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Research Report

A novel membrane protein, encoded by the gene covering KIAA0233, is transcriptionally induced in senile plaque-associated astrocytes

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ABSTRACT

Beta-amyloid (Aβ) deposition and senile plaque-associated astrocytes are common neuropathological features of Alzheimer's disease (AD). Although the molecular mechanisms by which Aβ contributes to the progression of neuropathologic changes have not been entirely established, there is little doubt that the association of Aβ with astrocytes, the predominant cell type in brain, significantly influences exacerbation of the disease. In an effort to identify astrocyte-derived molecules that may be intimately associated with progression of AD, we identified a novel Aβ-induced rat gene, designated Mib, whose human counterpart covers KIAA0233. Mib-transfected C6 cells express Mib protein in the endoplasmic reticulum and endplasmic reticulum–Golgi-intermediate compartment. To evaluate roles of Mib in AD, we investigated its expression in the AD brain. In non-AD brains, Mib mRNA has been detected in neurons but not in quiescent astrocytes. On the contrary, in AD brains, Mib mRNA is expressed in activated astrocytes associated with senile plaques, but not expressed in neurons around lesions. From these observations, Mib appears to be a novel Aβ-responsive gene that may play a role in astrocyte inflammatory activation around senile plaques in the AD brain.

1. Introduction

The neuropathological changes in the brains of patients with Alzheimer's disease (AD) include neuronal and synaptic loss, accumulation of abnormal tau filaments as intracellular neurofibrillary tangles, as well as the appearance of numerous senile plaques and reactive gliosis. A principal component of plaques is beta-amyloid (Aβ), which is demonstrated to induce astrocyte activation in addition to possessing direct toxicity to neurons, and this extracellular deposition is considered to be one of the most crucial events in the onset of AD (Yankner, 1996; Dickson, 1997; Selkoe, 2001). Numerous activated astrocytes observed in lesions are a common feature in the AD brain as well as in many other neurodegenerative disorders. They surround the Aβ-deposited plaques, exhibit morphological changes, and produce a variety of inflammatory media-

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tors in AD brains (Pike et al., 1995; McGeer and McGeer, 1995; Mrak et al., 1995; Johnstone et al., 1999; Wyss-Coray et al., 2003; Nagale et al., 2004).

These observations support the notion that the activated astrocytes in AD lesions have a significant influence on neighboring neurons and their environment in relation to progression of the disease. Apparently, Aβ has a critical role in the exacerbation of AD, and there is little doubt that the association of this molecule with astrocytes significantly affects the lesions of AD brains. Nevertheless, the molecular mechanisms by which Aβ contributes to the progression of astrocyte-mediated neuropathological changes and the actual influence these predominant cells in brain have on exacerbation of AD are not well established.

In an effort to identify key molecules that may be intimately associated with AD progression, we searched for Aβ-responsive mRNAs derived from astrocytes by using a cDNA subtraction technique. In this study, we identified a novel Aβ-induced gene covering KIAA0233, designated Mib. The rat and human protein domain structures, intracellular localization analysis, and evaluation of mRNA expression in AD brains are reported here. Our results give insights into the involvement of this novel gene in AD brain pathology.

2. Results and discussion

2.1. *Identification of a novel Aβ-induced gene*

One subtracted cDNA fragment that originated from a distinctly Aβ-induced astrocyte mRNA (Fig. 1) did not show any nucleotide sequence similarity with DDBJ/EMBL/GenBank data. We compiled an Aβ-treated rat astrocyte cDNA library, screened it using this fragment as a probe, and cloned a 7.0 kb cDNA. Determination of the nucleotide sequence revealed this cDNA to be a novel rat gene with an ORF of 6318 bp (registered in DDBJ/EMBL/GenBank with the accession number AB161229) initiated by an appropriate sequence (GCCATGATGG) for the eukaryotic consensus (Kozak, 1987). The subtracted cDNA fragment, harboring 200 bp nucleotides, was found to extend over the short 3′-terminal-end of the ORF and 3′-untranslated regions in the mRNA. No additional translation initiation sites in the 5′upstream region of the obtained cDNA were obtained by the 5′-RACE method (data not shown). The cDNA encodes 2106 amino acids, and the deduced protein has a typical signal sequence (residues 1–27), followed by 23 transmembrane regions (Fig. 2). Except for the numerous transmembrane regions, there were no distinctly predictive motifs in this protein, so that we designated this protein as Mib (a membrane protein induced by beta-amyloid-treatment). Aβ treatment of rat astrocytes resulted in an approximately 7-fold induction of Mib compared with nontreated cells, as quantified by the intensity of radioactive bands on Northern blot analysis and normalized to those of the β-actin (Figs. 1A and B).

2.2. *Determination of human counterpart, hMib*

Based on 82.9% amino acid identity (68.8% at the nucleotide level), rMib is thought to be the rat orthologue of the human gene covering KIAA0233 (Nagase et al., 1996). The observation

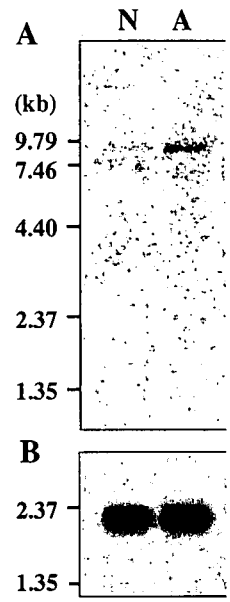

Fig. 1 – Northern blot analysis of rMib. Each lane contains 2 μg of poly(A)+RNA isolated from nontreated (N) and beta-amyloid (25 μM) treated (A) rat astrocytes. (A) The blot was hybridized with the random primed subtracted rMib cDNA probe and exposed for 3 h. (B) As an internal control, the blot was hybridized with the β-actin cDNA control probe and exposed for 2 h.

that the deduced amino acids of rMib have additional N-terminal sequences to that of KIAA0233, as mentioned in the N-terminal truncation warning on the HUGE web site (http://www.kazusa.or.jp/huge/gfpage/KIAA0233/), prompted us to determine the human counterpart cDNA, hMib. The terminal and internal nucleotide sequences of the hMib mRNA were determined by RACE and RT-PCR methods with oligonucleotide primers based on the sequences of KIAA0233 (see Experimental procedure). The compilation of nucleotide sequences revealed that hMib cDNA, with an ORF of 6270 bp (registered in DDBJ/EMBL/GenBank with the accession number AB161230) and the same translational initiation sequence as that of rMib mentioned above, encodes 2090 amino acids. The deduced protein has a typical signal sequence (1–27 residues) and 24 transmembrane domains (Fig. 2). Human Mib has 54 amino acids added to the N-terminus of KIAA0233 and shares 83.1% amino acid identity with the rat counterpart (69.4% at the nucleotide level).

2.3. *Tissue expression*

Rat Mib displayed moderate expression in lung and kidney, and very weak expression in heart, spleen, and liver (Fig. 3). The under-detectable expression of rMib in the brain suggests that the expression is controlled at low levels in the steady state and is consistent with the result shown in Fig. 1. The expression pattern of hMib in tissues was similar to the result displayed on the HUGE web site (http://www.kazusa.or.jp/huge/gfimage/northern/html/KIAA0233.html) (data not shown). As with the result from rat tissues, hMib expression is also regulated to be low in brain relative to other tissues.

2.4. *Mib intracellular localization*

The observation that Mib has a large number of transmembrane domains prompted us to evaluate a possibility that the protein may reside specifically in some organelles. To elucidate the intracellular localization of Mib, a plasmid containing the C-terminal GFP-fused rMib ORF region was transiently introduced into C6 rat glial cells. After 48 h posttransfection, the cells were analyzed by double immunofluorescence staining for each intracellular resident protein. Rat Mib was co-localized with SERCA2 (a marker for endoplasmic reticulum (ER); Fig. 4A) and β-COP (a marker for ER-Golgi-intermediate compartment (ERGIC) and cis-Golgi; Fig. 4B) but not with GM130 (a marker for cis-Golgi; Fig. 4C) or markers for mitochondria, early endosomes, and lysosomes (data not shown). These results indicate that Mib resides in the ER and ERGIC (also referred to as vesicular tubular clusters), but does not enter into the Golgi apparatus. Based on its numerous transmembrane domains, Mib appears to be a protein existing in membranes constituting the ER and ERGIC and might be involved in intracellular protein transport and/or modification.

2.5. *Mib expression in activated astrocytes adjacent to senile plaques of Alzheimer's disease brain*

Mib has been identified as an Aβ-responsive astrocyte gene through an effort to identify novel astrocyte molecules involved in Alzheimer's disease. To evaluate the pathological involvement of hMib in AD, localization of the gene transcripts was evaluated in cerebral cortex sections from the patients by in situ hybridization analysis. As shown in Fig. 5A, mRNA for hMib was expressed in only neurons, but not in astrocytes in age-matched non-AD cortex sections. In AD brain sections, there appeared areas where hMib mRNA expression was not observed in neurons or was observed in damaged-shaped neurons, and these areas are thought to be lesions. In these 'neuron-negative' areas, hMib mRNA expression was detected in some cells surrounding the nonspecifically stained senile plaques (Fig. 5B). These plaques, which were associated with hMib mRNA-positive cells, were verified to contain Aβ using an anti-Aβ antibody (Fig. 5C). Immunohistochemistry with anti-GFAP revealed that these senile plaque-associated hMib mRNA-positive cells were activated astrocytes (Fig. 5D). A review of five AD brain sections indicated that approximately half of the activated astrocytes located around classical senile plaques were positive for hMib mRNA expression. Control hybridization sections, which were incubated without the DNA probe or with a 1:1 mixture of the digoxigenin-11dUTP-labeled and nonlabeled PCR DNA probe, were negative. When a 10:1 mixture of the digoxigenin-11dUTP-labeled and nonlabeled PCR DNA probe was used, only a weak signal could be detected (data not shown). These results indicate that hMib is a distinctly inducible gene in human astrocytes in vivo similar to the result of in vitro studies on rMib in rat astrocytes (Fig. 1), because hMib mRNA was detected in activated astrocytes around senile plaques in AD brain, but not in quiescent astrocytes in non-AD brain nor in lesion-free areas of AD brains. In contrast, neurons express hMib mRNA in non-AD brains and in lesion-free areas of AD brains. These results suggest that hMib expression may be required for maintenance of homeostasis in neuronal activities, although it is not clear whether loss of hMib mRNA in neurons of AD brains is caused by cellular death or loss of homeostasis in diseased neurons. It is also possible that the roles of Mib may differ between astrocytes and neurons.

In inflammatory states, as in AD and other neurodegenerative disorders, numerous activated astrocytes are observed in lesions. Mib might be involved in astroglial inflammatory activation through intracellular protein transport and/or modification, based on analysis of its subcellular localization. There is a possibility that Mib may be involved in ER stress (Kondo et al., 2005), because inducible nitric oxide synthase, which produces the stress-causing nitric oxide (Oyadomari et al., 2001), has been predominantly screened from the same subtracted library (Satoh et al., 2002). To investigate whether expression of hMib mRNA in activated astrocytes occurs in other neurodegenerative disorders than AD, we evaluated localization of the mRNA in substantia nigral sections from Parkinson's disease (PD) patients by in situ hybridization analysis. In substantia nigra of neurologically normal control, hMib mRNA expression was observed only in neurons (Fig. 6A). In PD substantia nigra, the number of melanin-containing neurons was extremely decreased, and hMib mRNA expression was not detected in a few remaining melanin-containing neurons or activated astrocytes (Fig. 6B). Human Mib mRNA expression was observed in some nonmelanized neurons (Fig. 6C). Existence of activated astrocytes in PD sections used was confirmed by immunohistochemistry with anti-GFAP (data not shown). Taken together with the facts that rMib was screened out from Aβ-treated astrocyte cDNA library and hMib mRNA expression was observed in senile plaque-associated astrocytes, these results suggest induction of Mib mRNA expression in activated astrocytes may be caused by astrocytes' connection with Aβ. Further functional investigation into Mib may provide additional insights into its physiological role, particularly in neurodegenerative inflammation and disease pathways in AD.

3. Experimental procedures

3.1. *Astrocyte cell culture and Aβ peptides treatment*

Rat astrocytes were prepared from cerebral cortex by a previously described method (Kato et al., 1979). Briefly, cerebra of 18-day fetuses of Wistar rats (Charles River Laboratories) were isolated and dissociated with trypsin before culturing in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 20% fetal calf serum (FCS; Gibco) and antimycotic solution (Sigma) for 1 week. After trypsin treatment, the secondary culture was grown in DMEM containing 10% FCS. The preparation of aggregated Aβ was as previously described (Satoh et al., 2000). After the astrocytes (first passage cells) reached about 80% confluency, the cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with DMEM containing B27 (Gibco) supplement. After 48 h, aggregated Aβ (25 μM) or control medium was then added to the cells and incubated for 15 h.

3.2. *cDNA subtraction and screening*

Total RNA of Aβ-treated or nontreated astrocytes was purified (Chomczynski and Sacchi, 1987) and poly A (+) RNA was obtained using oligo (dT) latex resin (Roche). Aβ-treated cDNA was subtracted by using a PCR-select cDNA subtraction kit (Clontech) with nontreated astrocyte-derived cDNAs (forward subtraction), and the concurrent reverse

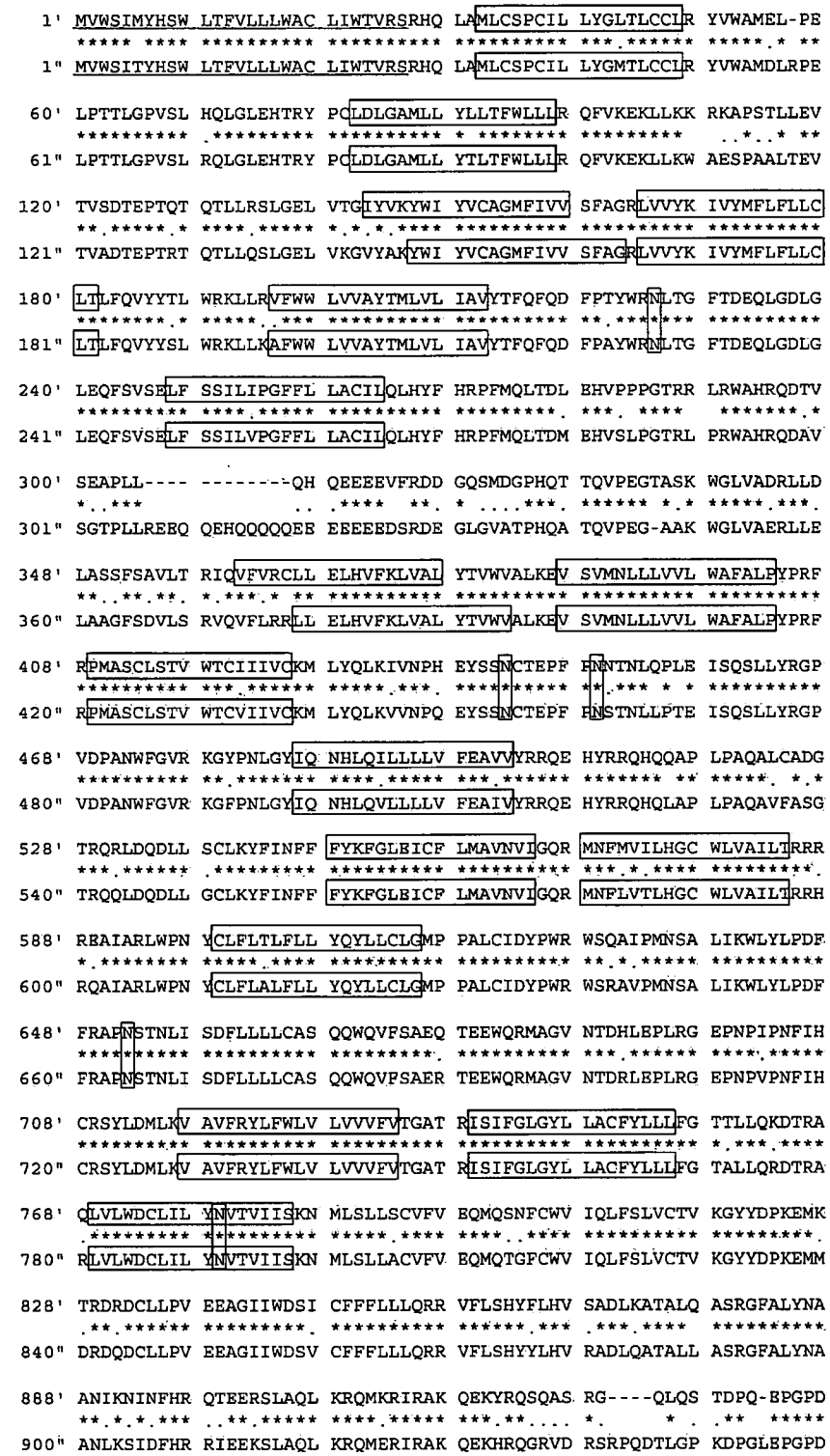

Fig. 2 – Comparison of the Mib amino acid sequences. The amino acid residues for rat (top line) and human (bottom line) Mib are shown and have 83% homology, based on identical matches. The numbers denoted at the left side of the sequences correspond to numbering of respective proteins. The Mib N-terminal region consists of a putative signal sequence (1–27 amino acids, underlined) followed by 23 transmembrane domains in rat or 24 in human. Seven potential N-linked glycosylation sites corresponding to the sequence N-X-S/T are boxed. Domain structure was predicted by PSORT II analysis.

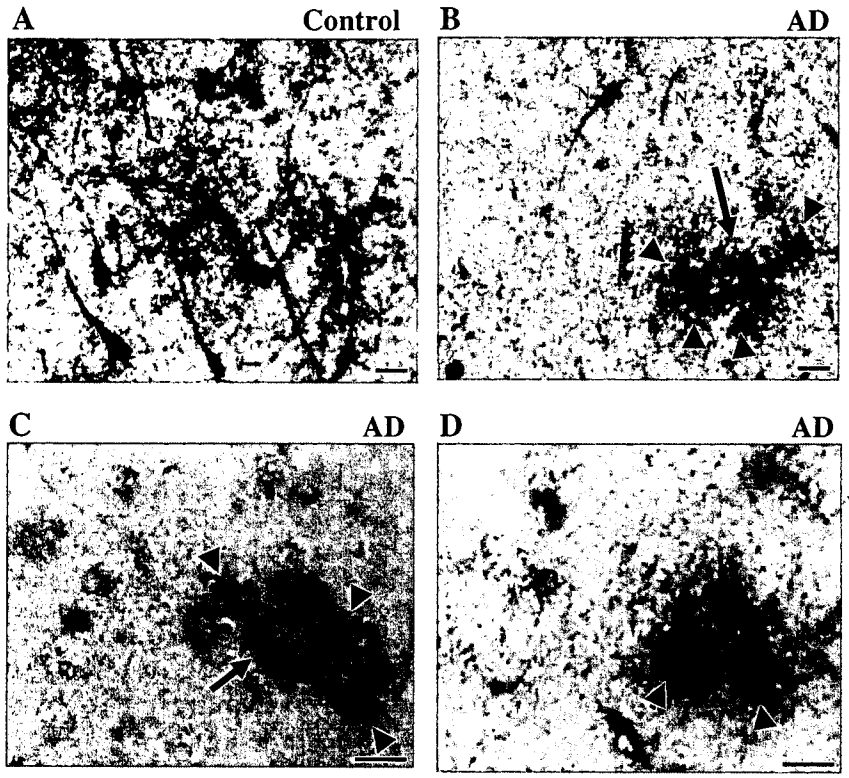

Fig. 5 – In situ localization of hMib mRNA in non-AD (A) and AD brain sections (B, C, D). (A) Positive signals for hMib mRNA were seen in neurons in non-AD cerebral cortex. (B) In the AD brain, unlike the non-AD one, areas devoid of hMib mRNA expression in neurons were observed. In these areas where hMib mRNA-positive neurons were not seen, signals for hMib mRNA were seen in several other nonneuronal cells (arrowheads). Positive cells surrounding nonspecifically stained senile plaques (arrow) were observed. Neuron was faintly stained (N). Nonspecific staining was seen in some vessels. (C) In situ hybridization histochemistry for hMib mRNA (blue black) followed by immunohistochemistry with anti-Aβ antibody (brown) in AD brain. Cells positive for hMib mRNA expression (arrowheads) directly surround senile plaques positive for Aβ (arrow). (D) In situ hybridization histochemistry for hMib mRNA (blue black) followed by immunohistochemistry with anti-GFAP antibody (brown) in AD brain. Cells positive for hMib mRNA expression (arrowheads) were also positive for GFAP. Scale bars: 20 μm.

3.3. *Determination of nucleotide sequence of the human Mib*

Nucleotide primers used here were generated based on the KIAA0233 nucleotide sequence. The terminal mRNA sequences were determined by 5′- and 3′-rapid amplification of cDNA ends (RACE) methods with a GeneRacer kit (Invitrogen) according to the supplier's instructions. The gene-specific primer used for the 5′-RACE reaction is 5′-GAACATGCCAGCACACACATAGATC-3′ and that for the 3′-RACE is 5′-TGCACGGCGGCAGCTG-3′. For determination of the remaining internal mRNA sequences by reverse transcriptase PCR (RT-PCR), the cDNA was divided into 6 regions to avoid DNA polymerization errors. Primer sequences used for amplification of respective regions were as follows: nt 357–1222, forward primer 5′-GGTCACCGTGGCAGACACAG-3′ and reverse primer 5′-CCACCAGCAGCAGGTTCATC-3′; nt 1152–1958, forward primer 5′-CAAGCTGGTGGCCCTGTAC-3′ and reverse primer 5′-CACTTGATGAGTGCGGAGTTCATG-3′; nt 1889–2706, forward primer 5′-CGGCCCTGTGCATTGATTATCC-3′ and reverse primer 5′-GAGGTTGGCAGCGTTGTAGAG-3′; nt 2618–3711, forward primer 5′-GCCATTACTACCTGCACGTCAG-3′ and reverse primer 5′-CCCCTCCGCAAACAGCTC-3′; nt 3618–4765, forward primer 5′-GTACCAGGGACTGATGCGG-3′ and reverse primer 5′-CGCGGTCAACCACCATGG-3′; nt 4668–5757, forward primer 5′-GGCCACAGACATCACGTCC-3′ and reverse primer 5′-CACAGACTGGTCCGAGGTG-3′ (nt: nucleotides number of hMib ORF sequence registered in DDBJ/EMBL/GenBank with the Accession No. AB161230). Human placenta poly A (+) RNA (Clontech), SuperScript II (Gibco), and KOD Plus DNA polymerase (Toyobo) were used as the cDNA template, reverse transcriptase, and high fidelity DNA polymerase, respectively. Each PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen), and each sequence was determined and verified from six independent clones.

3.4. *Tissue mRNA expression*

To determine the tissue expression profiles of the rat and human genes, Northern blots of poly(A)+RNA extracted from rat or human tissues (MTN blot; Clontech) were hybridized with a randomly ^{32}P-labeled rat subtracted cDNA probe or human cDNA probe covering nt 3352–3711, as described above.

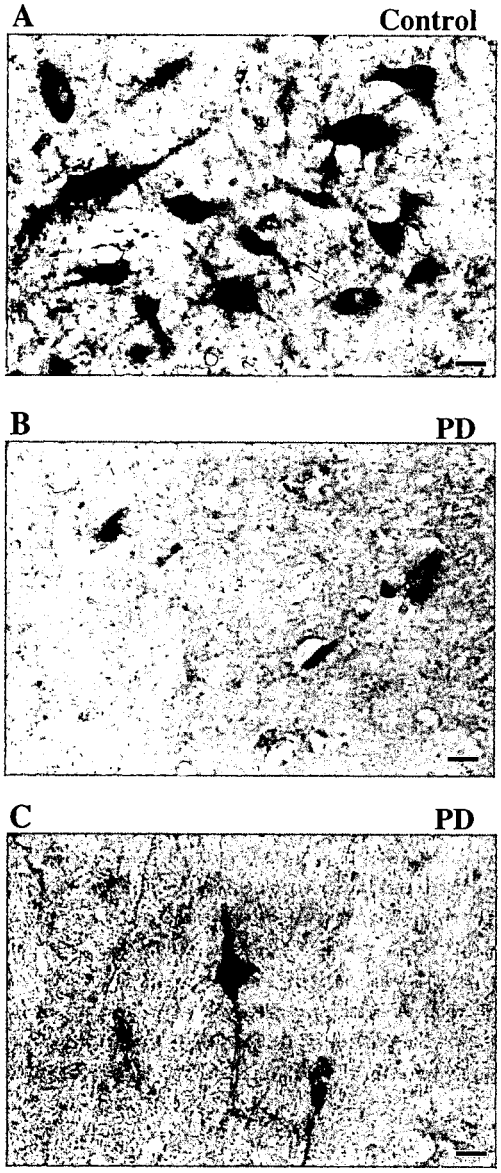

Fig. 6 – In situ localization of hMib mRNA in substantia nigral sections from neurologically normal control (A) and Parkinson's disease (PD) patients (B, C). (A) Positive signals for hMib mRNA were observed in neurons in control substantia nigra. (B) In PD, the number of melanin-containing neurons was extremely decreased, and hMib mRNA expression was not observed in a few remaining melanin-containing neurons. (C) Human Mib mRNA expression was detected in some nonmelanized neurons in PD. Scale bars: 20 μm.

3.5. *Intracellular localization analysis*

The ORF region of rMib was cloned into the phMGFP vector (Promega) and verified by sequencing. The resultant plasmid harboring C-terminal GFP-fused rMib was transfected into C6 rat glioma cells purchased from the American Type Culture Collection and maintained in DMEM containing 10% FCS at 37 °C under 5% CO_2 in a 35-mm dish with an attached coverslip, using FuGENE 6 (Roche). After 48 h, cells were washed with PBS and then fixed with 3.7% paraformaldehyde in PBS for 15 min, followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were then incubated with mouse monoclonal anti-SERCA2 (sarcoplasmic/endoplasmic reticulum calcium ATPases) antibody (clone IID8; Affinity Bioreagents), rabbit polyclonal anti-β–COP (β-coatmer-protein) antibody (Affinity Bioreagents) or mouse monoclonal anti-GM130 antibody (clone 35; BD Transduction Laboratories) in PBS for 1.5 h at room temperature. After this incubation, cells were washed and incubated for 1 h with either Alexa Flour 568-conjugated anti-mouse or anti-rabbit IgG antibody (Molecular Probes). The stained cells were observed with a confocal laser scanning microscopy (Leica TCS NT).

3.6. *Brains*

The brains were obtained from the brain bank of the Choju Medical Institute of Fukushimura Hospital and protocols used were approved by the ethics committee of Fukushimura Hospital. The scientific use of these human materials was conducted in accordance with the Declaration of Helsinki and informed consents were obtained from the guardians of the patients. The brains from five neurologically normal control patients in which Alzheimer's disease (AD)-type changes were lacking (ranged from 54 to 82 years), and those from five patients with AD (ranged from 67 to 80 years) were examined. The diagnosis of AD was established using the criteria recommended by the National Institute on Aging (Khachaturian, 1985) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991). The brains from three neurologically normal control patients (ranged from 61 to 85 years), and those from three patients with Parkinson's disease (PD) (ranged from 61 to 84 years) who fulfilled the diagnostic criteria (Calne et al., 1992) were examined. In all cases, brains were obtained within 2–13 h after death. Small blocks were dissected from the parietal lobes and stored at −80 °C until used. The frozen samples were thawed and fixed for 2 days in phosphate-buffered 4% paraformaldehyde. They were then transferred to a maintenance solution of 15% sucrose in 0.1 M phosphate buffer, pH 7.4, and kept in the cold until used. Sections were cut on a freezing microtome at 20-μm thickness.

3.7. *In situ hybridization*

Human Mib expression in AD brain tissue was evaluated by *in situ* hybridization histochemistry. An hMib cDNA fragment encompassing nt 3352–3711 of the ORF was amplified by PCR and cloned into pCR-Blunt II-TOPO (Invitrogen). A cDNA probe for hMib was constructed by PCR using T7 and SP sequences in the vector as primers. Amplifications were done in 100 μl PCR buffer containing 10 pM primer, 2 nM dNTPs, 200 pM digoxigenin-11-dUTP (Roche), 10 ng template plasmid, and 5 U ampli-Taq DNA polymerase with a thermal cycler (Perkin-ElmerGeneAmp PCR System 9600). Samples were denatured at 94 °C for 5 min, followed by 30 cycles of amplification for 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C. The final extension was at 72 °C for 5 min. Sections were hybridized at 37 °C for 2 days in buffer containing 50% formamide, 4× SSC, 0.2× Denhardt's solution, 21 ng/ml salmon sperm DNA, and 250 ng/ml digoxigenin-11-dUTP labeled and nonlabeled PCR DNA probes.

After hybridization, sections were rinsed three times in 1× SSC. Hybridization was detected by an enzyme-catalyzed color reaction with the DIG Nucleic Acid Detection kit (Boehringer Mannheim Biochemica) according to the supplier's instructions. Negative controls were pretreated with RNAase and processed by identical procedures. Other control experiments were done with mixtures of either 10:1 or 1:1 of the digoxigenin-11dUTP-labeled and nonlabeled PCR DNA probes. After detection of the mRNA signal for hMib by in situ hybridization, immunohistochemistry (Yamada et al., 1998) was used to characterize senile plaques or the labeled cells using antibody against anti-Aβ (1:1000, mouse monoclonal, Signet) or anti-glial fibrillary acidic protein (GFAP) (1:10,000, rabbit polyclonal, DAKO). The sections were mounted on glass slides and coverslips were sealed with liquid paraffin.

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Lymphocyte-specific protein tyrosine kinase is a novel risk gene for Alzheimer disease

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Abstract

Lymphocyte-specific protein tyrosine kinase (LCK) is a lymphoid-specific, Src family protein tyrosine kinase that is known to play a pivotal role in T-cell activation and interact with the T-cell coreceptors, CD4 and CD8. It has been shown to be significantly down-regulated in Alzheimer disease (AD) hippocampus compared with non-demented controls. Furthermore, it is located in a previously identified genetic linkage region (1p34-36) associated with AD. Therefore, we consider it to be a candidate gene for AD. We examined the relationship between AD and the LCK and apolipoprotein E (APOE) genes in 376 AD (including 323 late-onset AD (LOAD) cases and 53 early-onset AD (EOAD) cases) and 378 non-demented controls using a single nucleotide polymorphism (SNP). The polymorphism in intron 1 (+6424 A/G) was significantly associated with AD risk. The odds ratio (OR) for total AD associated with the GG genotype was 1.41 (95% CI=1.06–1.87) and that for LOAD was 1.37 (95%CI=1.02–1.85), while that for APOE-ε4 was 5.06 (95% CI=3.60–7.12). In the APOE-ε4 non-carrier subgroup, the GG genotype also showed significant association (OR=1.66; 95% CI=1.16–2.38). These results indicate that the LCK is a novel risk gene for AD regardless of the APOE genotype.

Keywords: Alzheimer disease; Lymphocyte-specific protein tyrosine kinase (LCK); Polymorphism; Association study; ApoE; Risk factor

1. Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disorder characterized by multiple cognitive deficits and progressive memory impairment in mid- to late-life. Both genetic and environmental factors have been implicated in the development of AD, but it is still unclear how these factors combine and ultimately lead to the neurodegenerative process [1–3]. A number of chemokines, as well as their related receptors, have been shown to be up-regulated in AD brain, supporting the hypothesis that lymphocytes are related to its pathogenesis [4–7].

Lymphocyte-specific protein tyrosine kinase (LCK) is a lymphoid-specific, Src family protein tyrosine kinase that is known to play a pivotal role in T-cell activation and interact with the T-cell coreceptors, CD4 and CD8 [8–10]. In situ hybridization and immunohistochemical studies indicate that the LCK gene is expressed in neurons throughout the brain in distinct regions, including hippocampus and cerebellum [11]. Immunohistochemical examination of brain tissue in mice revealed that its expression was highest in the hippocampus, particularly in dendrites of pyramidal cells [12]. It has also been shown to be significantly down-regulated in the hippocampus in Alzheimer disease (AD)

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patients compared with non-demented controls [13]. Furthermore, human LCK is located in a previously identified genetic linkage region (1p34-36) associated with AD [14]. It has 13 exons distributed across 35 kb of genomic DNA. Its expression is driven by two promoters (distal and proximal) that are active at different stages of development [15]. All of these data suggest that LCK contributes to the pathogenesis of AD. To date, the potential roles for LCK have been reported in T-cell leukemia, colon cancer, type 1 diabetes, systemic lupus erythematosus, relapsing–remitting multiple sclerosis, and rheumatoid arthritis [16–23]. However, there are no reports regarding the association of LCK gene polymorphism with AD. In this study, we investigated whether LCK gene polymorphism could contribute to the risk of sporadic AD.

2. Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected using NINCDS-ADRDA criteria for definite or probable AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination (MMSE)[24,25]. Brain and blood samples were obtained with informed consent from the patients (or their guardians) in the Chubu, Kansai and Ehime areas of Japan [26,27]. A total of 376 unrelated AD patients had been diagnosed previously, and 376 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence for each patient. The mean age±SD (years) at the time of this study was 78.2±8.3 for late-onset AD and 75.5±4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol-chloroform method [28].

During screening for LCK gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of +6424 A/G (C/T) (hCV1895446) in the intron 1 region (minor allele frequency: 0.34). It was consistent with the SNP database, NCBI build 34 Genome (Caucasian 0.14, African–American 0.01, Japanese 0.33, and Chinese 0.30). Genotyping of SNPs was performed using the TaqMan-PCR method. The primers and probes were obtained by ABI assay-on-demand C_1895446_10. Amplification was performed according to the manufacture's protocol. The fluorescent intensity of the PCR products was measured using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and 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 [26]. Allelic and genotypic distribution were analyzed by the usual Chi-squared test of association. The genotypic frequencies were compared by Chi-squared test with the values predicted under 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. The relation of genotypic factors and the effect of APOE-ε4 on AD were assessed by logistic regression analysis. Statistical analyses were performed with SPSS software version 11.0 (SPSS Inc., Chicago, IL).

3. Results

Table 1 shows the distribution of the three genotypes (GG, GA, AA). The distribution obtained for the patients and controls were in Hardy–Weinberg equilibrium. The GG genotype was found in 53% of the 376 total AD patients (57% of early-onset AD (EOAD) and 52% of late-onset AD (LOAD)) and 44% of the 378 control subjects. A significant association was observed between the +6424 A/G polymorphism and total AD ($p<0.02$), and LOAD ($p<0.05$). The odds ratio (OR) for AD associated with the GG genotype was 1.41 (95% CI=1.06–1.87; Table 2) and that for LOAD was 1.37 (95%CI=1.02–1.85). Stratifying AD patients by sex, no statistically significant differences in allele distribution were observed (data not shown). As expected, APOE-ε4 conferred an increased risk for AD (OR=5.06, 95% CI: 3.60–7.12; Table 2). After the logistic regression analysis, a co-dominant model (ε4 dose–effect)

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in LCK

Group	Genotype (frequency)				Allele (frequency)	
	GG	GA	AA	AA+GA	G	A
Control (378)	167 (0.44)	168 (0.44)	43 (0.12)	211 (0.56)	502 (0.66)	254 (0.34)
Total AD (376)	198 (0.53)	138 (0.37)	40 (0.10)	178 (0.47)**	534 (0.71)	218 (0.29)
EOAD (53)	30 (0.57)	15 (0.28)	8 (0.15)	23 (0.43)	75 (0.71)	31 (0.29)
LOAD (323)	168 (0.52)	123 (0.38)	32 (0.10)	155 (0.48)*	459 (0.71)	187 (0.29)

EOAD: early-onset AD, LOAD: late-onset AD.
* $p<0.05$.
** $p<0.02$.

Table 2
Relative risk for interaction between APOE-ε4 and +6424GG

		AD cases	Controls	Odds radio	95%CI
	+6424G/A				
	Non-GG	178	211	Reference	
	GG	198	167	1.41	1.06–1.87
APOE-ε4					
–		195	320	Reference	
+		185	60	5.06	3.60–7.12
APOE-ε4	GG				
–	–	86	181	Reference	
–	+	108	137	1.66	1.16–2.38
+	–	92	30	6.45	3.97–10.5
+	+	90	30	6.31	3.88–10.3

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

provided the best fit ($P=0.024$; Exp(β)=2.78; 95% CI=1.14–6.77), but a dominant model could not be rejected ($P=0.054$; Exp(β)=2.50; 95% CI=0.98–6.34). After logistic regression analysis, a combination of a recessive model of LCK and a co-dominant model of APOE-ε4 provided the best fit ($P=0.014$; Exp(β)=3.01; 95% CI=1.24–7.30). We then examined the GG genotype as a risk factor for AD, considering the APOE status. To quantify possible interactions between APOE-ε4 and LCK-GG, we analyzed the data with respect to various carrier status combinations, taking subjects who had neither APOE-ε4 nor LCK-GG as a reference (Table 2). Four categories were defined by the presence (+) or absence (–) of an ε4 or GG genotype. The GG genotype alone showed an increased risk (OR=1.66; 95% CI=1.16–2.38), and OR for APOE-ε4 and the GG genotype was 6.31. As for the interaction between the APOE-ε4 and LCK-G alleles for the risk of AD; logistic regression analysis did not indicate a significant effect ($P=0.61$). The synergistic effect of G allele in patients having APOE-ε4 was weak. Interestingly, the allele distribution was similar among the AD patients regardless of age at onset (EOAD and LOAD) in the APOE-ε4 non-carrier subgroup. The LCK +6424G allele frequency was also significantly higher in AD patients than in controls (0.66 vs. 0.70–0.73) (Table 3). The results showed that the LCK gene was associated with AD regardless of the APOE genotype.

4. Discussion

We carried out an association analysis of LCK polymorphism with AD. Our data showed that LCK GG homozygosity was associated with significantly increased risk of AD, especially in patients without the APOE-ε4 allele. Patients with the G allele had a higher risk of AD than those with the A allele. The association was obvious not only between total AD patients and controls but also between LOAD patients and controls, even excluding the effect of APOE-ε4. The APOE gene is the only established genetic risk factor for LOAD. However, 50% of LOAD cases carry no APOE-ε4 alleles, suggesting that there must be additional risk factors. Our preliminary data suggest that the LCK gene, or a nearby gene (1p35), is one of the additional risk factors, independent of the APOE gene in AD. We can also suppose that the GG genotype in intron 1 may influence the expression of LCK and could be involved in the selective vulnerability of neurons in AD. The LCK gene consists of 13 exons. The proximal promoter, like that of Src family members, is TATA-less and contains multiple start sites for initiation of transcription. Muise-Helmericks and Rosen determined a potentially important sequence located at positions −474 to −466 acts as a strong repressor of transcription [29]. Although the SNP studied here is located in intron 1, it lies only 7 kb downstream from the critical region of transcription regulation site. According to the SNPbrowser Version 2.0 (Applied Biosystems), strong linkage disequilibrium is shown around the LCK gene. Therefore, it is reasonable to think that +6424A/G polymorphism in intron 1 can contribute to promoter activity. +6424A/G may be the representative marker that influences gene expression. In our data, EOAD patients with the GG genotype did not show a significant difference compared with controls, but the P values are near the threshold. This may be due to a small sample size.

The results of this study support the hypothesis that immunological response contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of LCK immunoreactivity may play an important role in the pathophysiological processes of AD [13]. Although the detailed mechanism of the involvement of LCK in AD is unknown, our data raise the possibility that LCK contributes to the pathogenesis of AD.

Table 3
Genotype and allele numbers and frequencies for G/A polymorphism in LCK without APOE-ε4

Group	Genotype (frequency)				Allele (frequency)	
	GG	GA	AA	AA+GA	G	A
Control (318)	137 (0.43)	146 (0.46)	35 (0.11)	181 (0.57)	420 (0.66)	216 (0.34)
AD (194)	108 (0.56)**	65 (0.33)	21 (0.11)	86 (0.44)***	281 (0.72)*	107 (0.28)
EOAD (35)	20 (0.57)	9 (0.26)	6 (0.17)	15 (0.43)	49 (0.70)	21 (0.30)
LOAD (148)	88 (0.55)	56 (0.35)	15 (0.10)	71 (0.45)**	232 (0.73)*	86 (0.27)

EOAD: early-onset AD, LOAD: late-onset AD.
* $p<0.05$.
** $p<0.02$.
*** $p<0.01$.

LCK might be involved in a new signal transduction pathway. Five of the Src family members, lck, lyn, fyn, src, and yes, have been reported to be expressed in the CNS [30–32]. The adult fyn-deficient brain exhibits abnormal hippocampal development and impairment of long-term potentiation. Although lck knock-out mice have no obvious neurological disorder, a complementation mechanism which expresses a consistent increase in the amount of Src protein may mask its actual effect [33,34]. Furthermore, there is evidence that members of the Src PTK family play important roles in synaptic transmission and plasticity at excitatory synapses in the CNS [35]. In particular, src itself has been shown to up-regulate the activity of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor in the hippocampus and spinal cord [36,37]. The efficiency with which *N*-methyl-D-aspartate receptors (NMDARs) trigger intracellular signaling pathways governs neuronal plasticity, development, senescence, and disease [38]. To date, the potential roles for LCK have been reported in T-cell leukemia, colon cancer, type 1 diabetes, systemic lupus erythematosus, relapsing–remitting multiple sclerosis, and rheumatoid arthritis [16–23]. However, there are no reports regarding the association of LCK gene polymorphism with AD. Our data should be further examined by functional analysis of LCK polymorphisms in AD. A systematic survey in a larger cohort of subjects and family studies are required to evaluate the functional relevance of all SNPs, alone or in combination, in patients. Our study also provides a direction for further investigation of the function of p56lck in the central nervous system.

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

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

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

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

Subjects and Methods

Subjects

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

We compared allele frequencies between sporadic late-onset AD and healthy control subjects. Because *ApoE-ε4* is a risk factor for AD, we stratified the population by ε4 carrier status. *ApoE* genotyping was performed as described previously. Allelic and genotypic distributions were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. p values less than 0.05 were considered significant. Odds ratios (ORs) were calculated with two-tailed p values and 95%

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

Up-Regulated Genes	Down-Regulated Genes
PPP3CB (10q22); calcineurin A beta; rs12644	*HMMR* (5q34); hyaluronan-mediated motility receptor; rs299290
RANBP1 (22q11); RAN binding protein 1; hCV2613312	*LAMB1* (7q31); laminin, beta 1; rs2237685
GNA11 (19p13); guanine nucleotide binding protein 11; rs308064	*POU2F1* (1q24.2); POU domain, class 2, transcription factor 1; rs1407814
CSN1 (4q13); casein, alpha; rs2279526	*MYH8* (17p13); myosin, heavy polypeptide 8, skeltal muscle; rs2024076
FCER1G (1q23); Fc fragment of IgE, high affinity 1; rs11421	*TM4SF5* (17p13); transmembrane 4 superfamily member 1; rs3851
ART3 (4q21); ADP-ribosyltransferase 3; hCV450363	*ADORA2B* (17p12); adenosine A2b receptor; rs1076424
FGL2 (7q11); fibrinogen-like 2; rs2075761	*COL11A1* (1p21.1); collagen, type XI alpha; rs3753841
ZAP128 (14q24.3); peroxisomal long-chain acyl-CoA thioesterase; hCV11164654	*PDCD11* (10q24.33); human mRNA for KIAA0185 gene; rs2986014
CLCNKB (1p36); chloride channel, kidney, B; hCV1814709	*TGM4* (3p21); transglutaminase 4; rs1995641
MCM3AP (21q22); minichromosome maintenance 3-associated protein; rs3788252	*PCK2* (14q11.2); phosphoenolpyruvate carboxykinase 2; rs2071586
FACL4 (Xq23); fatty acid CoA ligase, long chain 4; rs1324805	*HSPC242* (22q12); Homo sapiens PAC clone DJ130H16; rs2072158
RPS15 (19p13.3); ribosomal protein S15; rs1847602	*LCK* (1p35); lymphocyte-specific protein tyrosine kinase; hCV1895446
GBP2 (1p22); guanylate binding protein 2, interferon-inducible; rs4656097 (hCV2431431)	*TNFRSF8* (1p36); tumor necrosis factor receptor superfamily, member 8; hCV9567
PHKG2 (16p11.2); phosphorylase kinase, gamma 2; hCV27530858	*DPYS* (8q22); dihydropyrimidinase; rs2246815
AVPR1A (12q14.2); arginine vasopressin receptor 1A; rs1042615	*EGR2* (10q21.3); early growth response 2; rs2297489
PSMB7 (9q33.3); proteasome subunit, beta-type 7; hCV3112402	*CD36* (7q21); CD36 antigen; rs1358337
EFEMP1 (2p16); EGF-containing fibulin-like extracellular matrix protein 1; rs1344733	*CAV2* (7q31); caveolin 2; rs2270189
	AKAP8 (19p13.12); A kinase anchor protein 95; hCV2596739

confidence intervals (CIs). Bonferroni correction was applied to reduce type I error. The relation of genotypic factors and the effect of *ApoEε4* to AD was assessed with logistic regression analysis. Statistical analyses were performed with SPSS software version 11.0 (SPSS, Chicago, IL).

Genotyping

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

Results

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

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

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

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

	Gene Name	rs number	Celera	Location		AD : Control (Genotype)	*P* value	AD : Control (allele)	*P* value	OR (95% CI) APOE4(−) Subjects
Down-regulated	*POU2F1*	rs1407814	hCV1622428	intron 2	9943T/C	TT/TC/CC = 11/90/275 : 2/65/309	0.0022**	T/C = 112/640 : 69/683	0.0007**	1.73 (1.11–2.69)
	MYH8	rs2024076	hCV2179107	intron 18	13583T/C	TT/TC/CC = 9/132/235 : 23/110/243	0.017*	T/C = 150/602 : 156/596	0.700	1.98 (0.78–5.06)
	CD36	rs1358337	hCV1803785	intron 3	12329A/G	GG/GA/AA = 44/152/180 : 51/182/143	0.024*	G/A = 240/512 : 284/468	0.017*	1.35 (0.79–2.29)
	DPYS	rs2246815	hCV9595720	intron 5	38687A/G	GG/GA/AA = 174/163/39 : 137/192/47	0.023*	G/A = 511/241 : 466/286	0.015*	1.45 (0.80–2.64)
	COL11A1	rs3753841	hCV2947954	exon 52	193817A/G	AA/AG/GG = 178/160/38 : 158/156/62	0.030*	A/G = 516/236 : 472/280	0.017*	1.62 (0.95–2.75)
Up-regulated	*GNA11*	rs308064	hCV3149364	intron 2	18527T/C	TT/TC/CC = 144/176/56 : 156/186/34	0.046*	T/C = 464/288 : 498/254	0.068	1.02 (0.70–1.46)
	FCER1G	rs11421	hCV1841966	exon 5	3825T/C	TT/TC/CC = 132/159/85 : 140/183/53	0.009**	T/C = 423/329 : 463/289	0.036*	1.05 (0.73–1.52)
	MCM3AP	rs3788252	hCV3270916	intron 25	43966T/C	TT/TC/CC = 18/144/214 : 31/115/230	0.026*	T/C = 180/572 : 177/575	0.856	1.19 (0.83–1.71)
	GBP2	rs4656097	hCV2431431	intron 6	6000T/A	TT/TA/AA = 17/155/204 : 22/122/232	0.041*	T/A = 189/563 : 166/586	0.162	1.14 (0.84–1.54)

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

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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Clinical Features of Argyrophilic Grain Disease

A Retrospective Survey of Cases With Neuropsychiatric Symptoms

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Objective: *Although argyrophilic grain disease (AGD) appears common in post-mortem series, its clinical features are not widely known. The aim of this study was to explore such clinical features in neuropathologically-confirmed AGD cases.* **Methods:** *After completing a neuropathological assessment of 386 patients, 33 cases (8.5%) were diagnosed as having AGD; 10 were diagnosed as "pure" cases. These subjects had been admitted to geriatric wards of mental hospitals because of behavioral or neuropsychiatric symptoms requiring medical management. Assessment of the clinical features of the pure cases was based on the evaluations in medical records.* **Results:** *The average age at onset was 82.2 years. Amnesia was the most common initial symptom; irritability and agitation were also common as initial symptoms. During the course of the illness, irritability was the most frequently observed, followed by delusions (mostly delusions of persecution), dysphoria, and then agitation, and apathy. In contrast to the severity of amnesia, other cognitive functions were relatively spared, and the sensorimotor symptoms were not remarkable.* **Conclusions:** *AGD is a late-onset dementing disorder clinically characterized by amnesia, with other cognitive functions relatively spared, and prominent neuropsychiatric features. These features may correlate with the high level of AGD seen in limbic structures. Future studies are needed to elucidate whether these features are common to all AGD patients or to a clinical subtype with neuropsychiatric symptoms.* (Am J Geriatr Psychiatry 2005; 13:1083–1091)

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Argyrophilic grains (AGs) were first reported as a novel pathological finding in the hippocampal region in brains of late-onset dementia patients.[1,2] Although it is still controversial whether argyrophilic grain disease (AGD) should be considered to be a disease entity, in the proper sense of the word, a series of studies have characterized the morphologic, biochemical, and genetic features of AGD, suggesting the validity of AGD as a distinct pathologic entity. AGs are immunoreactive to a series of anti-tau antibodies, suggesting that AGD is a tauopathy.[3] Recent studies have also revealed that AGs are composed of tau isoforms with four repeats (4R) in the microtubule-binding domain, showing that AGD is a 4R tauopathy, which also includes progressive supranuclear palsy and corticobasal degeneration.[4–6] It may be reasonable to characterize AGD as a "limbic 4R tauopathy," since most of the lesions in AGD are prominent in the limbic system, particularly in the hippocampal region and the amygdala.[4]

AGD is found in 5%–10% of consecutive autopsies,[3,7,8] rising to over 40% when the very early stage of AGD is included.[9] Although AGD is therefore not a rare pathological entity, little is known about its clinical features, except the finding that AGD causes cognitive decline even in the presence of a concurrent mild Alzheimer-type pathology, suggesting that AGD contributes to the dementing process. A few reports have also described AGD as being clinically characterized by neuropsychiatric symptoms. Braak and Braak[7] reported that the clinical features of AGD closely resemble those of Alzheimer disease (AD), with the subtle difference that personality changes tend to precede memory failure in AGD. Jellinger,[10] on the other hand, reported that personality changes, memory loss, and frontal-lobe signs are much more prominent in AGD than dementia, indicating the importance of differentiating between AD and Pick's disease, yet these reports did not include details of the cases. Ikeda et al.[11] also reported detailed clinical records of four cases of AGD that showed memory disturbance, with relative preservation of other functions, and personality change characterized by emotional disorders, including aggression or ill temper.

In the present study, over 300 cases from our brain banks were screened for the presence of AGs by use of Gallyas silver staining of the hippocampus. After carrying out detailed neuropathological evaluations, the clinical features of pure AGD cases were assessed from the patients' medical records. The materials investigated for this study were the records of patients who had been admitted to the geriatric wards of mental hospitals because of behavioral or neuropsychiatric symptoms requiring medical management. Therefore, it is possible that the patients enrolled here constitute a clinical subtype of AGD, with prominence of neuropsychiatric symptoms. Nevertheless, this study reveals some key clinical findings of AGD that we believe can assist in the clinical diagnosis of this poorly understood pathological entity.

METHODS

Sections from 386 brains in the brain banks of the Department of Psychiatry of Yokohama City University School of Medicine and the Fukushimura Hospital were examined for AGs. The series included 187 men and 199 women, ranging in age from 48 to 102 years, with a mean age of 79.9 years. For routine neuropathological evaluation, formalin-fixed, paraffin-embedded sections from each area of the whole brain were stained with hematoxylin-eosin, Klüver-Barrera, and methenamin-silver stains. Also, α-synuclein immunostain was routinely used to detect Lewy bodies, and tau immunostains were used, as described,[12,13] to evaluate the tau pathologies if neurofibrillary lesions were detected by the methenamin-silver staining. These sections were also used to re-evaluate the concomitant pathologies in AGD cases for the present study. The main pathological diagnoses in the present series included 113 cases of AD (with Braak tangle Stages ranging from IV to VI, combined with plaque Stage C),[14] including 45 men and 68 women, with a mean age of 82.2 years; there were 66 cases of vascular dementia,[15] 41 cases of dementia with Lewy bodies,[16] and 30 cases of physiological aging, with mild age-associated Alzheimer pathology of Braak tangle Stage III or less. Gallyas silver staining[17] was applied to sections of the anterior hippocampal region, including the cornu ammonis, the dentate gyrus, the subiculum, the entorhinal/transentorhinal cortex, and the adjacent temporal cortex, to screen for the presence of AGs. In cases with AGs, an additional section of the basal forebrain, including the amygdala, was also stained with Gallyas silver stain. Diagnosis of AGD was determined by the presence of massive AGs in both the hippocampus and

the amygdala (Figure 1 [A]); additional pathologies, including coiled bodies under the diseased cortex (Figure 1 [B]) and ballooned neurons in the limbic area (Figure 1 [C]).[8,18] Also, the cases were characterized as "pure" AGD in this study only if they involved concurrent mild Alzheimer pathology corresponding to Braak tangle Stage III or less.

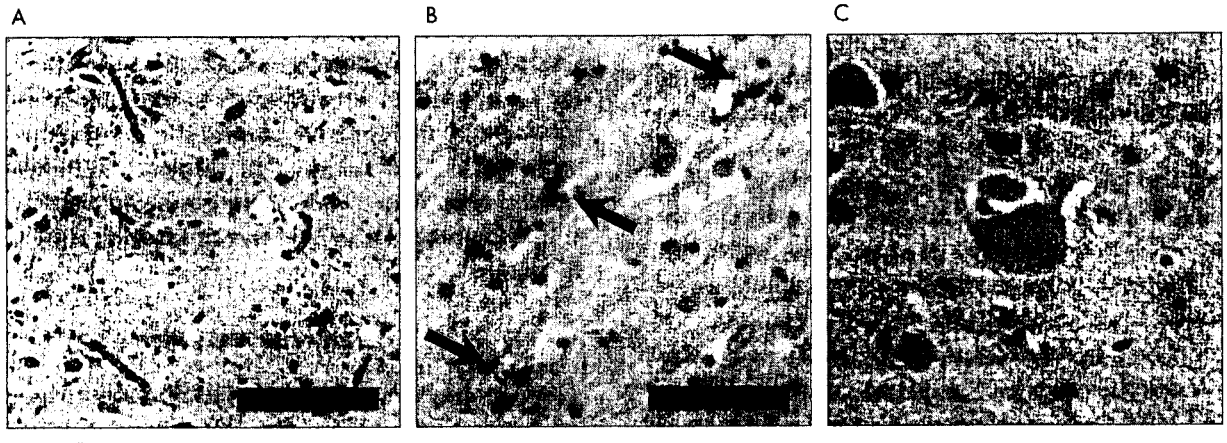
The brains used in this study were from individuals who had been admitted to the Fukushimura Hospital or other mental hospitals because of the appearance of behavioral or neuropsychiatric symptoms needing medical management. Therefore, all cases had undergone a clinical geriatric evaluation, which comprised a detailed history, physical and neuropsychiatric examination, routine biochemical and hematological investigations, and functional assessment by nurses. During hospitalization, the patients were evaluated once weekly by psychiatrists highly skilled in the management of dementia. They had all received psychiatric residency training and had at least 2 years' experience in medical residency. Typically, the patients' cognitive functioning was assessed by Mini-Mental State Exam (MMSE)[19] or other dementia rating scales.[20] Also, nurses evaluated patients' activities on a daily basis. Every patient underwent brain-imaging by means of computed topography (CT) one or more times, but without magnetic resonance imaging (MRI). This study was approved by the Human Subjects Review Committee of Yokohama City University and Fukushimura Hospital.

Assessment of the neuropsychiatric features in the present study was based on the evaluations in medical and nursing records. Three trained geriatric psychiatrists (TT, DI, and HU) independently reviewed the records. Cognitive functioning and neuropsychiatric symptoms were assessed mainly from descriptions in the medical records. The descriptions of neuropsychiatric symptoms were categorized retrospectively in accordance with the items in the Neuropsychiatric Inventory (NPI).[21,22] In this study, the symptoms were considered clinically significant if both the frequency and the severity were regarded as applying to rating 2: Often; about once per week for frequency; and moderate in severity) or higher, by the definitions of NPI. If there was a discrepancy in the evaluations by the three psychiatrists, the assessments were then determined through consensus discussion. Also, the attending physicians were consulted as needed.

Functional ability was assessed through interview of the nursing staff who directly cared for the patient, in accordance with the items on the Disability Assessment for Dementia Scale (DAD).[23] Nursing charts were also referred to for this evaluation. Ability on admission was evaluated, since the patients' performance had been carefully assessed and recorded in detail at that time.

Atrophy of the medial temporal lobe was assessed by CT, through measurement of the radial width of the temporal horn of the lateral ventricles, as described by Frisoni et al.[24] This is a measure of enlarge-

FIGURE 1. Neuropathological Findings of Case 1



Note: Gallyas silver stain of the parahippocampal gyrus (A, B) reveals massive argyrophilic grains in the neuropil (A), coiled bodies in the white matter underlying the affected cortex (B; arrows), and a ballooned neuron in the amygdala stained with hematoxilin-eosin (C).
A: bar = 50 μm; B and C: bar = 100 μm.

ment of the temporal horn owing to shrinkage of the hippocampus, and it has been shown to be an accurate marker of AD.[24] The measure was taken from a section in which 1) the temporal horn could be better appreciated throughout its antero-posterior extension (from the trigone of the lateral ventricle to the tip of the temporal horn); and 2) the tip of the temporal horn was largest. Assessment was performed by using a CT of each patient that had been taken on admission. After measurement of the radial widths of both right and left temporal horns, we calculated the mean for each patient.

The difference in age at onset between "pure" AGD cases, AGD cases with complications, and the 113 cases with AD was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test, with pure-AGD as a control. The differences between two independent variables were analyzed by a two-sample *t*-test. The difference in brain weight between pure-AGD and AD was analyzed by two-way analysis of covariance (ANCOVA), with sex as a covariant. The radial width of the temporal horn was compared in pure-AGD and AD cases by two-sample *t*-test, since sex-related differences have not been reported to be significant with this measure.[24] The data were analyzed with SPSS for Windows, Version 11.0J (SPSS Inc., Chicago, IL), and a p value <0.05 was considered statistically significant.

RESULTS

Age at Onset and Duration of AGD

Thirty-three cases of AGD, including 15 men and 18 women, with a mean age of 83.7 years at autopsy (standard deviation [SD]: 7.4), were identified from a total of 386 brains (8.5%). Of the 33 AGD cases, 23 had additional pathological findings, including 11 cases with cerebrovascular disease, 5 cases with dementia with Lewy bodies, 3 cases with AD (with Braak Stage IV of neurofibrillary pathology), and 4 cases with other neurological diseases. Ten cases, including 3 men and 7 women, with a mean age of 88.0 years at autopsy (SD: 6.6), were diagnosed as having "pure" AGD, involving concurrent mild Alzheimer pathology corresponding to Braak tangle Stage III or less. Significant differences in age at onset were observed between pure-AGD (mean age: 82.2 years; SD: 5.4), AGD cases with complications (mean age: 75.9 years; SD: 7.0), and AD (mean age: 75.0 years; SD: 8.6; $F_{[2]}=3.52$; $p=0.03$), and the subsequent Dunnett's test revealed significant differences between pure-AGD and AD ($p=0.02$). The age at onset in AGD-with-complications tended to be lower than in pure-AGD cases, although the difference did not reach statistical significance ($p=0.08$). The mean duration of pure-AGD cases was 5.8 years, which was not significantly different from AD cases (7.3 years; $t=-0.98$, $p=0.33$). The patients had been hospitalized for a mean duration of 1.8 years, although it must be noted that it is common in Japan for patients to stay at the same psychiatric hospital even after improvement in their psychiatric symptoms because of deterioration in activities of daily living or lack of appropriate caregivers.

Neuropsychiatric Symptoms and Differential Diagnosis of AGD

The clinical features in the pure-AGD cases are summarized in Table 1. Case 7 was referred to Yokohama City University Hospital from Soga Hospital, a mental hospital with a geriatric ward; the other cases were referred from Fukushimura Hospital.

In pure-AGD cases, amnesia was the most common initial symptom (70%). Irritability and agitation were also often found as initial symptoms. In the whole clinical course, irritability was the most frequently observed neuropsychiatric symptom (80%), followed by delusions (70%; mostly delusions of persecution), dysphoria (60%), and then agitation, apathy, and aberrant motor behavior (50% for each). In some cases, the emergence of these neuropsychiatric symptoms preceded the appearance of amnesia. On the other hand, prominent sensorimotor symptoms were not recorded in these cases, except for contractures of joints in the late stages. The clinical diagnosis was senile dementia of the Alzheimer type in 8 of 10 cases, suggesting that AD is the most important differential diagnosis.

Functional Abilities

An evaluation of functional abilities was performed on admission; the patients' mean MMSE score was 20.1 (SD: 5.9). Since the evaluation was mainly based on the assessment in the hospital, items such as "meal preparation" and "going on outings"