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Influence of *APOE* Genotype and the Presence of Alzheimer's Pathology on Synaptic Membrane Lipids of Human Brains

Naoto Oikawa,[1] Hiroyuki Hatsuta,[2] Shigeo Murayama,[2] Akemi Suzuki,[3] and Katsuhiko Yanagisawa[1]*

[1]Department of Drug Discovery, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu, Japan
[2]Department of Neuropathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan
[3]Institute of Glycoscience, Tokai University, Kanagawa, Japan

The *APOE* genotype is the major risk factor for Alzheimer's disease (AD); however, it remains unclarified how the ε4 allele accelerates whereas the ε2 allele suppresses AD development, compared with the more common ε3 allele. On the basis of the previous finding that the assembly of the amyloid-β protein (Aβ) into fibrils in the brain, an early and invariable pathological feature of AD, depends on the lipid environment, we determined the levels of synaptic membrane lipids in aged individuals of different *APOE* genotypes. In the comparison between amyloid-free ε2/ε3 and ε3/ε3 brains, the presence of the ε2 allele significantly decreased the level of cholesterol. Alternatively, in the comparison among ε3/ε3 brains, the presence of AD pathology substantially decreased the levels of cholesterol. This study suggests that the ε2 allele suppresses the initiation of AD development by lowering the cholesterol levels in synaptic membranes.

Key words: Alzheimer's disease; cholesterol; ganglioside; synaptic plasma membrane; microdomain

The ε4 allele of the apolipoprotein E gene (*APOE*) increases the risk of Alzheimer's disease (AD), whereas the ε2 allele decreases it, compared with the most common ε3 allele (for review see Holtzman et al., 2012; Liu et al., 2013). Despite intensive efforts, the mechanisms underlying *APOE*-allele-linked modulation of AD development still remain to be clarified. However, it is at least confirmed that the *APOE* alleles can affect the risk of AD through a wide range of biological functions of apolipoprotein E, not through a simple function, such as the regulation of lipid transport, glucose metabolism, or neuroinflammation (for review see Holtzman et al., 2012; Liu et al., 2013). In addition, it is assumed that the *APOE* alleles modulate AD development upstream and downstream of amyloid deposition, a fundamental core of AD pathology, or through their effects on amyloid-dependent and amyloid-independent processes (Liu et al., 2013).

With regard to amyloid deposition in the brain, postmortem neuropathological examination and clinical amyloid imaging by positron emission tomography revealed that it is enhanced in the presence of the ε4 allele (Schmechel et al., 1993; Oyama et al., 1995; Polvikoski et al., 1995; Reiman et al., 2009) but suppressed in the presence of the ε2 allele (Benjamin et al., 1994; Nagy et al., 1995; Polvikoski et al., 1995; Lippa et al., 1997; Tiraboschi et al., 2004). Although the *APOE*-genotype-dependent effects on amyloid deposition also remain unknown, such effects likely are due to modulation of fibrillogenesis and/or clearance of the amyloid-β protein (Aβ), a proteinaceous component of amyloid (for review see Holtzman et al., 2012).

Accumulating evidence suggests that Aβ assembles into fibrils in the brain upon interaction with cellular membranes, especially through specific binding to gangliosides (for review see Yanagisawa, 2007; Ariga et al., 2008; Matsuzaki et al., 2010; Di Paolo and Kim, 2011). In our early studies with human *APOE* knock-in mouse brains, the ε4 knock-in brains, compared with the ε3 knock-in brains, showed significant increases in cholesterol levels in the exofacial leaflet of synaptic membranes (Hayashi et al., 2002) and ganglioside levels in an age-dependent manner in synaptic membrane microdomains (Yamamoto et al., 2004). Taken together with the evidence that amyloid deposition in the brain starts at

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*Correspondence to: Katsuhiko Yanagisawa, Department of Drug Discovery, Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, Obu 474-8522, Japan. E-mail: katuhiko@ncgg.go.jp

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presynaptic terminals (Bugiani et al., 1990; Probst et al., 1991), it is reasonable to assume that the effect of *APOE* genotype on amyloid deposition is attributable to the modulation of synaptic membrane lipids.

This study examined the frontal cortices of autopsied brains of aged individuals carrying the ε2/ε3, ε3/ε3, ε3/ε4, and ε4/ε4 alleles. Available brains from individuals carrying the ε4 allele all showed advanced AD pathology, making it difficult for us to perform fine analyses of synaptic membrane lipids. In contrast, available brains from individuals carrying the ε2 allele all lacked amyloid deposition. The extent of AD pathology varied in the brains with the ε3/ε3 allele; therefore, we selected the brains lacking or showing amyloid deposition. We focused on cholesterol and gangliosides because the major role of apolipoprotein E is the transport of cholesterol between cells in brains, and gangliosides likely are involved in the acceleration of Aβ assembly (Matsuzaki et al., 2010). Here we report the following. First, in the comparison between amyloid-free ε2/ε3 and ε3/ε3 brains, the presence of the ε2 allele decreased the levels of cholesterol in synaptic membranes and synaptic membrane microdomains. Second, in the comparison among ε3/ε3 brains, the presence of AD pathology decreased the levels of cholesterol in synaptic membranes and synaptic membrane microdomains and the levels of some species of gangliosides in synaptic membrane microdomains. This study suggests that ε2 allele suppresses AD development by keeping cholesterol in synaptic membranes and/or synaptic membrane microdomains under a certain level, which is prerequisite for the initiation of Aβ assembly into fibrils.

MATERIALS AND METHODS

Materials

The following antibodies were used: synaptophysin (Synaptic Systems, Gottingen, Germany), SNAP25 (Enzo Life Science, Farmingdale, NY), Bip/GRP78 and flotillin-1 (BD Transduction Laboratories, San Jose, CA), LAMP-1 (Millipore, Billerica, MA), transferrin receptor (TfR; Life Technologies, Carlsbad, CA), prion protein (PrP; Sigma, St. Louis, MO), anti-β amyloid 11–28 (12B2; IBL, Maebashi, Japan), and antiphosphorylated tau (AT8; Innogenetics, Temse, Belgium). Cholera toxin B subunit–peroxidase conjugate (CTB-HRP) and an Amplex-red cholesterol assay kit were purchased from Sigma-Aldrich and Life Technologies, respectively. Synthetic GM1(d18:1-^{13}C16:0) and GM3(d18:1–14:0) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Avanti Polar Lipids (Alabaster, AL), respectively. All the organic solvents used in liquid chromatography-mass spectrometry (LC-MS) were of LC-MS grade (Fluka, Sigma-Aldrich).

Tissue Source

Human brain specimens were obtained from the Brain Bank for Aging Research at the Tokyo Metropolitan Institute of Gerontology. This study was approved by the Ethics Committees of the National Center for Geriatrics and Gerontology and Tokyo Metropolitan Institute of Gerontology. Each specimen was taken from Brodmann area 8 or 9. Neuropathological analysis was performed by modified methenamine and Gallyas-Braak silver staining and immunohistochemical analysis with anti-Aβ 11–28 (12B2, monoclonal) and antiphosphorylated tau (AT8, monoclonal) antibodies as previously reported (Adachi et al., 2010). AD neuropathologies were classified in accordance with the criteria of Braak and Braak (1991).

Preparation of Synaptosomes

Synaptosomes were prepared as previously reported (Igbavboa et al., 1996; Yamamoto et al., 2008). Briefly, after removal of the white matter from a tissue piece, the gray matter was homogenized in ice-cold buffer A (10 mM HEPES, 0.32 M sucrose, 0.25 mM EDTA, pH 7.4) by motor-driven homogenization at 1,000 rpm with 10 strokes. After the removal of the nuclei by centrifugation at 580*g* for 8 min at 4°C, a crude mitochondrial pellet (CMP) was collected by centrifugation of the postnuclear supernatant (PNS) at 14,600*g* for 20 min at 4°C. The CMP was suspended in buffer B (10 mM HEPES, 0.32 M sucrose, pH 7.4) by hand homogenization, layered over 7.5% and 14% Ficoll in buffer B, and then centrifuged at 87,000*g* (SW50.1 rotor, Beckman) for 30 min at 4°C. The interface between 7.5% and 14% Ficoll solutions rich in synaptosomes was collected in 10 ml buffer B and then centrifuged at 18,550*g* for 15 min at 4°C.

Preparation of Synaptic Plasma Membranes

Synaptic plasma membranes (SPMs) were prepared as previously reported (Cotman and Matthews, 1971; Fontaine et al., 1980; Igbavboa et al., 1996). Briefly, synaptosomes were osmotically shocked by suspension in ice cold 5 mM Tris buffer (pH 8.5) and stirring on ice for 90 min with vortex mixing every 30 min. After centrifugation of the suspension at 43,700*g* for 20 min at 4°C, the resultant pellet was suspended in buffer B, layered over 25% and 32.5% sucrose in 10 mM HEPES (pH 7.4), and then centrifuged at 41,000*g* (SW50.1 rotor; Beckman) for 30 min at 4°C. The interface between 25% and 32.5% sucrose solutions rich in SPMs was collected in 9 volumes of 10 mM HEPES buffer (pH 7.4) and then centrifuged at 50,380*g* for 20 min at 4°C.

Isolation of Low-Density Membrane Microdomains From SPMs

Low-density membrane microdomains (LDMs), or lipid rafts, were isolated by a detergent-free method based on a previous report (Persaud-Sawin et al., 2009). Briefly, SPMs were suspended in buffer C (25 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing Complete protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany) and sonicated five times for 8 sec each on ice at a high power output with an ultrasonic disruptor (UD201; Tomy Seiko, Tokyo, Japan) at 1–2-min intervals. After adjusting to 40% sucrose by mixing with buffer C and 80% sucrose in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.4), the suspension was placed at the bottom of a tube, and a discontinuous sucrose gradient was prepared by pouring 35%, 25%, 15%, and 5% sucrose in TBS sequentially on top of the suspension.

The gradient was ultracentrifuged at 261,000*g* (SW41 rotor; Beckman) for 18 hr at 4°C. Fractions (1 ml each) were collected from the top. Fractions 4, 5, and 6 were collected in 7 volumes of water and centrifuged at 450,000*g* for 20 min at 4°C (type 70.1 rotor; Beckman). The resultant pellets were used as LDM samples. In characterizations of the fractions by Western blotting, each sample was subjected to trichloroacetic acid (TCA) precipitation. The samples were mixed with an equal amount of 20% TCA solution and incubated for 1 hr at −20°C. After centrifugation at 20,400*g* for 15 min at 4°C, the pellets were washed with ice-cold 100% ethanol, and recentrifuged at 20,400*g* for 15 min at 4°C. The resultant pellets were air dried and analyzed by SDS-PAGE and Western blotting.

Measurement of Cholesterol Level

Cholesterol level was measured using an Amplex-red cholesterol assay kit, as previously reported (Oikawa et al., 2012).

Lipid Extraction

Lipids were extracted as previously reported (Oikawa et al., 2012). Briefly, whole lipids were extracted from SPMs and LDMs with chloroform–methanol (2:1; v/v) and chloroform–methanol–water (1:2:0.8; v/v/v). Gangliosides were separated by two-phase partitioning, as previously reported (Folch et al., 1957). After evaporation under N_2 gas, whole-lipid extract was redissolved in chloroform–methanol–water (8:4:3; v/v/v) and then centrifuged at 1,500 rpm for 10 min, and the upper phase was collected. Pure-solvent upper phase prepared from chloroform–methanol–water (8:4:3; v/v/v) was added to the resultant lower phase, followed by centrifugation at 1,500 rpm for 10 min. The upper phase was then collected. This step was repeated twice. The collected upper phases were combined, evaporated under N_2 gas, and redissolved in methanol. Synthetic GM1(d18:1-^{13}C16:0) and GM3(d18:1–14:0) were added to SPM and LDM samples, respectively, as internal standards for MS.

LC-MS

Gangliosides were analyzed by LC-MS, as previously reported, with slight modification (Nagafuku et al., 2012). Briefly, gangliosides were separated by LC using a Develosil ε30 column (1 mm i.d. × 50 mm; Nomura Chemical) and elution solvents, solvent A (12.5% [SPMs] or 20% [LDMs] water, 10 mM ammonium formate, and 0.1% formic acid in methanol) and solvent B (2.5% [SPMs and LDMs] water, 10 mM ammonium formate, 0.1% formic acid, 50% isopropyl alcohol in methanol), using a gradient elution of 20% B in A for 5 min, from 20% to 100% B in A for 20 min, and 100% B for 5 min. The flow rate in LC was 50 μl/min. MS was performed using a Shimadzu LC-IT-MS in the negative ion and automode with the mass range from m/z 200 to 2,000 and the detector voltage at 1.9 kV. The ratio of ganglioside signals to internal standard signals was measured using mass chromatograms monitoring $[M–H]^-$ ions. Ganglioside structures were confirmed by MS2 with collision-induced dissociation (CID) energy at 50% arbitrary, and ceramide structures were characterized by MS3 or MS4 with manual mode detection monitoring corresponding m/z values.

Statistical Analyses

All statistical analyses were performed in GraphPad Prism version 5. Data are presented as mean ± SEM (in cholesterol analysis) or mean (in ganglioside analysis). For comparisons between groups with different *APOE* genotypes and AD pathology, Student's *t*-test was used, and $P < 0.05$ were considered significant.

RESULTS

Demographics and Neuropathological Characteristics of Autopsied Brains

There were no significant differences between groups in any of the demographics, including age and postmortem delay (Table I). Histopathological analysis showed no Aβ deposition in the amyloid-free ε2/3 and ε3/3 brains (Fig. 1A,B). In the ε3/3 brains with AD pathology, numerous senile plaques and neurofibrillary tangles were observed (Fig. 1C,D).

SPM and LDM Preparation From Brain Tissues

We first attempted to extract synaptosomes and SPMs from brain cortical tissues by density gradient fractionation (Igbavboa et al., 1996; Yamamoto et al., 2008). As previously reported, synaptic proteins, such as synaptophysin and SNAP25, were abundant in the synaptosome fraction, whereas an endoplasmic reticulum-localized protein, Bip/GRP78, and a lysosomal protein, LAMP-1, were limited in the fraction (Fig. 2A). Subsequently, SPMs were prepared from the collected synaptosomes by osmotic shock and sucrose density gradient fractionation, as previously reported (Cotman and Matthews, 1971; Fontaine et al., 1980). Next, to determine whether lipid distribution was also affected in synaptic membrane microdomains by the *APOE* genotype and the presence of AD pathology, LDMs were collected from SPMs by the detergent-free method, which potently prevents the artificial domain formation that commonly occurs in the conventional detergent method (Lichtenberg et al., 2005; Lingwood and Simons, 2007). For the detergent-free isolation of LDMs, we employed an original method (Persaud-Sawin et al., 2009), with minor modification as follows. The samples

TABLE I. Demographics and Neuropathologic Characteristics of the Autopsied Brains*

Group	ε2/ε3 (AF)	ε3/ε3 (AF)	ε3/ε3 (AD)
N (male/female)	8 (6/2)	8 (7/1)	8 (2/6)
Age (years; mean ± SD)	79 ± 2	78 ± 2	81 ± 2
PMD (hr; mean ± SD)	14.7 ± 8.0	16.0 ± 8.5	15.2 ± 19.9
Senile plaque stage	0	0	C
Braak stage (male/female)	I (5/0) II (1/1) III (0/1)	I (4/1) II (3/0)	V (2/6)

*N, number of brain; AF, amyloid free; AD, neuropathologically diagnosed as Alzheimer's disease; PMD, post-mortem delay.

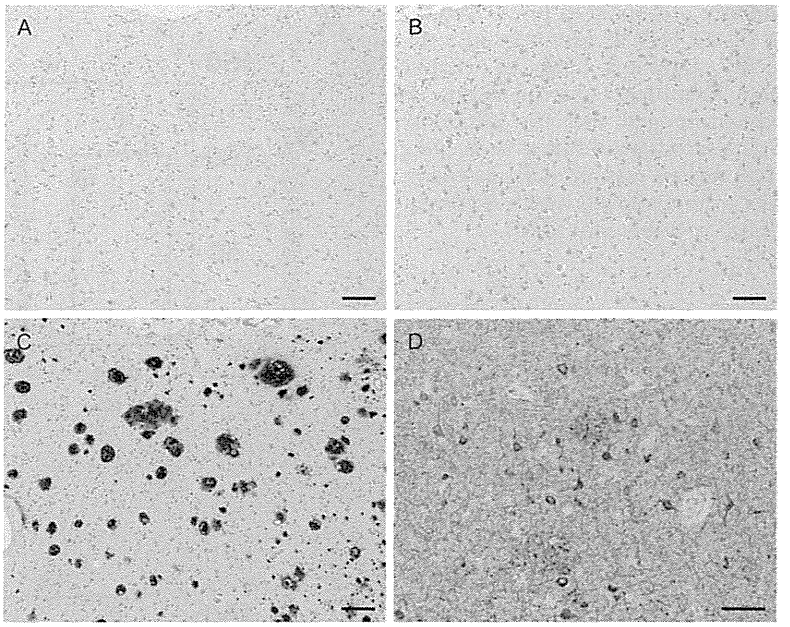


Fig. 1. Pathological profiles of the brains. Representative images of immunohistochemical analysis of the cortices of ε2/ε3 without amyloid (amyloid-free, AF; **A**), ε3/ε3 (AF; **B**), and ε3/ε3 with Alzheimer's disease pathology (AD; **C,D**) are shown. Immunohistochemical analysis was performed with an anti-Aβ 11–28 antibody (12B2; A–C) and antiphosphorylated tau antibody (AT8; D). Scale bars = 100 μm in A–C; 50 μm in D.

were extensively homogenized by sonication and the number of fractions for discontinuous sucrose-density-gradient centrifugation was increased (Fig. 2B). In comparison with the conventional detergent method using Triton X-100, in which the LDM marker protein and lipid (prion protein and GM1 ganglioside, respectively) were clearly separated from a non-LDM marker protein, the transferrin receptor (data not shown), the present detergent-free method provided satisfactorily pure LDMs; the levels of the LDM markers are relatively high in the upper phase (fractions 4–6) compared with a low level of the transferrin receptor (Fig. 2C).

Cholesterol Levels in SPMs and LDMs

We analyzed the cholesterol levels in SPMs and LDMs. The cholesterol levels in SPMs prepared from amyloid-free ε2/ε3 brains were significantly lower than those prepared from amyloid-free ε3/ε3 brains (Fig. 3A). The cholesterol levels in SPMs prepared from ε3/ε3 brains with AD pathology were significantly lower than those prepared from amyloid-free ε3/ε3 brains (Fig. 3A). Differences between amyloid-free ε2/ε3 brains and amyloid-free ε3/ε3 brains and between amyloid-free ε3/ε3 brains and ε3/ε3 brains with AD pathology were more pronounced in LDMs (Fig. 3B).

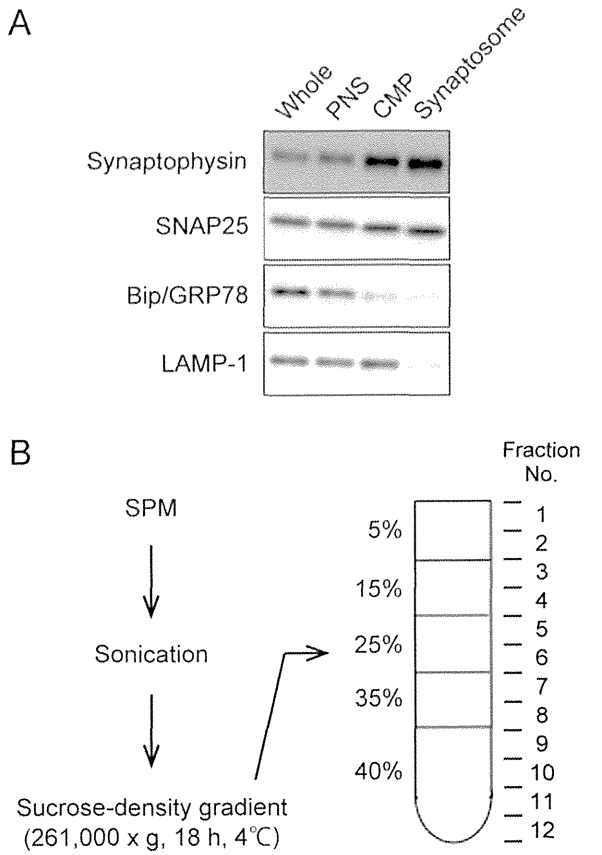
Ganglioside Levels in SPMs and LDMs

We analyzed ganglioside levels in SPMs and LDMs by quantitative LC-MS analysis. In SPMs, no changes in the levels of gangliosides were detected (Fig. 4A). In LDMs, the levels of some species of gangliosides, including GT1(d20:1–18:0) and OAc-GT1(d20:1–18:0), decreased in the ε3/ε3 brains with AD pathology in comparison with the amyloid-free ε3/ε3 brains (Fig. 4B). Previous studies showed a decrease in the levels of major gangliosides, including GM1, GD1, and GT1, in the cortices of AD brains (Op Den Velde and Hooghwinkel, 1975; Crino et al., 1989; Kracun et al., 1991). In this study, although the difference between amyloid-free ε3/ε3 brains and ε3/ε3 brains with AD pathology was not statistically significant, the levels of GD1(d20:0–18:0) tended to decrease in the presence of AD pathology. However, the levels of GM1 were apparently unchanged.

DISCUSSION

It remains to be clarified how the *APOE* genotype increases or decreases the risk of AD development. Despite intensive efforts, it is poorly understood how the *APOE* genotype is linked to the initiation of amyloid deposition, a fundamental core of AD pathology, in the brain. Previous studies suggested that, first, amyloidogenic proteins, including Aβ, likely assemble into fibrils through interaction with lipids (for review see Gorbenko and Kinnunen, 2006); second, microdomains or lipid rafts are deeply involved in the pathogenesis of AD, including Aβ assembly (for review see Rushworth and Hooper, 2010; Hicks et al., 2012); and, third, amyloid deposition in the brain starts at presynaptic terminals (Bugiani et al., 1990; Probst et al.,

1991). These lines of evidence prompted us to explore the pathological significance of the *APOE* genotype in amyloid deposition by directly examining lipids of synaptic membranes and synaptic membrane microdomains of autopsied brains. Our results suggest that the inhibitory effect of ε2 on amyloid deposition is attributed to keeping cholesterol in synaptic membranes and/or synaptic membrane microdomains under a certain level, which is prerequisite for the initiation of Aβ assembly into fibrils.



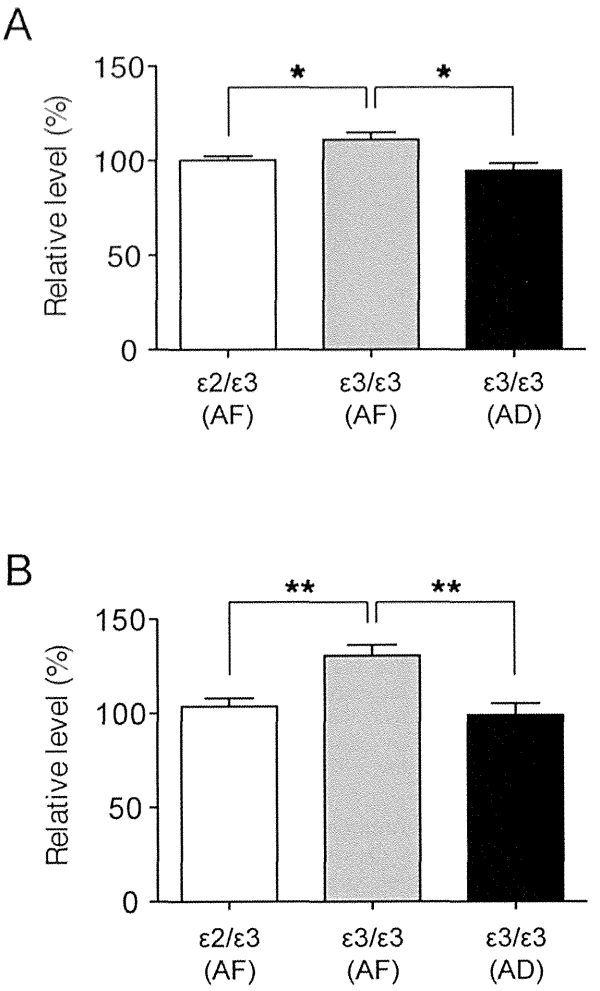


Fig. 3. Cholesterol levels in synaptic plasma membranes (SPMs) and low-density membrane microdomains (LDMs). SPMs (**A**) and LDMs (**B**) were prepared from the cortices of ε2/ε3 without amyloid (amyloid-free; AF), ε3/ε3 (AF), and ε3/ε3 with Alzheimer's disease pathology (AD). The levels of cholesterol were determined by using an Amplex-red cholesterol assay kit. Each column indicates mean ± SEM (n = 8, $*P < 0.05$, $**P < 0.01$).

Fig. 2. Preparation and characterization of synaptosomes and low-density membrane microdomains (LDMs). **A:** Collected whole homogenates, postnuclear supernatant (PNS), crude mitochondrial pellet (CMP), and synaptosomes were analyzed by Western blotting using the antibodies to the following subcellular compartment markers: synaptophysin and SNAP25 for synapses, Bip/GRP78 for the endoplasmic reticulum, and LAMP-1 for lysosomes. **B:** Outline of LDM preparation from synaptic plasma membrane (SPM). After centrifugation, fractions (1 ml) were sequentially collected from top (fraction 1) to bottom (fraction 12). **C:** Collected fractions and a sonicated sample without fractionation (whole) were analyzed by Western blotting using antibodies to the following compartment markers: transferrin receptor (TfR) for non-LDMs and flotillin-1 (Flot-1), prion protein (PrP), and choleratoxin B subunit (CTB) for LDMs. Fractions 4–6 (indicated by an asterisk) were collected as LDM samples.

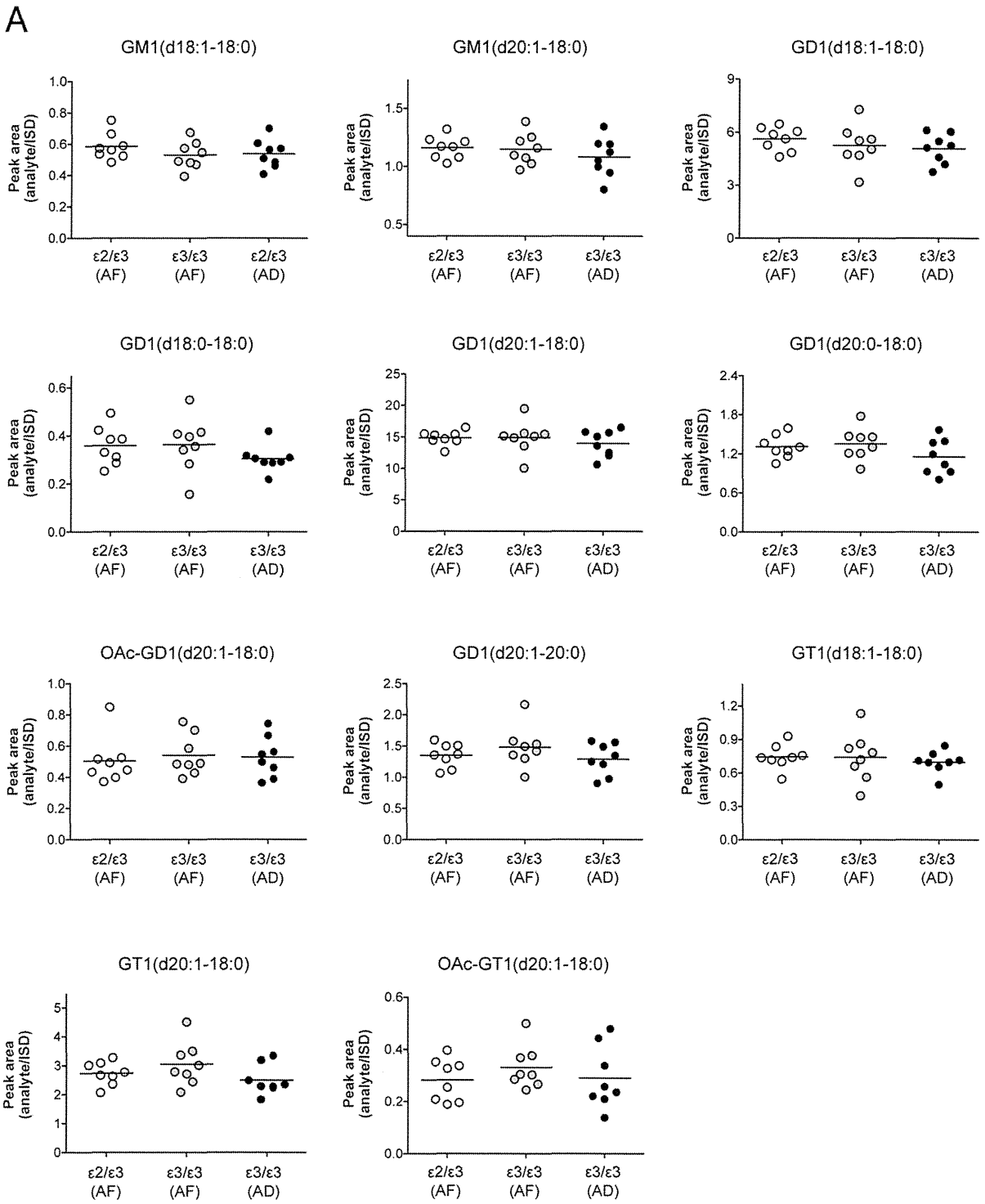


Fig. 4. Ganglioside levels in synaptic plasma membranes (SPMs) and low-density membrane microdomains (LDMs). SPMs (**A**) and LDMs (**B**) were prepared from the cortices of ε2/ε3 without amyloid (amyloid-free; AF), ε3/ε3 (AF), and ε3/ε3 with Alzheimer's disease pathology (AD). Gangliosides were analyzed by liquid chromatograpy-mass spectroscopy (LC-MS). The peak area of each ganglioside was determined with GM1(d18:1-^{13}C16:0; A) or GM3(d18:1–14:0; B) as an internal standard (ISD). Each bar represents the mean value in the group (n = 8).

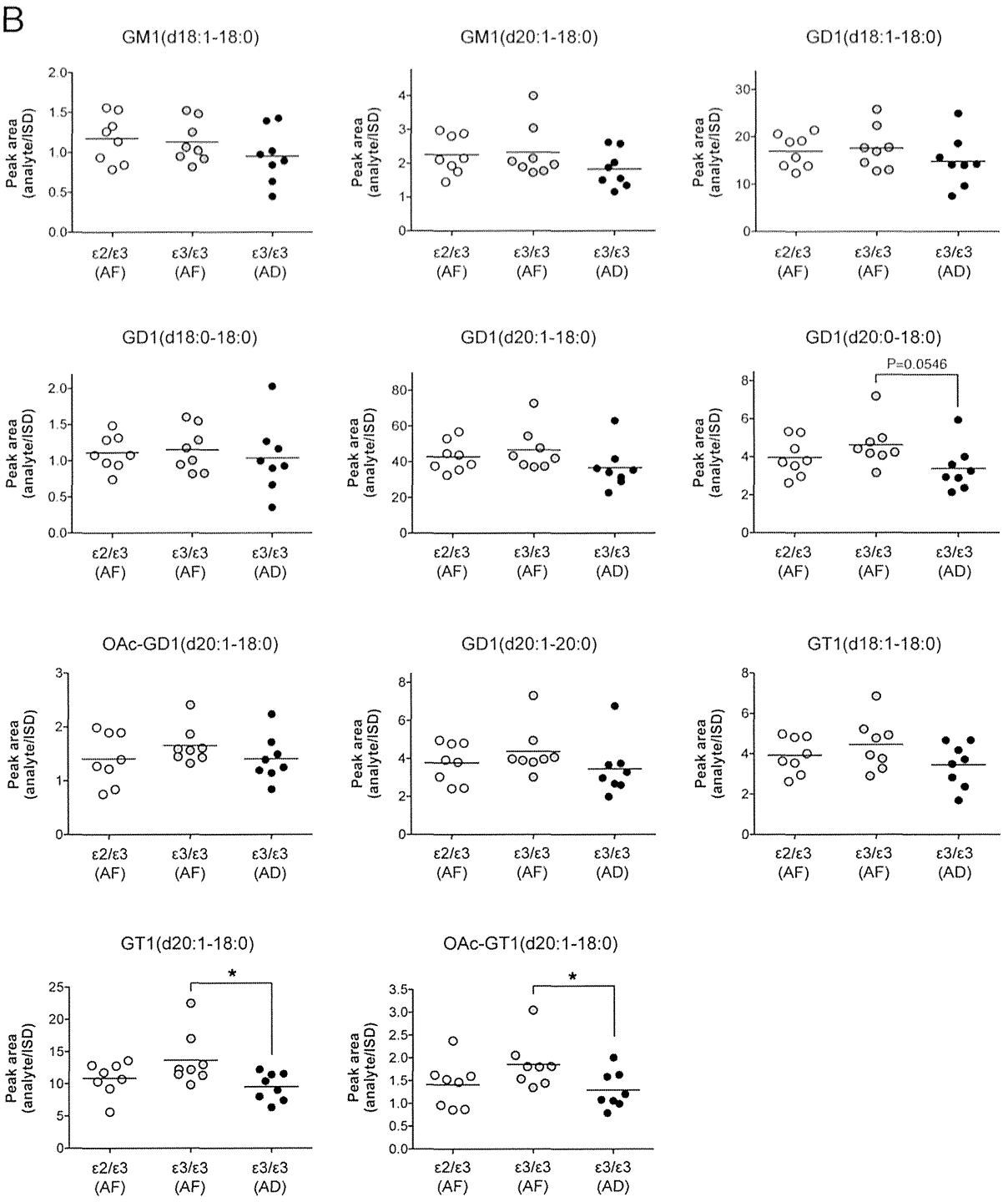


Fig. 4. Continued

In our early study, we quantitatively analyzed synaptic membrane lipids using human *APOE* knock-in mouse brains (ε3 knock-in and ε4 knock-in). In that study, the levels of cholesterol significantly increased in the exofacial leaflet of the synaptic membrane of ε4 knock-in mouse brains compared with ε3 knock-in and wild-type mouse brains (Hayashi et al., 2002), suggesting that the pathological significance of ε4 is linked to an increase in the levels of cholesterol in synaptic membranes. Although the scope of the current study is only the comparison between ε2 and ε3, note that ε2 decreases the levels of cholesterol in synaptic membranes and synaptic membrane microdomains. Overall, the *APOE* genotype likely has an impact on the regulation of cholesterol levels at presynaptic terminals and thereby modulates amyloid deposition in the brain.

Although it remains to be clarified how ε2 decreases the level of cholesterol in synaptic membranes and synaptic membrane microdomains, apolipoprotein E-dependent cholesterol regulation in neurons has been studied in vitro by our group and other groups (Michikawa et al., 2000; Rapp et al., 2006). Notably, cholesterol efflux from neurons was found to be regulated by apolipoprotein in an isoform-dependent manner; the order of potency was E2 > E3 > E4, i.e., apolipoproteins encoded by ε2, ε3, and ε4, respectively (Michikawa et al., 2000). On the other hand, apparently there was no isoform-dependent difference between E2 and E3 in the extent of cholesterol supply to neurons (Rapp et al., 2006). Thus, although further studies are needed, it may be possible to conclude that ε2 provides a negative balance of cholesterol dynamics in neuronal membranes compared with ε3 and ε4.

In this study, the presence of AD pathology decreased the level of cholesterol and some species of gangliosides in synaptic membranes and/or synaptic membrane microdomains. Elucidation of the mechanisms underlying alterations in neuronal lipid levels under various pathological conditions will be a challenging task. Many studies of AD have been carried out concerning on this subject (Molander-Melin et al., 2005; Gylys et al., 2007); however, results reported to date are not consistent but rather varied. The discrepancy probably is due to differences in the method of preparing neuronal membranes and, to a greater extent, to differences in the pathological stages of brains or brain regions examined. In addition, as shown in this study and another study (Bandaru et al., 2009), the *APOE* genotype substantially affects the levels of lipids, or at least cholesterol, in neuronal membranes. Thus, in lipid-chemical studies of autopsied brains, attention should be paid to the *APOE* genotype of the individuals from whom specimens are obtained.

There are several implications of our findings from the viewpoint of the roles of cholesterol in AD development. First, cholesterol in membranes accelerates the formation of GM1 ganglioside-bound Aβ (GAβ), an endogenous seed for Alzheimer's amyloid (Yanagisawa et al., 1995; Hayashi et al., 2004), through facilitation of the formation of GM1 ganglioside clusters, which are required for GAβ generation (Kakio et al., 2001), and through tuning of GM1 ganglioside conformation (Fantini et al., 2013). Second, cholesterol increases the activities of secretases, which are involved in Aβ production in membrane microdomains or lipid rafts (for review see Di Paolo and Kim, 2011). Third, cholesterol enhances internalization of the amyloid precursor protein, leading to increased Aβ secretion (Cossec et al., 2010b) in association with the enlargement of early endosomes (Cossec et al., 2010a), which is the earliest cellular pathologic feature of AD (Cataldo et al., 2000). These findings strongly suggested that the decrease in cholesterol level by ε2 suppresses amyloid deposition. In addition, the decrease in cholesterol levels can also be beneficial in prevention of the clinical onset of AD, because pathological processes of AD, such as amyloid pore formation by Aβ oligomers, result in cognitive dysfunction of AD (Esparza et al., 2013), likely dependenjt on cholesterol levels in membranes (Di Scala et al., 2013). Alternatively, membrane microdomains or lipid rafts are critical for the function and even survival of neurons; thus, alterations in the levels of lipids in the presence of AD pathology, as observed in this study, likely lead to the perturbation of microdomains or lipid raft integrity, seriously and detrimentally affecting neurons in AD (for review see Hicks et al., 2012). In summary, this study, together with our previous study of human *APOE* knock-in mouse brains (Hayashi et al., 2002), suggests that the *APOE*-genotype-linked modulation of AD development is attributed to the regulation of cholesterol levels in synaptic membranes and/or synaptic membrane microdomains.

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The authors have no conflicts of interest to disclose.

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Clinical features of genetic Creutzfeldt-Jakob disease with V180I mutation in the prion protein gene

Temu Qina,[1] Nobuo Sanjo,[1] Masaki Hizume,[1] Maya Higuma,[1] Makoto Tomita,[2] Ryuichiro Atarashi,[3] Katsuya Satoh,[3] Ichiro Nozaki,[4] Tsuyoshi Hamaguchi,[5] Yosikazu Nakamura,[6] Atsushi Kobayashi,[7] Tetsuyuki Kitamoto,[7] Shigeo Murayama,[8] Hiroyuki Murai,[9] Masahito Yamada,[5] Hidehiro Mizusawa[1]

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For numbered affiliations see end of article.

Correspondence to
Dr Nobuo Sanjo;
n-sanjo.nuro@tmd.ac.jp

ABSTRACT

Objectives: Genetic Creutzfeldt-Jakob disease (CJD) due to V180I mutation in the prion protein gene (*PRNP*) is of great interest because of the differences from sporadic CJD and other genetic prion diseases in terms of clinical features, as well as pathological and biochemical findings. However, few systematic observations about the clinical features in patients with this unique mutation have been published. Therefore, the goal of this study was to relate this mutation to other forms of CJD from a clinical perspective.

Design: We analysed clinical symptoms, prion protein genetics, biomarkers in cerebrospinal fluid (CSF) and MRI of patients.

Participants: 186 Japanese patients with the V180I mutation in *PRNP*.

Results: Our results indicate that the V180I mutation caused CJD at an older age, with a slower progression and a lower possibility of developing myoclonus, cerebellar, pyramidal signs and visual disturbance compared with classical sporadic CJD with methionine homozygosity at codon 129 of *PRNP*. Cognitive impairment was the major symptom. Diffuse hyperintensity of the cerebral cortex in diffusion-weighted MRI might be helpful for diagnosis. Owing to the low positivity of PrP^{Sc} in the CSF, genetic analysis was often required for a differential diagnosis from slowly progressive dementia.

Conclusions: We conclude that the V180I mutation in *PRNP* produces a late-developing and slow-developing, less severe form of CJD, whose lesions are uniquely distributed compared with sporadic and other genetic forms of CJD.

Strengths and limitations of this study

- The study used the largest V180I prion protein mutation cohort yet to be published to improve statistical power.
- The study compared the V180I variant of CJD to other genetic and sporadic variants of CJD, not just to non-CJD controls, allowing comparisons to be made across the spectrum of prion diseases.
- The study compared the V180I variant with regard to other mutations (the 129 and 219 codon polymorphisms) known to alter disease progression in other variants.
- The study was limited by focusing primarily on clinical features and retrospective data, making interpretation of the potential mechanisms differentiating disease progression in V180I and other CJD variants difficult or impossible without future studies.

INTRODUCTION

Prion diseases are transmissible and lethal neurodegenerative diseases that affect humans and animals.[1] In humans, prion disease can be categorised into sporadic, acquired and genetic forms.[2] The genetic form of prion disease (gPrD) that is caused by mutations in the prion protein gene (*PRNP*) accounts for 10.2% of cases in Europe and 16.7% in Japan.[3 4]

The epidemiological distributions of patients with gPrD were reported to be different between European countries and Japan. While the E200K mutation occurs most frequently in Europe,[3] the V180I mutation is the most frequent mutation in Japan.[5] Currently, several reports indicate that the V180I mutation in *PRNP* accounts for specific clinical and pathological findings.[5–8] Because patients with V180I rarely have a family history of the disease, the question of whether this mutation causes prion disease persists. On the other hand, patients with V180I show several specific clinical features different from those of sporadic Creutzfeldt-Jakob disease (sCJD) or other gPrDs.[9] We have previously reviewed clinical symptoms and cerebrospinal fluid (CSF) markers of several *PRNP* mutations, including V180I.[5] Patients with V180I are readily

distinguishable from patients with other dementia because they show specific hyperintensity in the cerebral cortex in diffusion-weighted MRI. We present some clinical features in genetic CJD (gCJD) with V180I and Alzheimer's disease in table 1.

In the current study, in order to better elucidate the clinical characteristics of the V180I mutation, we analysed the surveillance data of 186 patients with V180I, including the occurrence rate of neurological symptoms, the period of time between disease onset and the occurrence of these symptoms, biomarkers in the CSF, MRI and EEG data, and codon 129 polymorphism in *PRNP*. Our study indicates that myoclonus and periodic sharp wave complexes (PSWCs) in the EEG, which are included in the diagnostic criteria of CJD, occur less frequently in patients with V180I.

METHODS

Patients

The Prion Disease Surveillance Committee in Japan diagnosed gPrDs in accordance with the WHO Case Definition Criteria for epidemiological surveillance. Information on each patient was collected between April 1999 and September 2013, after the current Prion Disease Surveillance Committee of Japan began the comprehensive surveillance on prion diseases in Japan. In the current study, we analysed the surveillance data of 186 patients with definite or probable gPrD with a V180I mutation. In order to differentiate clinical features of patients with V180I from sCJD, we compared the V180I patient group with patients having sCJD with type-1 PrP^{Sc} and methionine homozygosity at codon 129 (sCJD-MM1) of *PRNP*, a classical type of prion disease. In this study, 59 patients with sCJD-MM1 with definitive diagnosis were included as a control.

Clinical analysis

We collected information on age of onset, sex, family history, clinical duration of each sign or symptom (duration from onset to death, or to the point when we confirmed the condition of the patient if he or she was alive and to the point when clinical signs were observed) and the clinical signs themselves (first symptom, dementia, psychological disturbance, cerebellar disturbance, visual disturbance, pyramidal or extrapyramidal signs, myoclonus and akinetic mutism). The appearance of PSWCs in the EEG and hyperintensities in the MRI was examined as previously described[4] The open reading frame and polymorphisms of codons 129 and 219 of the *PRNP* gene were analysed after genomic DNA was extracted from the patients' blood, as previously described.[10]

Table 1 Comparison of clinical characteristics between gPrD-V180I and Alzheimer's disease (AD)

	gPrD-V180I	AD
Age at onset (years)	Late 70s	Early 70s
Period from onset to death (years)	2–3	4–8
Myoclonus	+	Late stage
PSWCs on EEG	+–	–
MRI findings	Cortical hyperintensity	Hippocampal atrophy
CSF findings	Total τ↑↑↑, PrP^{Sc} (+)	Aβ42↓

CSF, cerebrospinal fluid; gPrD, genetic form of prion disease; PSWCs, periodic sharp wave complexes.

CSF biomarkers

CSF analysis of all patients was performed at Nagasaki University.[11] We evaluated 14-3-3 and total τ (t-τ) protein levels in the CSF by western blotting as previously described.[5] PrP^{Sc} in the CSF was detected by real-time quaking induced conversion (RT-QUIC), as previously described.[12] Briefly, CSF was incubated with recombinant human prion protein (residues 23–231 of human PrP with methionine at codon 129) at 37°C with intermittent shaking. Four wells were tested twice for each CSF sample and the sample was decided as positive when two or more of the four wells showed more than 1000 reactive fluorescence units (thioflavin T) within 48 h. The kinetics of fibril formation was monitored by reading the fluorescence intensity every 10 min.

Statistical analysis

The Mann-Whitney U test was used for the statistical comparisons of age of onset, disease duration and the level of τ protein in the CSF. Fisher's exact probability test was used for the comparisons of sex, the rate of occurrence of each clinical sign, presence of PSWCs in the EEG, presence of hyperintensity in the MRI and rate of positive detection of 14-3-3 and PrP^{Sc} proteins. For analysis of the correlation between CSF markers and each clinical parameter, analysis of variance or multiple comparison tests (χ^2 and Kruskal-Wallis) were used. Significance was defined as $p<0.05$. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, California, USA) and IBM SPSS Statistics (IBM, New York, New York, USA).

Ethical issues

Informed consent from the family of each patient was obtained for the current study. The study was performed in accordance with the ethical standards laid down by the 2013 Declaration of Helsinki.

RESULTS

Comparison of clinical features between patients with V180I-MM and sCJD-MM1

Since gCJD with V180I mutation contains codon 129 methionine homozygosity (129MM) and methionine/valine heterozygosity (129MV), we compared patients with V180I and 129MM (V180I-MM) and sCJD-MM1 or MM2 (table 2), and V180I-MV and sCJD-MV (there are no pathologically defined MV1 cases in Japan; table 3).

Table 2 Clinical features of codon 129 homozygosity of methionine among V180I, sCJD-MM1 and sCJD-MM2

	V180I-MM n=139	sCJD-MM1 n=59	p Value (vs V180I-MM)	sCJD-MM2 n=8	p Value (vs V180I-MM)
Male/female	58/81	25/34		5/3	0.53
Codon 219	135 EE; 4 NA	54 EE; 5 NA		8 EE	
Age at onset (years)*	77.3±6.8 (78, 44–93, n=139)	68.9±9.1 (70, 40–89, n=59)	<0.001	60.3±11.9 (63, 43–74, n=8)	<0.001
Period from onset to death (months)*	23.1±15.1 (19, 5–70, n=75)	17.2±12.5 (15, 1–60, n=57)	0.032	22.3±12.0 (20, 10–50, n=8)	0.98
Myoclonus†	46/130 (35.4%)	52/59 (88.1%)		4/8 (50%)	<0.001
Period from onset to myoclonus (months)*	6.4±6.1 (5, 0–36, n=38)	2.0±2.4 (1, 0–13, n=49)	<0.001	7.3±4.0 (8, 3–11, n=3)	0.92
Cognitive impairment†	138/138 (100%)	59/59 (100%)		8/8 (100%)	1
Period from onset to cognitive impairment (months)*‡	0.5±1.4 (0, 0–7, n=121)	0.6±1.0 (0, 0–6, n=55)	1	15.6±40.3 (0, 0–115, n=8)	<0.001
Pyramidal signs†	66/132 (50%)	40/54 (74.1%)		2/7 (28.6%)	0.004
Period from onset to pyramidal sign (months)*	3.9±5.8 (2.5, 0–36, n=58)	2.9±4.4 (2, 0–24, n=38)	0.53	12 (n=1)	
Extrapyramidal signs†	71/133 (53.4%)	30/52 (57.7%)		2/8 (25.0%)	0.23
Period from onset to extrapyramidal signs (months)*	3.8±3.5 (3, 0–19, n=58)	2.2±4.3 (1, 0–24, n=29)	0.13	13.0±1.4 (13, 12–14, n=2)	0.002
Cerebellar dysfunction†	40/119 (33.6%)	32/45 (71.1%)		3/7 (42.9%)	<0.001
Period from onset to cerebellar dysfunction (months)*	2.9±2.7 (3, 0–9, n=33)	0.7±0.9 (1, 0–3, n=31)	<0.001	12.7±1.2 (12, 12–14, n=3)	<0.001
Visual disturbance†	10/109 (9.2%)	28/49 (57.1%)		2/7 (28.6%)	<0.001
Period from onset to visual disturbance (months)*	2.2±1.5 (2, 0–4, n=10)	0.7±1.7 (0, 0–7, n=26)	0.036	0 (n=2)	0.15
Psychiatric symptoms†	68/130 (52.3%)	32/51 (62.7%)		5/7 (71.4%)	0.36
Period from onset to psychiatric symptoms (months)*	1.6±3.0 (0, 0–19, n=62)	0.8±0.9 (1, 0–3, n=29)	0.32	5.3±7.1 (3, 0–15, n=4)	0.024
Akinetic mutism†	74/137 (54.0%)	44/57 (77.2%)		2/8 (25.0%)	0.001
Period from onset to akinetic mutism (months)*	9.8±6.6 (8, 1–27, n=64)	3.6±4.3 (2, 0–23, n=42)	<0.001	18 (n=2)	
PSWCs on EEG†	10/131 (7.6%)	55/59 (93.2%)		2/7 (28.6%)	<0.001
Hyperintensities on MRI	135/136 (99.3%)	57/57 (100%)	<0.001	5/8 (62.5%)	0.092
Positive rate of 14-3-3 protein in CSF†	46/53 (86.8%)	27/31 (87.1%)	1	NA	
Positive rate of t-τ protein in CSF†	48/53 (90.6%)	27/31 (87.1%)	0.72	NA	
Amount of t-τ protein in CSF (pg/mL) *	2965±1712 (2400, 146.0–9940.0, n=53)	7950±8423 (5450, 150.0–40120.0, n=29)	<0.001	NA	
Positive rate of PrP^{Sc} in CSF†	36/53 (67.9%)	27/30 (90.0%)	0.032	NA	

Codon 219 is presented with total cases of that polymorphism type. EE means glutamic acid homozygous. NA means data not available.
Medians are compared using analysis of variance with Dunnett's post hoc test for age of onset, the period from disease onset to death or the appearance of each symptom and sign, the two-tailed Mann-Whitney U test for the period from onset to akinetic mutism and the CSF biomarker level. Frequencies of positive cases are compared using the two-tailed Fisher's exact test.
*Age of onset, period of time from disease onset to death or the appearance of each symptom and sign and CSF biomarker level are presented as mean±SD (median, range, cases).
†Frequencies of positive cases are presented as positive cases/total cases (percentage).
‡These were zero-inflated.
CSF, cerebrospinal fluid; PSWCs, periodic sharp wave complexes; sCJD, sporadic Creutzfeldt-Jakob disease; t-τ, total τ.

Table 3 Clinical features of codon 129 heterozygosity of methionine/valine between V180I and sCJD-MV

	V180I-MV n=45	sCJD-MV n=7	p Value
Type 1 or 2		5 type 2; 2 NA	
Male/female	20/25	3/4	1
Codon 219	44 EE; 1 EK	7 EE	
Age at onset (years)*	76.7±7.6 (78, 57–92, n=43)	62.0±7.0 (62, 51–73, n=7)	<0.001
Period from onset to death (months)*	27.8±16.3 (25, 7–64, n=23)	26.2±12.9 (21, 12–43, n=6)	0.98
Myoclonus†	21/43 (48.8%)	5/7 (71.4%)	0.42
Period from onset to myoclonus (months)*	9.2±7.2 (7, 2–30, n=18)	8.5±4.7 (7.5, 4–15, n=4)	0.86
Cognitive impairment†	43/44 (97.7%)	7/7 (100%)	1
Period from onset to cognitive impairment (months)*‡	0.6±1.4 (0, 0–5, n=38)	3.0±4.5 (0, 0–10, n=5)	0.26
Pyramidal signs†	14/42 (33.3%)	2/6 (33.3%)	1
Period from onset to pyramidal sign (months)*	5.2±4.2 (5, 0–14, n=11)	12 (n=1)	
Extrapyramidal signs†	23/40 (57.5%)	5/6 (83.3%)	0.23
Period from onset to extrapyramidal signs (months)*	3.8±4.5 (2, 0–16, n=18)	5.5±6.6 (4, 0–15, n=4)	0.58
Cerebellar dysfunction†	12/38 (31.6%)	6/6 (100%)	0.003
Period from onset to cerebellar dysfunction (months)*	3.4±4.1 (3, 0–12, n=8)	5.8±5.4 (4, 0–14, n=5)	0.50
Visual disturbance†	1/34 (2.9%)	1/5 (20%)	0.24
Period from onset to visual disturbance (months)*	(n=0)	(n=0)	
Psychiatric symptoms†	16/38 (42.1%)	3/7 (42.9%)	1
Period from onset to psychiatric symptoms (months)*	2.0±2.6 (0, 0–7, n=13)	4.5±2.1 (5, 3–6, n=2)	0.24
Akinetic mutism†	30/44 (68.2%)	3/7 (42.9%)	0.23
Period from onset to akinetic mutism (months)*	13.2±10.9 (9, 0–49, n=23)	12.5±5.0 (13, 9–16, n=2)	
PSWCs on EEG†	5/39 (12.8%)	2/6 (33.3%)	0.23
Hyperintensities on MRI	44/44 (100%)	7/7 (100%)	1
Positive rate of 14-3-3 protein in CSF†	11/18 (61.1%)	NA	
Positive rate of t-τ protein in CSF†	12/18 (66.7%)	NA	
Amount of t-τ protein in CSF (pg/mL)*	2025±1441 (1689, 170.0–6430.0, n=18)	NA	
Positive rate of PrP^{Sc} in CSF†	7/18 (38.9%)	NA	

Codon 219 is presented with total cases of that polymorphism type. EE and EK mean glutamic acid and glycine homozygous, respectively. NA means data not available.
Medians are compared using the two-tailed Mann-Whitney U test for age of onset, the period from disease onset to death or the appearance of each symptom and sign and the CSF biomarker level. Frequencies of positive cases are compared using the two-tailed Fisher's exact test.
*Age at onset, period of time from disease onset to death or the appearance of each symptom and sign and CSF biomarker level are presented as mean±SD (median, range, cases).
†Frequencies of positive cases are presented as positive cases/total cases (percentage).
‡These were zero-inflated.
CSF, cerebrospinal fluid; PSWCs, periodic sharp wave complexes; sCJD, sporadic Creutzfeldt-Jakob disease; t-τ, total τ.

The average age of onset in V180I-MM and V180I-MV was about 10 years older than those of sCJD-MM1 and sCJD-MM2, and MV2, respectively. The period from disease onset to death was longer in V180I-MM than in sCJD-MM1, as previously reported,[4] but was almost the same as in sCJD-MM2 (table 2). Among the 16 total autopsied patients with V180I in this cohort, few patients had additional neuropathological alterations such as Alzheimer's disease.[6 7] There was no difference between definite and probable or possible cases with V180I mutation. The periods from onset to the occurrence of myoclonus, cerebellar dysfunction, visual disturbance and akinetic mutism in V180I-MM were significantly longer than those in sCJD-MM1. However, except for visual disturbance, the length of onset to the occurrence of all other signs was shorter than those in sCJD-MM2 (table 2). As for the clinical features of 129MV, there was no significant difference between V180I-MV and sCJD-MV, except for age of onset (table 3).

The analysis of the probability of occurrence of neurological symptoms and signs similarly demonstrated reduced severity in patients with V180I-MM compared to those with sCJD-MM1. While 88.1% of patients with sCJD-MM1 developed myoclonus, only 35.4% of patients with V180I developed myoclonus. Pyramidal signs, cerebellar dysfunction, visual disturbance and akinetic mutism were also less frequent in patients with V180I-MM1 than in patients with sCJD-MM1. However, as previously reported,[9] cerebellar and visual systems were not completely spared in patients with V180I.

EEG and MRI findings

PSWCs were observed in only 7.3% of patients with V180I-MM, but in over 90% of patients with sCJD-MM1 (table 2). MRI revealed hyperintensities with a similar positive rate in V180I-MM and sCJD-MM1, but was observed less frequently in patients with sCJD-MM2. The

Table 4 Effects of the codon 129 polymorphism on the clinical features of V180I

	129MM n=139	129MV n=45	p Value
Male/female	58/81	20/25	0.862
Age at onset (years)*	77.3±6.8 (78, 44–93, n=139)	76.7±7.6 (78, 57–92, n=45)	0.701
Period from onset to death (months)*	23.1±15.1 (19, 5–70, n=75)	27.8±16.3 (25, 7–64, n=23)	0.159
Myoclonus†	46/130 (35.4%)	21/43 (48.8%)	0.149
Period from onset to myoclonus (months)*	6.4±6.1 (5, 0–36, n=38)	9.2±7.2 (7, 2–30, n=18)	0.154
Cognitive impairment†	138/138 (100.0%)	43/44 (97.7%)	0.242
Period from onset to cognitive impairment (months)*	0.5±1.4 (0, 0–7, n=121)	0.6±1.4 (0, 0–5, n=38)	0.456
Pyramidal signs†	66/132 (50.0%)	14/42 (33.3%)	0.075
Period from onset to pyramidal signs (months)*	3.9±5.8 (3, 0–36, n=58)	5.2±4.2 (5, 0–14, n=11)	0.136
Extrapyramidal signs†	71/133 (53.4%)	23/40 (57.5%)	0.719
Period from onset to extrapyramidal signs (months)*	3.8±3.5 (3, 0–19, n=58)	3.8±4.5 (2, 0–16, n=18)	0.460
Cerebellar dysfunction†	40/119 (33.6%)	12/38 (31.6%)	1.000
Period from onset to cerebellar dysfunction (months)*	2.9±2.7 (3, 0–9, n=33)	3.4±4.1 (3, 0–12, n=8)	0.973
Visual disturbance†	10/109 (9.2%)	1/34 (2.9%)	0.460
Period from onset to visual disturbance (months)*	2.2±1.5 (2, 0–4, n=10)	(n=0)	NA
Psychiatric symptoms†	68/130 (52.3%)	16/38 (42.1%)	0.357
Period from onset to psychiatric symptoms (months)*	1.6±3.0 (0, 0–19, n=62)	2.0±2.6 (0, 0–7, n=13)	0.576
Akinetic mutism†	74/137 (54.0%)	30/44 (68.2%)	0.116
Period from onset to akinetic mutism (months)*	9.8±6.6 (8, 1–27, n=64)	13.2±10.9 (9, 0–49, n=23)	0.190
PSWCs on EEG†	10/131 (7.6%)	5/39 (12.8%)	0.339
Hyperintensities on MRI†	135/136 (99.3%)	44/44 (100.0%)	1.000
Positive rate of 14-3-3 protein in CSF†	46/53 (86.8%)	11/18 (61.1%)	0.035
Positive rate of t-τ protein in CSF†	48/53 (90.6%)	12/18 (66.7%)	0.014
Amount of t-τ protein in CSF (pg/mL)*	2965±1712 (2400, 146.0–9940.0, n=53)	2025±1441 (1689, 170.0–6430.0, n=18)	0.022
Positive rate of PrP^{Sc} in CSF†	36/53 (67.9%)	7/18 (38.9%)	0.049

*Age of onset, period from disease onset to death or the appearance of each symptom and sign and CSF biomarker level are presented as mean±SD (median, range, case number).
†Frequencies of positive cases are presented as positive cases/total cases (percentage).
Medians are compared using the two-tailed Mann-Whitney U test for age of onset, the period from disease onset to death or the appearance of each symptom and sign and the CSF biomarker level. Frequencies of positive cases are compared using the two-tailed Fisher's exact test.
CSF, cerebrospinal fluid; PSWCs, periodic sharp wave complexes; t-τ, total τ.

pattern of hyperintensities in patients with V180I was uniquely distributed in the cerebral cortex.

Effect of *PRNP* polymorphism on clinical symptoms and signs of V180I

We also analysed the effect of the codon 129 polymorphism on the clinical symptoms of patients with V180I (table 4).

In a total of 184 patients with V180I, 139 patients (75.5%) were 129MM, while the remaining 45 (24.5%) were 129MV. We detected only three patients with the V180I mutation on the same allele as valine. In this case, the clinical features were no different from those with the mutation on the same allele as methionine. We also analysed the codon 219 polymorphism in 179 patients with V180I and 54 patients with sCJD-MM1, all of whom showed glutamic acid homozygosity (table 2). When analysing the influence of the codon 129 polymorphism on clinical symptoms and signs in patients with V180I, no symptoms or signs, in terms of the occurrence rate and the speed to develop them after disease onset, were affected by codon 129MV.

CSF biomarkers

Positive tests of the CSF 14-3-3 protein and t-τ proteins in patients with V180I-MM were similar to those of patients with sCJD-MM1 (table 2). However, the median value of t-τ proteins in the CSF of patients with V180I-MM was significantly lower than that of patients with sCJD-MM1. In patients with V180I-MM, we found fewer positive tests for PrP^{Sc} in the CSF than in patients with sCJD-MM1. Patients with V180I-MV, in particular, showed significantly fewer positive tests for 14-3-3, t-τ protein and PrP^{Sc} in the CSF, as well as in the amount of CSF t-τ (p=0.034, 0.023, 0.001 and <0.001, respectively; multiple comparison using Fisher's exact and Kruskal-Wallis tests).

Effect of age

In order to exclude whether any variables were age dependent, we compared some laboratory and CSF findings in patients with V180I and with sCJD older than 75 years (table 5). We found that positive rates of PrP^{Sc} were comparable, although whether the greater percentage of older patients with PrP^{Sc} is age dependent or due to the small sample size is currently unclear.

Table 5 Laboratory and CSF findings of gPrD-V180I compared to sCJD older than 75 years

	V180I n=186	sCJD (>75 years) n=11	p Value
Male/female	78/108	25/34	1.00
Age at onset (years)*	77.2±6.9 (78, 44–93)	80.9±4.2 (80, 76–89)	0.056
PSWCs on EEG†	16/172 (9.3%)	10/11 (90.9%)	<0.001
Positive rate of 14-3-3 protein in CSF†	57/71 (80.2%)	7/8 (87.5%)	1.00
Positive rate of t-τ protein in CSF†	61/71 (85.9%)	6/8 (75.0%)	0.60
Amount of t-τ protein in CSF (pg/mL)*	2727±1688 (2400, 146.0–9940.0, n=71)	6569.8±4270.3 (8995.0, 150.0–10290.0, n=8)	0.035
Positive rate of PrP^{Sc} in CSF†	44/71 (62.0%)	7/8 (87.5%)	0.246

Medians are compared using the two-tailed Mann-Whitney U test for age at onset, the period from disease onset to death or the appearance of each symptom and sign and the CSF biomarker level. Frequencies of positive cases are compared using the two-tailed Fisher's exact test.
*Age at onset and the appearance of CSF biomarker level are presented with mean±SD (median, range, case number).
†Frequencies of positive cases are presented with positive case number/total case number (percentage).
CSF, cerebrospinal fluid; gPrD, genetic form of prion disease; PSWCs, periodic sharp wave complexes; sCJD, sporadic Creutzfeldt-Jakob disease; t-τ, total τ.

DISCUSSION

With a very rare incidence in Europe,[3] the V180I mutation was geoepidemiologically discovered mainly in Japan, and has turned out to be the most common cause of gPrD in Japan.[5] The reason for this geographical distribution difference is currently unclear, but racial and/or environmental factors are most likely involved.

Of the patients with V180I gPrD, 78 cases were of male patients and 108 cases were female, indicating a possible gender influence on the susceptibility of this mutation in the disease. Similar to other mutations in *PRNP*, women appear more susceptible.[3] Patients with sCJD-MM1 were characterised by fast, severe progression of the disease, and neurological malfunctions resulting from extensive brain lesions appeared in a period of less than 3 months (table 2). However, V180I progressed relatively slowly. Myoclonus, cerebellar signs and visual dysfunctions occurred less frequently and with greater latency in patients with V180I (table 2). PSWCs in the EEG, a frequent finding in patients with sCJD-MM1, were rarely detected in patients with V180I (table 2). While a triad of dementia, myoclonus and PSWCs in the EEG is typical of sCJD, patients with V180I mainly presented with cognitive impairment and a very low rate of myoclonus in the early stages, along with rarely detectable PSWCs. Instead of possible CJD, these cases tended to be misdiagnosed as dementia due to Alzheimer's disease. MRI could facilitate the diagnosis of V180I when a specific pattern of ribbon hyperintensity lesions is detected.[8 13] However, it may still be difficult to distinguish patients with V180I from patients with sCJD-MM1 because hyperintensity was similarly detected in patients with sCJD-MM1, necessitating direct testing of the *PRNP* gene.

Previous reports suggested that there were no visual and cerebellar clinical symptoms in V180I, and neuroimaging of the medial occipital lobes posterior to the parieto-occipital sulcus and the cerebellum revealed that they were not involved until the terminal stage.[9] These data posit V180I as a comparative analogue of sCJD[14] or a cortical form of sCJD with type-2 PrP^{Sc} and methionine homozygosity at codon 129.[15] In our current study of 186 patients with V180I, we found that 34% demonstrated clinical cerebellar dysfunction, and 8.3% presented with visual disturbances (tables 2 and 3). Although no detailed description of the exact manifestations of cerebellar and visual symptoms was recorded, and a subjective bias in identifying the true origins of these symptoms should be taken into consideration, our finding indicates that in order to confirm whether the cerebellum is actually spared in patients with V180I, it is critical to analyse the pathological and immunohistochemical features including PrP^{Sc} deposition and spongiform changes in a topological manner.

The penetrance of V180I was very low. Only 11 out of 186 patients (5.9%) had a family history of dementia, while family member involvement in the case of other gPrD mutations, such as E200K, P102L and P105L, was frequently noted.[3 5] Within the 11 patients with V180I in the current study who had a recorded family history, 3 patients had one family member each diagnosed with CJD. The remaining eight cases had family members of one generation above, or the same generation, who had dementia due to an unknown cause. The low penetrance of V180I, specific clinical features and MRI findings was intriguing, and leads us to speculate whether the V180I mutation is causative for the disease or is actually a disease-associated factor accompanying other protective or toxic factors. The V180I mutation is reported to have significantly higher proportions of overall prion disease (n=881, both p<0.001),[4] compared with the genotypes of *PRNP* in the general Japanese population (n=466; isoleucine allele at codon 180 was not detected).[16] These findings indicate that the V180I mutation is not simply a polymorphism, but is indeed disease related.

Different PrP^{Sc} glycotypes might lead to differential distributions of PrP^{Sc} throughout the brain,[17 18] and may account for the disparate affects on brain regions

underlying cerebral cortical symptoms as opposed to cerebellar symptoms, for instance. In this cohort, western blotting of brain homogenates from patients with V180I indicated only weak bands of the monoglycosylated and unglycosylated fragments.[5] In the future, studies should examine the role of factors that influence lesion topology on the disease's clinical expression and progression. We hypothesise that the different pattern of clinical and pathological features with V180I may represent different, but still topologically defined, neuronal loss when compared with sCJD-MM1. Elucidating this mechanism would require systematic pathological, immunochemical and biochemical studies of PrP^{Sc}.

The codon 129 polymorphism in *PRNP* plays an important role in determining the disease phenotype and the type of PrP^{Sc} present in sCJD.[14 19 20] It was also reported that the codon 129 polymorphism affects the phenotype in gPrD.[21–23] In our study, while 75.5% had methionine in the normal allele (MM homozygous), 24.5% had valine in the normal allele (MV heterozygous). We observed that when the codon 129 polymorphism occurred in the allele opposite to the V180I mutation, its influence on the clinical symptoms and signs were similar to the wild type MV polymorphism (table 4). However, the MV polymorphism in codon 129 significantly lowered the positive test rate and amount of CSF biomarkers such as the 14-3-3 protein, τ protein and PrP^{Sc} positivity, suggesting that the codon 129 polymorphism may contribute to the severity and/or speed of neurological degeneration. Moreover, there might be other unknown disease modifying factors that contribute to the clinical features and course of genetic prion disease. In addition, the codon 129 and 219 polymorphisms have been reported to be risk and protective factors, respectively, for sCJD.[24–27] In our study, similar to the study of patients with sCJD-MM1, all patients with V180I tested for the codon 219 polymorphism were glutamic acid homozygous (table 2). This result further suggests that codon 219 heterozygosity would be a protective factor in resisting prion disease onset. Interestingly, the frequency of codon 129 in MV heterozygous patients with the V180I mutation is greater than that in the general Japanese population, creating a discrepancy in the hypothesis that codon 129 homozygosity increases the susceptibility to prion disease.

Although there are several reports describing V180I in terms of its clinical features, imaging characteristics, pathology, immunohistochemistry and biochemistry, most were either case reports or analyses of a small number of cases.[6–8 28–31] To the best of our knowledge, the current study is the first large cohort clinical study of V180I. From this study, we conclude the clinical features of V180I to be as follows: (1) a late age of onset and slow progression; (2) a relatively low occurrence rate, and slow development of symptoms such as myoclonus, cerebellar abnormalities and visual disturbances; (3) a low detectable rate of PSWCs in EEGs, and a high detectable rate of hyperintensity in diffusion-weighted or fluid-attenuated inversion recovery imaging; (4) lower t-τ protein levels in the CSF versus sCJD-MM1 and (5) an extremely low likelihood of a family history of V180I.

Author affiliations
[1]Department of Neurology and Neurological Science, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences, Tokyo, Japan
[2]Faculty of Medicine, Clinical Research Center, Tokyo Medical and Dental University, Tokyo, Japan
[3]Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[4]Department of Neurology, National Hospital Organization Iou Hospital, Kanazawa, Japan
[5]Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan
[6]Department of Public Health, Jichi Medical University, Tochigi, Japan
[7]Division of CJD Science and Technology, Department of Prion Protein Research, Tohoku University Graduate School of Medicine, Miyagi, Japan
[8]Department of Neurology and Neuropathology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan
[9]Department of Neurology, Neurological Institute, Kyushu University Graduate School of Medicine, Fukuoka, Japan

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Contributors TQ drafted the manuscript and analysed the data. NS and M Hizume revised the manuscript, designed the study and analysed the data. NS and MT performed the statistical analysis and obtained funding. RA and KS analysed the CSF samples. M Higuma, IN, TH, SM and AK coordinated the study. TK analysed the prion protein gene and supervised the study. YN collected patient data. MY and HM supervised this study.

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