly from the frozen preparations with an ISOGEN solution (Nippongene, Tokyo, Japan). The first strand cDNA was synthesized from total RNA (2.0 μg) with SuperScriptsIII™ (Invitrogen, CA, USA) and random hexamers in a total volume of 20 μl according to manufacturer's protocol. The synthesized cDNA solution was diluted 1:20 and then used for quantitative real-time PCR amplification with TaqMan Gene Expression Assays (Applied Biosystems) and an ABI PRISM 7900HT instrument in a total volume of 10 μl according to manufacturer's instructions. Briefly, 2.5 μl of a diluted cDNA solution (corresponding to 12.5 ng of total RNA) was mixed with 5.0 μl of 2 × TaqMan Universal PCR Master Mix (Applied

Biosystems), 0.5 μl of 20 × TaqMan Gene Expression Assay and 2.0 μl of distilled water on a 384-well optical PCR plate. The PCR conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All measurements were performed in quadruplicate. The threshold cycle was determined in the linear range and relative gene expression was calculated as the cycle difference. Each measurement of *DNMBP* mRNA (Celera assay ID, Hs00324375_m1) was normalized to the expression levels of *GUSB* (Celera assay ID, Hs99999908_m1) and 18S rRNA (Celera assay ID, Hs99999901_s1), which were selected from among the 11 housekeeping genes [acidic ribosomal protein, beta-actin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerokinase, beta-2-microglobulin, GUSB, hypoxanthine ribosyl transferase, transcription factor IID (TATA binding protein), transferring receptor genes and 18S rRNA] on a TaqMan Human Endogenous Control Plate (Applied Biosystems) as internal standards.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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APPENDIX

The members of JGSCAD who participated in the collection of blood samples from AD patients and controls were as follows. All the authors of this paper, Akihiko Nunomura, MD, and Shigeru Chiba, MD, Department of Psychiatry and Neurology, Asahikawa Medical College, Asahikawa; Satoshi Takahashi, MD, Department of Neurology, Iwate Medical University, Morioka; Naoki Tomita, MD, Department of Geriatric and Complementary Medicine, Tohoku University Graduate School of Medicine, Sendai; Jyunzo Ito, MD, Alpine Kawasaki, Kawasaki, Miyagi; Haruo Hanyu, MD, Department of Geriatric Medicine, Tokyo Medical University, Tokyo; Hideo Kimura, PhD, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira; Shin Kitamura, MD, Second Department of Internal Medicine, Nippon Medical School, Tokyo; Hitoshi Shinotoh, MD, Asahi Hospital for Neurological Disease, Chiba; Hiroyuki Iwamoto, MD, Department of Neurology, Hatsuishi Hospital, Kashiwa; Masahiko Takahashi, MD, Department of Old Age Psychiatry and Memory Clinic, Tokyo Metropolitan Geriatric Medical; Yasuo Harigaya, MD, Department of Neurology, Maebashi Red Cross Hospital, Gunma; Masaki Ikeda, MD, and Masakuni Amari, MD, Department of Neurology, Gunma University Graduate School of Medicine, Maebashi; Takeo Takahashi, MD, Ina Neurological Hospital, Ina; Ryoichi Nakano, MD, and Masatoyo Nishizawa, MD, Department of Neurology, Brain Research Institute, Niigata University, Niigata; Masaichi Suga, MD, Higashi Niigata Hospital, Niigata; Makoto Hasegawa, MD, Niigata Shin-ai Hospital, Niigata; Yasuhiro Kawase, MD, Kawase Neurology Clinic, Sanjo; Kenichi Honda, MD, Honda Hospital, Uonuma; Toshiro Kumanishi, MD, and Yukiyosi Takeuchi, MD, Niigata Longevity Research Institute, Shibata; Atsushi Ishikawa, MD, Department of Neurology, Brain Disease Center, Agano Hospital, Agano; Masahiro Morita, MD, Department of Psychiatry, Mishima Hospital, Mishima; Fumihito Yoshii, MD, Department of Neurology, Tokai University School of Medicine, Isehara; Hiroyasu Akatsu, MD, and Kenji Kosaka, MD, Choju Medical Institute, Fukushimura Hospital, Toyohashi; Masahito Yamada, MD, and Tsuyoshi Hamaguchi, MD, Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa; Satoshi Masuzugawa, MD, Department of Neurology, Mie Prefectural Shima Hospital, Shima; Etsuro Matsubara, MD, and Takeshi Kawarabayashi, MD, Department of Neurology, Okayama University Graduate School of Medicine and Dentistry, Okayama; Takeo Takao, MD, and Nobuko Ota, Kurashiki Heisei Hospital, Kurashiki; Ken Sasaki, MD, Yoshikatsu Fujisawa, MD, and Kenji Nakata, MD, Kinoko Espoir Hospital, Kasaoka; Ken Watanabe, MD, Watanabe Hospital, Tottori; Yosuke Wakutani, MD, and Kenji Nakashima, MD, Department of Neurology, Institute of

Neurological Science, Tottori University, Yonago; Toshiyuki Hayabara, MD, Iwaki Hospital, Kagawa; Terumi Ooya, Town Office, Mizuho, Shimane; Mitsuo Takahashi, MD, Department of Clinical Pharmacology, Fukuoka University, Fukuoka; Tatsuo Yamada, MD, Fifth Department of Internal Medicine, Fukuoka University, Fukuoka; Taihei Miyakawa, MD, Labour Welfare Corporation Kumamoto Rosai Hospital, Yatsushiro; Eiichiro Uyama, MD, Department of Neurology, Graduate School of Medical Science, Kumamoto University, Kumamoto; Takefumi Yuzuriha, MD, Department of Psychiatry, National Hospital Organization Hizen Psychiatric Center, Sefuri; Ryuji Nakagawa, MD, Shizushi Yoshimoto, MD, and Kayoko Serikawa, MD, Ureshino-Onsen Hospital, Saga.

特集 遺伝環境相互作用

認知症と遺伝環境相互作用

浦上克哉*

KEY WORDS

・アポリポ蛋白E（ApoE）
・栄養
・運動
・モデル動物
・双生児研究

SUMMARY

アルツハイマー病（AD）や血管性認知症をはじめとする認知症は，高齢化社会になるに伴いその頻度が増加してきており，その発症には遺伝因子と環境要因の相互作用が重要であると推定されている．しかし，疫学的に得られる危険因子・防御因子と遺伝学的に得られる危険因子・防御因子は相互に研究対象となってきたものの，統合的な研究はまだ少数にとどまっている．本稿では認知症，特にADにおける遺伝環境相互作用の理解を進めるうえで重要と考えられる知見について概観する．

はじめに

アルツハイマー病（Alzheimer disease：AD）や血管性認知症をはじめとする認知症は，高齢化社会になるに伴いその頻度が増加してきており，その発症には遺伝因子と環境要因の相互作用が重要であると推定されている．一方，ADの分子遺伝学的研究の飛躍的な進歩によって，AD発症の分子メカニズムが明確になりつつある．特にアミロイドβ蛋白（Aβ）を中心としたアミロイドカスケードに焦点が当てられ，Aβの生合成，分解，重合・線維形成，神経活動，細胞機能への影響，およびそれにかかわる分子の研究が重点的・多面的に研究されている（図1）．それらの分子のなかには，食事，精神・身体活動，ライフスタイル，ストレスなどの環境因子の影響を受ける可能性があるものが数多くあると想定されており，危険因子・防御因子としておもに疫学的手法を用いて解析されている．しかし，環境因子が遺伝子発現や機能にどのような影響を与えることがADの発症につながるのかは，いまだ明確になってはいない．

本稿ではそれらに焦点を当てた，疫学研究，遺伝学的研究・遺伝疫学的調査およびモデル動物を用いた研究を紹介する．

1．遺伝子―アポリポ蛋白E遺伝子（*ApoE*）

*ApoE*遺伝子多型は，ADの発症に最も強力に影響を与える遺伝子多型として非常に重要である[1]．ApoEε4アレルを一つもつと約2～4倍，2つもつと10～30倍のAD発症への危険度をもつといわれている．ApoEε4アレルの遺伝子頻度は，若干人種によって異なるものの，基本的に人種を超えた遺伝的危険因子と考えられており，今のところ*ApoE*遺伝子多型と同等あるいはそれを凌駕する因子は報告されていない．

しかし，最近の研究では同じ遺伝的背景（人種）をもつ場合でも，環境（広い意味での）が異なることで*ApoE*遺伝子多型のAD発症に対する危険度は異なることが報告されている．たとえばアフリカ人では，ApoEε4のアレル頻度は非常に高いにもかかわらずADの発症率は非常に低いが，米国に在住する同じ遺伝

URAKAMI Katsuya/＊鳥取大学医学部保健学科生体制御学講座

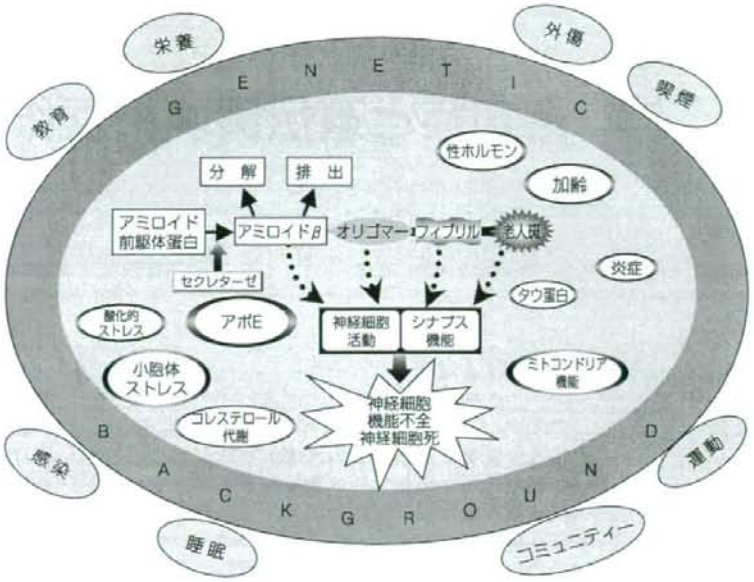

図 1. アルツハイマー病の発症機序

(筆者作成)

的な背景を共有するコミュニティー（アフリカ系アメリカ人）においては，ADの発症率は高く，ApoEε4がADの発症に寄与している[2]．この現象には生活習慣が深く関連していると考えられており，特に後述する食事因子・栄養因子が深く関連していると想定されている．

2．その他の遺伝子・遺伝子多型

ADにおける危険因子・防御因子としての遺伝子多型の研究は，世界中で行われている．たとえば，Alzheimer Research ForumのAlzGene（http://www.alzforum.org/res/com/gen/alzgene/default.asp）には，これまでに報告されているAD関連遺伝子の研究が網羅されており，登録されている遺伝子は250を超えている．これらのADとの関連が検討された遺伝子のなかには，ADと種々の環境要因を研究した疫学研究に端を発したものも数多くみられる．特に，最大の遺伝的危険因子である*ApoE*と同様に，コレステロール代謝に深く関与している遺伝子や酸化的ストレスに関連する遺伝子研究が多い．しかし，前述のとおり*ApoE*のようにはっきりとしたコンセンサスが得られた遺伝的危険因子・防御因子は，明らかとなっていない．もし，環境要因がAD，特に遅発性ADの発症に深く関与していると考えるならば，遺伝子多型の解析のみでは不十分で，前向き研究によって長期間にわたり種々の環境要因をモニターしながら遺伝子解析を行っていく必要があるであろう．

3．食事因子・栄養因子とAD

前述のアフリカ人とアフリカ系アメリカ人の食事因子の比較では，アフリカ人では脂質の摂取は少なく，抗酸化物の摂取は反対に多く，アフリカ系アメリカ人では反対に高脂肪・高カロリーであり，肥満も非常に多い．糖尿病，脳梗塞，高血圧，心筋梗塞などのいわゆる生活習慣病の頻度も非常に高くなるが，ADの発症にもこれらの食生活の違いが寄与していると考えられている[3]．

また，ADの有病率と総エネルギー・総脂質摂取の増加は高い相関をもつといわれており，糖尿病や耐糖能障害もAD発症の独立した危険因子であるとされている．総カロリー摂取の増加や糖尿病・耐糖能障害のAD発症に対する分子メカニズムとしては，インスリン代謝やフリーラジカルの関与が研究されている．高インスリン血症やいわゆるインスリン抵抗性が，Aβの合成・放出・分解の調節機構や海馬神経細胞におけるインスリン受容体

を介したシグナル伝達を障害することが報告され，総カロリー摂取の増加は脳内のフリーラジカルの増加，サイトカインの活性化・炎症反応の促進を引き起こす．

食事因子に焦点を当てたロッテルダムやシカゴなどにおける欧米の疫学研究では，魚の摂取がADの発症に予防的であるとされ，特にエイコサペンタエン酸(eicosapentaenoic acid：EPA)やドコサヘキサエン酸(docosahexaenoic acid：DHA)といったn-3系（ω-3系）の不飽和多価脂肪酸(polyunsaturated fatty acid：PUFA)の摂取が重要であるとされている．ADに対する予防的な分子メカニズムとしては，脳内のアラキドン酸代謝やサイトカイン活性化抑制を介した抗炎症作用や，抗動脈硬化作用などの血管系を介した作用が主として報告されている．

最近では，n-3系PUFAの作用はモデル動物を用いた研究も行われている．AD病理を再現するトランスジェニックマウス（TGM）にDHAを大量に与えて飼育すると，脳内のAβ量が著しく減少し，老人斑の出現も著明に抑制される[4]．また，Hashimotoら[5]の研究によると，脳内にAβペプチドを人工的に投与して認知機能障害を引き起こすモデルラットにおいて，あらかじめDHAを豊富に含む食事を投与しておくと，明らかに認知機能の低下が抑制できることを報告している．脳内のDHAの増加に従い，Aβペプチドの投与によって引き起こされる過酸化脂質や反応性活性酸素の上昇も抑制が可能であったという．

脂質摂取以外の研究では，おもにビタミンC，Eといった抗酸化作用をもつビタミンの摂取や，ビタミンB_{12}，葉酸といったホモシステイン代謝に関連するビタミンの研究も数多く行われており，これらのビタミン群の摂取はADの発症に対して防御的に働いていることが推定されているが，今のところ一定のコンセンサスは得られていない．最近の，葉酸・ホモシステインと認知症の発症に関する縦断的な疫学調査では，高ホモシステイン血症および低葉酸血症はいずれも2倍のAD発症に対する危険率があると報告されている[6]．実験的な研究では，これらのビタミン群はADに関与しているAβ代謝，炎症，酸化的ストレス応答に対して防御的に働くことが，ほぼ確実視されていると思われる．

4．運動・身体活動・社会活動

AD発症に対する運動の予防的効果を検討したいくつかの報告では，歩行や定期的な運動などの身体活動レベルの高い群では，低い群にくらべて加齢に伴う認知機能の低下ならびに認知症発症の危険度が低下することが示されている[7][8]．脳機能画像を用いた検討でも，運動の継続により脳血流および脳代謝の改善が特に前頭葉においてみられることが報告されている．その他の身体活動や社会活動も，活動度の高いほうが認知機能に対して防御的であることが疫学的なコンセンサスとなっていると思われる．運動・身体活動・社会活動の活発度は，精神状態，特にうつ的気分・うつ状態に対しても同時に防御的である．

これらの活動の認知機能に対する防御的な効果は，基礎研究ではおもにノルアドレナリン，セロトニン，ドパミンなど上行性脳幹網様体賦活系にかかわる因子やシナプスの可塑性にかかわる因子がターゲットとなって研究されてきたが，実際の脳内での分子メカニズムやそれらにかかわる遺伝子の相互作用や発現の変化は明らかとなっていなかった．

ごく最近では，運動・身体活動・社会活動の認知機能や認知症発症・進行に対する影響を実験的に証明するため，TGMも用いられている．Lazarovら[9]は，身体活動の活発化が保証されている“environment enrichment”の状態に置かれたTGM（APPswe X PS1 ΔE9）と通常の飼育状態に置かれたTGMとを比較しているが，“environment enrichment”の状態に置かれたTGMは脳内のAβの量および沈着が著しく抑制されることを示した．さらにDNAマイクロアレイ法を用いて，脳内（特に海馬領域）において学習・記憶，血管・神経形成および神経細胞の生存にかかわる遺伝子の発現が実際に変化していることを示した．ヒトにおいても経験的あるいは疫学的に推察されていた身体活動の活発さとAD発症の関係を，はじめて動物モデルにおいて証明した重要な知見である．さらに，ADモデル動物と同様にハンチントン病のモデル動物においても“environment enrichment”の状態が，発症および進行を遅らせることが示されている[10]．

マウスよりも大型の動物においては，Milgram ら[11]はイヌを用いて“behavioral enrichment”（トレーニングを十分に行ったりする）とビタミンC，Eおよび果物や野菜からの抽出物を用いた栄養因子の長期にわたる複合的な効果を検討しており，それらはイヌにおける加齢による認知機能の低下を抑制し，さらに，病理学的にはイヌにおいてもみられる脳内のアミロイドの沈着を抑制していた．

これら栄養因子や身体活動の，アミロイド沈着や神経活動低下に対する防御的な効果の分子メカニズムの解明は今後の重要な課題ではあるが，むしろ，それらの効果はAβ代謝やシナプス・神経活動などの特定のステップに効果を発現しているというよりは，各種の代謝過程に対して全般的に良好な“場”を提供しているのかもしれない．

さらに，各種の疫学研究によると，前述の食事・栄養，運動のバランスを改善させることは，心筋梗塞，脳卒中などの血管性，動脈硬化性疾患の予防につながることが示されており，認知症予防の観点からもこれらの環境要因への医学的な介入を積極的に行うことが，今後の医学的な課題になると考えられる．

5．双生児研究

双生児研究は，同一の遺伝子をもつ一卵性双生児と，出生は同時であるものの兄弟と同程度の遺伝子の類似度をもつ二卵性双生児を比較することによって，各種の疾患に対する遺伝と環境の影響度を明らかにしていこうとする方法論であるが，認知機能の加齢による変化や認知症の発症に関しても各種の検討が行われている．スウェーデンで1960年代初頭に設立されたSwedish Twins Registryでの研究では，一卵性双生児のAD発症の一致率は約6割に上り，二卵性双生児では3割であったことから，遺伝的背景はAD発症に大きく関与していると報告されている．また，認知症に対する環境要因の研究も行われており，社会的活動・娯楽活動の参加，定期的な運動，複雑な知的作業を要する就労，教育歴の高さなどが，認知症ならびにAD発症の防御因子になっていると考えられている[12][13]．

おわりに

Nun Study（http://www.mc.uky.edu/nunnet/）は，米国の修道院の修道女の協力を得て，食事，精神・身体活動など種々の環境因子や遺伝的因子の影響を長期間追跡し，最終的な病理学的検討も加えて，認知症を含めた精神・神経疾患について詳細に検討している研究である．そのなかで，剖検脳ではADとしか考えられないのに，臨床的には認知症の兆候を示さなかった症例が報告されている．その修道女は，終生自身の生活を律し，何事にも興味を示し，バランスの良い食事をとり，日課を欠かさなかったという．そのような個別の症例の詳細な検討からも，食事・身体活動を中心とした環境因子の重要性が推察される．

一方，ヒトゲノム計画や国際ハプマップ計画の推進によってヒトの遺伝子多型の詳細が明らかにされ，さらに，一塩基変異多型（single nucleotide polymorphisms：SNP）チップなどの遺伝子多型を迅速・網羅的にタイピングする技術が開発されたことにより，近い将来，種々の疾患につながるヒトのいわゆる“体質”にかかわる“遺伝子”あるいは“遺伝子型”の“組み合わせ”が明らかにされるかもしれない．また，前述のADモデル動物を用いて，DNAマイクロアレイやプロテオミクス，あるいはこれから開発されると期待される細胞機能の変化を網羅的に一望できる手法を使うことにより，環境因子の遺伝子・蛋白質の発現や相互作用に対する総合的・網羅的な解析が行われるものと考えられる．

疫学的には，個人個人から得られる網羅的なデータを集めた前向き調査に高度な統計学的手法を応用することにより，ADの発症にかかわる明確な「遺伝環境相互作用」が解明されるかもしれない．わが国でも，環境因子・遺伝的因子を総合的に観察可能な長期縦断前向き研究がいくつか行われており，認知症に対する遺伝環境相互作用の影響が日本人において明らかにされるものと期待される．

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ORIGINAL ARTICLE

Mild cognitive impairment: biological diagnostic markers for early stages of Alzheimer's disease

Katsuya URAKAMI

Section of Environment and Health Science Department of Biological Regulation, Faculty of Medicine, Tottori University

Correspondence: Professor Katsuya Urakami, MD, PhD, Department of Biological Regulation, Faculty of Medicine, Tottori University, Tottori: 683-8503, Japan. Email: kurakami@grape.med. tottori-u.ac.jp

ABSTRACT

Background: With the aging population in Japan, the inevitable increase in the prevalence of Alzheimer's disease (AD) and the difficulty in diagnosing the condition. Reliable early diagnostic biomarkers for early stage of AD are needed.

Method: We examined a total 570 CSF samples from a variety of diseases, including AD, other types of dementia and controls to quantitate levels of tau protein phosphorylated at serine 199 by sandwich ELISA. WGA-binding glycoproteins were measured by western blot analysis.

Results: The CSF phosphorylated tau protein levels in the AD group were significantly elevated compared to those in all the other non-AD groups. CSF phosphorylated tau protein levels in the AD progressed from mild cognitive impairment (MCI) group were significantly elevated compared to those in no progression AD from MCI groups and controls. A comparison of the phosphorylated tau protein: WGA-glycoprotein fragment A ratio for AD and other tauopathies showed that this ratio has potential for differentiating between AD and other tauopathies, particularly dementia with Lewy bodies.

Conclusion: Our data showed that measuring cerebrospinal fluid (CSF) levels of phosphorylated tau protein may be useful as an early diagnostic marker in AD and MCI. Furthermore, the measurement of WGA-binding glycoprotein in CSF may provide a useful tool for differentiating AD from dementia of Lewy bodies and other tauopathies.

Key words: CSF, dementia with Leuy body, phosphorylated tau, tauopathy, total tau, WGA binding glycoprotein.

With the aging population in Japan, the inevitable increase in the prevalence of Alzheimer's disease and the difficulty in diagnosing the condition, reliable biological diagnostic biomarkers for both mild cognitive impairment (MCI) and Alzheimer's disease are needed.

PHOSPHORYLATED TAU IN ALZHEIMER'S DISEASE

The measurement of cerebrospinal fluid (CSF) levels of tau, a microtubule-associated protein that is an important component of neurofibrillary tangles, is helpful for diagnosing Alzheimer's disease when used in conjunction with other investigations. However, some overlap has been found between levels of total tau in patients and healthy controls.[1] To further improve and extend the diagnostic value of tau, a new enzyme immunoassay (EIA) system has been developed to measure phosphorylated tau (p-tau). While total tau is thought to reflect neuronal degeneration, p-tau levels are considered a marker for hyperphosphorylation of tau and, possibly, for the formation of neurofibrillary tangles.

Phosphorylated tau 199 was discovered by our group and an EIA was developed in collaboration with Mitsubishi Chemical Company (Tokyo, Japan). The selectivity and specificity of the p-tau assay was compared with the existing total tau assay in a study of CSF samples from over 550 patients (Table 1).[2]

Total tau levels were significantly higher in CSF samples from Alzheimer's disease than normal con-