

# Co-Occurrence of Types 1 and 2 PrP<sup>res</sup> in Sporadic Creutzfeldt-Jakob Disease MM1

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**The genotype (M/M, M/V, or V/V) at polymorphic codon 129 of the human prion protein (PrP) gene and the type (1 or 2) of protease-resistant PrP (PrP<sup>res</sup>) in the brain are major determinants of the clinicopathological phenotypes of sporadic Creutzfeldt-Jakob disease (sCJD). According to this molecular typing system, sCJD has been classified into six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2). Besides these pure subgroups, mixed cases presenting mixed neuropathological phenotypes and more than one PrP<sup>res</sup> type have been found in sCJD. To investigate the frequency of the co-occurrence of types 1 and 2 PrP<sup>res</sup> in sCJD patients classified as MM1, we produced type 2 PrP<sup>res</sup>-specific antibody Tohoku 2 (T2) that can specifically detect the N-terminal cleavage site of type 2 PrP<sup>res</sup> after protease treatment and examined brain samples from 23 patients with sCJD-MM1. Western blot analysis using the T2 antibody revealed that the minority type 2 PrP<sup>res</sup> could be detected in all sCJD-MM1 brain samples including those of the cerebellum where sCJD-MM2 prions rarely accumulate. These results show that the co-occurrence of types 1 and 2 PrP<sup>res</sup> within a single sCJD-MM1 patient is a universal phenomenon. The general co-occurrence of multiple PrP<sup>res</sup> fragments within a single prion strain questions the validity of the conventional molecular typing system. (*Am J Pathol* 2011, 178:1309–1315; DOI: 10.1016/j.ajpath.2010.11.069)**

Creutzfeldt-Jakob disease (CJD) is a lethal transmissible neurodegenerative disease caused by an abnormal isoform of prion protein (PrP<sup>Sc</sup>), which is converted from the

normal cellular isoform (PrP<sup>C</sup>).<sup>1</sup> The genotype (M/M, M/V, or V/V) at polymorphic codon 129 of the human prion protein (PrP) gene and the type (type 1 or type 2) of PrP<sup>Sc</sup> in the brain are major determinants of the clinicopathological phenotypes of sporadic CJD (sCJD).<sup>2–5</sup> Type 1 and type 2 PrP<sup>Sc</sup> are distinguishable according to the size of the proteinase K (PK)-resistant core of PrP<sup>Sc</sup> (PrP<sup>res</sup>) (21 and 19 kDa, respectively), reflecting differences in the PK-cleavage site (at residues 82 and 97, respectively).<sup>2,5</sup> According to this molecular typing system, sCJD has been classified into six subgroups (MM1, MM2, MV1, MV2, VV1, or VV2).

Besides these “pure” subgroups, “mixed” cases presenting mixed neuropathological phenotypes and more than one PrP<sup>res</sup> type have been reported.<sup>4,6–10</sup> At first, the co-occurrence of types 1 and 2 PrP<sup>res</sup> within one individual was found in five of 14 patients with sCJD.<sup>6</sup> Recently, a systematic regional study in a series of 225 patients revealed that 35% of the sCJD patients presented both PrP<sup>res</sup> types.<sup>9</sup> In addition to these neuropathologically and biochemically mixed cases, monoclonal antibodies recognizing an epitope between residues 82 and 96 of human PrP (ie, specifically detecting type 1 PrP<sup>res</sup> after PK digestion), revealed that all CJD patients formerly classified as type 2 contained the minority type 1 PrP<sup>res</sup> despite the lack of mixed neuropathological phenotypes.<sup>11,12</sup> The co-occurrence of multiple PrP<sup>res</sup> fragments without mixed neuropathological phenotypes has remained controversial. To investigate accurately the frequency of the co-occurrence of types 1 and 2 PrP<sup>res</sup>, we produced type 2 PrP<sup>res</sup>-specific polyclonal antibody Tohoku 2 (T2)<sup>13</sup> and examined brain samples from 23 patients formerly classified as sCJD-MM1. Here we report

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**Table 1.** Summary of Clinical Features

	Age at onset (years)	PSWC on EEG (months)	Myoclonus (months)*	Akinetic mutism (months)*	Duration of illness (months)	PrP deposition
MM1 (n = 23)	68.6 ± 7.8	2.0 ± 2.6 (23/23) <sup>†</sup>	2.2 ± 2.5 (23/23) <sup>†</sup>	3.0 ± 3.2	13.5 ± 7.8	Synaptic
MM1+2 (n = 9)	65.8 ± 10.2	5.3 ± 5.8 (8/9) <sup>†</sup>	4.8 ± 5.4 (9/9) <sup>†</sup>	5.7 ± 6.3	11.8 ± 10.9	Synaptic + perivacuolar

Values are mean ± SD.

\*Duration until appearance of PSWC, myoclonus, or akinetic mutism from onset.

<sup>†</sup>Positive rate.

that the minority type 2 PrP<sup>res</sup> could be detected with type 1 in all sCJD-MM1 patients examined.

## Materials and Methods

### Patients

All CJD cases included in this study were patients with clinically, genetically, and neuropathologically proven sCJD. The diagnosis of CJD and the type of PrP<sup>res</sup> were confirmed by neuropathological examination, PrP immunohistochemistry, and conventional Western blotting using monoclonal antibody 3F4 as described.<sup>14,15</sup> The genotype and the absence of mutations in the open reading frame of the PrP gene were determined by sequence analysis.<sup>16</sup> All subjects were homozygous for methionine at codon 129 of the PrP gene and were classified as follows: MM1, 23 cases; MM1+2 (MM1-dominant form), nine cases; MM2 (cortical form), one case. The clinical features of the patients are summarized in Table 1. Detailed information of this sCJD-MM2 patient has been reported previously.<sup>17</sup> Four age-matched control subjects were included in this study and were also homozygous for methionine at codon 129 of the PrP gene.

### Sample Preparation and Western Blotting

Brain tissues were obtained at autopsy from the patients after receiving informed consent for research use. PrP<sup>res</sup> was extracted from brain tissues with collagenase treatment as described<sup>18</sup> with some modifications. Samples were subjected to 13% SDS–polyacrylamide gel electrophoresis and Western blotting as described.<sup>19</sup> The production of type 2 PrP<sup>res</sup>-specific polyclonal antibody T2 has been reported previously.<sup>13</sup> The monoclonal antibody 3F4<sup>20</sup> and the T2 antibody were used as the primary antibodies. Goat–antimouse immunoglobulin polyclonal antibody labeled with the peroxidase-conjugated dextran polymer, EnVision+ (Dako, Carpinteria, CA) and antirabbit EnVision+ were used as the secondary antibodies. The signal intensities of the Western blots were quantified with Quantity One software using an imaging device, VersaDoc 5000 (Bio-Rad Laboratories, Hercules, CA).

### Immunohistochemistry

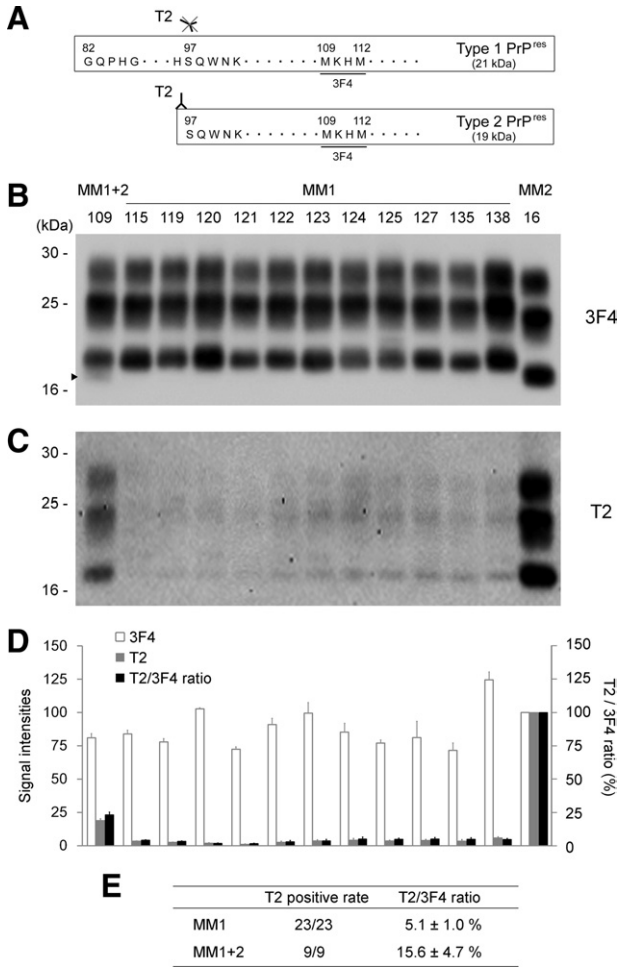
Formalin-fixed brain tissues were treated with 99% formic acid for 1 hour to inactivate the infectivity and embedded

in paraffin. Tissue sections were pretreated by hydrolytic autoclaving before PrP immunohistochemistry.<sup>14</sup> The 3F4 antibody and the monoclonal antibody #71<sup>21,22</sup> were used as the primary antibodies. Antimouse EnVision+ was used as the secondary antibody.

## Results

### T2-Reactive PrP<sup>res</sup> Fragments in the Cerebrums from sCJD-MM1 Patients

To determine whether type 2 PrP<sup>res</sup> could be detected in the sCJD patients formerly classified as type 1, we examined the cerebral samples from 23 patients with sCJD-MM1 by Western blotting using type 2 PrP<sup>res</sup>-specific polyclonal antibody T2 (Figure 1A). The T2 antibody is a proteolytic cleavage site–specific antibody and can specifically react with the N-terminally cleaved type 2 PrP<sup>res</sup> after PK digestion.<sup>13</sup> Although the N-terminal cleavage of type 2 PrP<sup>res</sup> by PK treatment can occur at several sites,<sup>5</sup> the T2 antibody detects the major cleavage products cleaved at residue 97.<sup>13</sup> As the positive control for the T2 antibody, we also examined nine patients with sCJD-MM1+2 (MM1-dominant form) and one patient with sCJD-MM2 (cortical form). In the conventional Western blot analysis using the monoclonal antibody 3F4, which is the most popular anti-PrP antibody that reacts with all PrP<sup>res</sup> types, the sCJD-MM1 brains showed only type 1 PrP<sup>res</sup> (Figure 1B). In contrast, in the Western blot analysis using the T2 antibody, all sCJD-MM1 brains contained T2-reactive PrP<sup>res</sup> fragments (Figure 1C). The sizes of the T2-reactive fragments were identical to those of type 2 PrP<sup>res</sup> in the sCJD-MM2 brain. In the quantitative analysis, the mean signal intensities of PrP<sup>res</sup> in the sCJD-MM2 brain were assigned as 100/mm<sup>2</sup> in each experiment using the 3F4 or T2 antibody (Figure 1D). To compare the amounts of the T2-reactive PrP<sup>res</sup> fragments among the patients, the mean signal intensities of the T2-reactive fragments were divided by those of the 3F4-reactive PrP<sup>res</sup> fragments, and the percentage of the T2-reactive fragments was calculated in each case (T2/3F4 ratio). The mean T2/3F4 ratio of the sCJD-MM1 patients was significantly lower than that of the sCJD-MM1+2 patients (Figure 1E). Thus, small but significant amounts of T2-reactive PrP<sup>res</sup> fragments could be detected in all sCJD-MM1 patients examined.

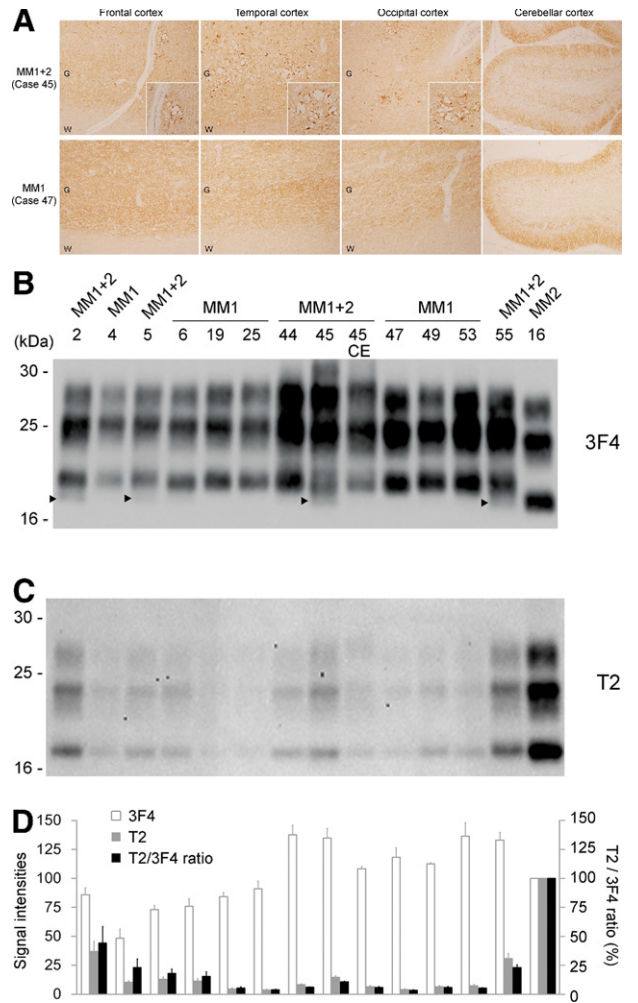


**Figure 1.** Tohoku 2 (T2)-reactive PrP<sup>res</sup> fragments in the cerebrums from sCJD-MM1 patients. **A:** The epitope for type 2 PrP<sup>res</sup>-specific antibody T2. The T2 antibody specifically detects the N-terminal cleavage site of type 2 PrP<sup>res</sup>.<sup>13</sup> In contrast, 3F4 antibody detects both PrP<sup>res</sup> types. **B:** The conventional Western blot analysis using the 3F4 antibody was used for the classification of sCJD patients included in this study. The subgroup and case number of each patient are described above each lane. A faint type 2 PrP<sup>res</sup> band (arrowhead) was detected with the majority type 1 PrP<sup>res</sup> bands in the sCJD-MM1+2 brain (case 109). **C:** T2-reactive PrP<sup>res</sup> fragments could be detected in all sCJD-MM1 brains examined. **D:** The signal intensities of the 3F4-reactive PrP<sup>res</sup> fragments (white bars) or those of the T2-reactive PrP<sup>res</sup> fragments (gray bars). The mean signal intensities of PrP<sup>res</sup> in the sCJD-MM2 brain (case 16) were assigned as 100/mm<sup>2</sup> in each experiment. To compare the amounts of the T2-reactive PrP<sup>res</sup> fragments among the patients, the mean signal intensities of the T2-reactive fragments were divided by those of the 3F4-reactive fragments, and the percentage of the T2-reactive fragments was calculated in each case (T2/3F4 ratio, black bars). The data are expressed as mean ± SEM (*n* = 3). **E:** Summary of the Western blot analysis using the T2 antibody. Values are means ± SEM.

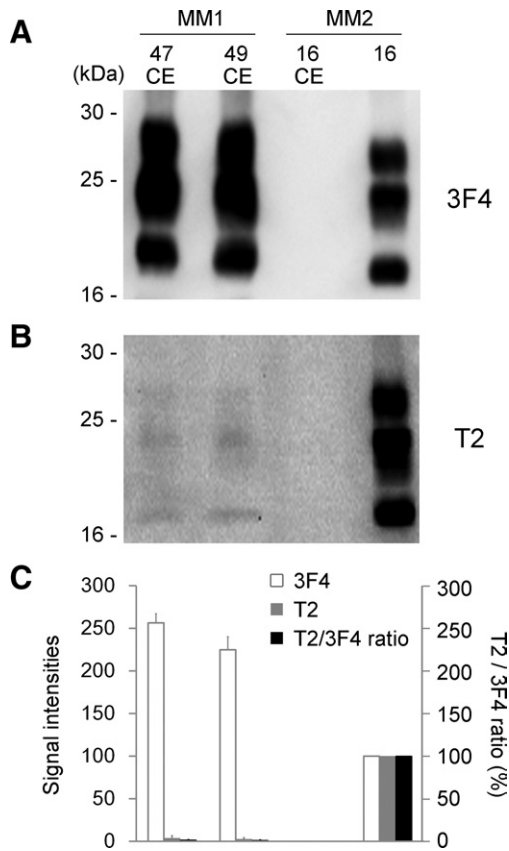
### T2-Reactive PrP<sup>res</sup> Fragments in the Cerebellums from sCJD-MM1 Patients

The consistent detection of the T2-reactive PrP<sup>res</sup> fragments in the cerebrums from sCJD-MM1 patients led us to suppose that co-occurrence of types 1 and 2 PrP<sup>res</sup> fragments might be a universal phenomenon. Indeed, the T2-reactive PrP<sup>res</sup> fragments were also detected in the cerebellum of a sCJD-MM1+2 patients in the present study. In most sCJD-MM1+2 patients, type 2 PrP<sup>res</sup> is rarely detected in the cerebellum by conventional Western blot analysis.<sup>9</sup> In addition, the cerebellums from

sCJD-MM1+2 patients lack the perivacuolar PrP deposition that is characteristic of sCJD-MM2 prions, whereas the cerebrums show a mixed PrP deposition pattern (ie, synaptic plus perivacuolar PrP deposition).<sup>9,10</sup> In accordance with these reports, the cerebellums from the sCJD-MM1+2 patients in the present study lacked perivacuolar PrP deposition and showed only the synaptic-type deposition, whereas the cerebral cortices showed perivacuolar PrP deposition in addition to the synaptic-type deposition (Figure 2A). Furthermore, conventional Western blot analysis using the 3F4 antibody showed the lack of type 2 PrP<sup>res</sup> accumulation in the cerebellum of the sCJD-



**Figure 2.** T2-reactive PrP<sup>res</sup> fragments in the cerebellum from the sCJD-MM1+2 patient. **A:** Immunohistochemistry using the anti-PrP antibody #71 showed that perivacuolar PrP deposition was restricted to within the cerebral cortices in the sCJD-MM1+2 patient (cases 45; ×40, insets ×200). The sCJD-MM1 patient showed only synaptic-type PrP deposition (case 47; ×40). G = gray matter; W = white matter. **B:** In the conventional Western blot analysis using the 3F4 antibody, faint type 2 PrP<sup>res</sup> bands (arrowheads) were detected in the cerebrums from the sCJD-MM1+2 patients (cases 2, 5, 45, and 55) but not in the cerebellum (case 45 CE). In case 44, although neuropathological examination showed focal perivacuolar PrP deposition, the conventional Western blot analysis failed to detect type 2 PrP<sup>res</sup>. **C:** T2-reactive PrP<sup>res</sup> fragments could be detected also in the cerebellum of the sCJD-MM1+2 patient (case 45 CE). **D:** The signal intensities of the 3F4-reactive PrP<sup>res</sup> fragments (white bars) or those of the T2-reactive PrP<sup>res</sup> fragments (gray bars), and the T2/3F4 ratios (black bars). The mean signal intensities of PrP<sup>res</sup> in the sCJD-MM2 brain (case 16) were assigned as 100/mm<sup>2</sup> in each experiment. Data are expressed as mean ± SEM (*n* = 3).



**Figure 3.** T2-reactive PrP<sup>res</sup> fragments in the cerebellums from the sCJD-MM1 patients. **A:** In the conventional Western blot analysis using the 3F4 antibody, PrP<sup>res</sup> was not detected in the cerebellum of the sCJD-MM2 patient (case 16 CE). **B:** The T2-reactive PrP<sup>res</sup> fragments could be detected in the cerebellums from the sCJD-MM1 patients (cases 47 CE and 49 CE), but not in the cerebellum from the sCJD-MM2 patient (case 16 CE). **C:** The signal intensities of the 3F4-reactive PrP<sup>res</sup> fragments (white bars) or those of the T2-reactive PrP<sup>res</sup> fragments (gray bars), and the T2/3F4 ratios (black bars). The mean signal intensities of PrP<sup>res</sup> in the sCJD-MM2 cerebrum (case 16) were assigned as 100/mm<sup>2</sup> in each experiment. Data are expressed as mean ± SEM (*n* = 3).

MM1+2 patient (case 45 CE) (Figure 2B). Nevertheless, Western blot analysis using the T2 antibody revealed that the cerebellum of the sCJD-MM1+2 patient (case 45 CE) contained T2-reactive PrP<sup>res</sup> fragments (Figure 2C). The sizes of the T2-reactive fragments were identical to those of type 2 PrP<sup>res</sup> in the sCJD-MM2 brain. In the quantitative analysis, the T2/3F4 ratio of the cerebellum (case 45 CE) was lower than that of the cerebrum (case 45) (Figure 2D). Thus, small amounts of T2-reactive PrP<sup>res</sup> fragments could be detected also in the cerebellum of the sCJD-MM1+2 patients.

For further analysis, we also examined the cerebellums from two sCJD-MM1 patients. In most sCJD-MM2 patients, the cerebellum lacks significant pathology, and PrP deposition is seldom or never observed.<sup>4</sup> In line with this report, the cerebellum of the present sCJD-MM2 patient (case 16 CE) did not contain any PrP<sup>res</sup> signals when probed with the 3F4 or T2 antibody (Figure 3, A and B).<sup>17</sup> In contrast, the cerebellums from the sCJD-MM1 patients contained the T2-reactive PrP<sup>res</sup> fragments (cases 47 CE and 49 CE). These T2-reactive PrP<sup>res</sup> fragments showed the exact type 2 PrP<sup>res</sup> migration pattern. In the quantitative analysis, the T2/3F4 ratios of the

cerebellums (case 47 CE, 1.5% ± 0.8%; case 49 CE, 1.1% ± 0.5%) were lower than those of the cerebrums (case 47, 3.4% ± 0.5%; case 49, 5.9% ± 0.8%) (Figure 3C). Thus, trace amounts of T2-reactive PrP<sup>res</sup> fragments could be detected in all sCJD-MM1 brain samples including those of the cerebellum.

### T2-Reactive Truncated PrP<sup>C</sup> Fragments in Non-CJD Control Brains

The normal cellular isoform of PrP, denoted as PrP<sup>C</sup>, is cleaved by endogenous proteases and can generate various N-terminally truncated PrP<sup>C</sup> molecules.<sup>23,24</sup> This raised the possibility that T2-reactive PrP<sup>C</sup> fragments (97-PrP<sup>C</sup>) might pre-exist in the brains and might be incorporated into MM1 PrP<sup>res</sup> aggregates in sCJD-MM1 patients. To determine whether the 97-PrP<sup>C</sup> fragments can be generated in normal brains, we examined brain samples from four age-matched control subjects without PK treatment. Western blot analysis using the T2 antibody showed that the cerebral cortices from the control subjects contained 97-PrP<sup>C</sup> (Figure 4, A and B). The glycosylation patterns of these 97-PrP<sup>C</sup> fragments were different from those of the T2-reactive PrP<sup>res</sup> fragments observed in the sCJD patients. After deglycosylation with PNGase F, these 97-PrP<sup>C</sup> fragments migrated at 19 kDa. Moreover, the cerebellums from the control subjects also contained the 97-PrP<sup>C</sup> (Figure 4, C and D). Thus, the T2-reactive truncated PrP<sup>C</sup> fragments could be detected in all control brain samples including those of the cerebellums.

### Patchy Plaque-Type PrP Deposition in the sCJD-MM1 Patients with High T2/3F4 Ratios

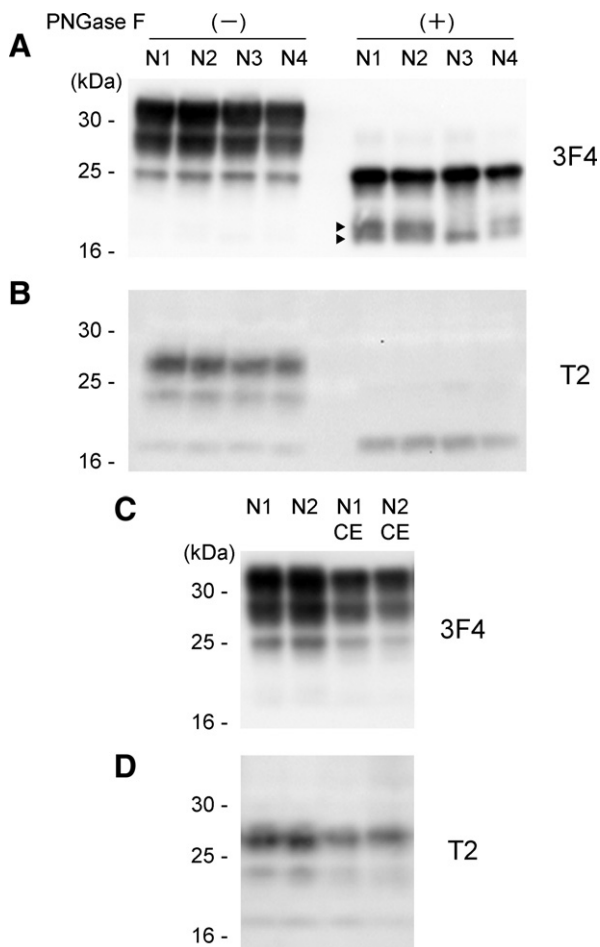
In the 23 patients with sCJD-MM1 examined in the present study, two patients (cases 4 and 6) showed high T2/3F4 ratios similar to those of the sCJD-MM1+2 patients (Figure 2D). The clinical courses of cases 4 and 6 were not significantly different from those of the other sCJD-MM1 patients (Figure 5A). Indeed, the duration until the appearance of akinetic mutism and total duration of illness in case 6 were rather shorter than the mean values in the sCJD-MM1 patients. However, the high T2/3F4 ratios raised the possibility that these two sCJD patients might contain a focal deposition of MM2 prions overlooked in the previous examination. Therefore, we re-examined all brain sections from these patients by PrP immunohistochemistry. The cerebral and cerebellar cortices from the two patients lacked perivacuolar PrP deposition and showed diffuse synaptic-type PrP deposition. However, careful re-examination revealed a very focal accumulation of small PrP plaques in the cerebral cortices (Figure 5B). These patchy plaque-type PrP deposits were not accompanied by large vacuoles, in contrast to the perivacuolar PrP deposition (Figure 5, C and D). Meanwhile, besides perivacuolar and synaptic-type deposition, coarse PrP deposits similar to the patchy plaque-type PrP deposition were observed in the cerebral cortices from the sCJD-MM1+2 patients. These coarse PrP deposits in the sCJD-MM1+2 patients were



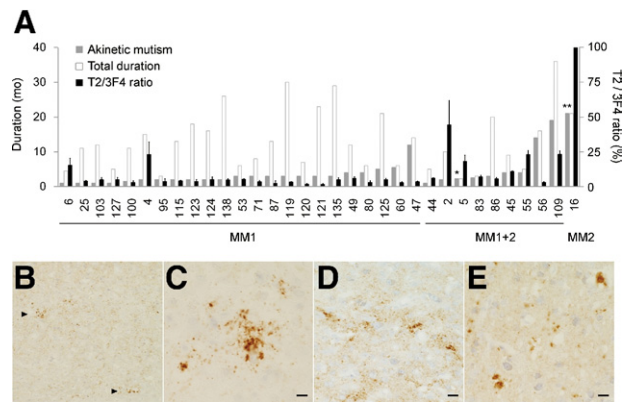
also independent of vacuoles (Figure 5E). Thus, the sCJD-MM1 patients with the high T2/3F4 ratios showed the patchy plaque-type PrP deposition similar to the coarse PrP deposition in the sCJD-MM1+2 patients. Although these sCJD patients had been classified into MM1 by the conventional classification system because the lack of perivacuolar PrP deposition, the patchy plaque-type PrP deposition might indicate the existence of MM2 prions.

### Discussion

Our data comprised three major findings. First, using the novel antibody T2 that can specifically react with the N-terminal cleavage site of type 2 PrP<sup>res</sup> after PK digestion, type 2 PrP<sup>res</sup> could be detected in all sCJD-MM1 brain samples including those of the cerebellum where sCJD-MM2 prions rarely accumulate. Second, T2-reactive truncated PrP<sup>C</sup> fragments (97-PrP<sup>C</sup>) could be detected in



**Figure 4.** T2-reactive truncated PrP<sup>C</sup> fragments in non-CJD control brains. **A:** The cerebral cortices from age-matched control subjects (N1 to N4) were examined by conventional Western blot analysis using the 3F4 antibody without PK treatment. After deglycosylation with PNGase F, major PrP<sup>C</sup> fragments migrated at 25 kDa. In addition, various truncated PrP<sup>C</sup> fragments (arrowheads) were also observed. **B:** T2-reactive truncated PrP<sup>C</sup> fragments (97-PrP<sup>C</sup>) could be detected in all control brains. The 97-PrP<sup>C</sup> fragments migrated at 19 kDa after the deglycosylation. **C and D:** The 97-PrP<sup>C</sup> fragments could be detected also in the cerebellums from the control subjects (N1 CE and N2 CE).



**Figure 5.** Patchy plaque-type PrP deposition in the sCJD-MM1 patients with high T2/3F4 ratios. **A:** The duration until the appearance of akinetism (gray bars), total duration of illness (white bars), and the T2/3F4 ratio (black bars) of the sCJD patients. The sCJD-MM1 or sCJD-MM1+2 cases are arranged in order of the duration until the appearance of akinetism. \*The patient became unconsciousness and bedridden 1 month after the initial symptom because of respiratory failure. \*\*The patient did not present akinetism throughout the clinical course. **B:** PrP immunohistochemistry using the 3F4 antibody revealed patchy plaque-type PrP deposition (arrowheads) besides diffuse synaptic-type PrP deposition in the cerebral cortices from the sCJD-MM1 patient with the high T2/3F4 ratio (case 4; temporal cortex;  $\times 40$ ). **C:** The patchy plaque-type PrP deposition was independent of vacuoles (case 4; occipital cortex;  $\times 400$ ). **D:** The patchy plaque-type deposition in the other sCJD-MM1 patient with the high T2/3F4 ratio (case 6; frontal cortex;  $\times 400$ ). **E:** The cerebral cortices from the sCJD-MM1+2 patient showed coarse PrP deposition similar to the patchy plaque-type PrP deposition (case 5; parietal cortex;  $\times 400$ ). Scale bars = 10  $\mu$ m.

age-matched control brains without PK treatment. Third, two sCJD-MM1 patients with relatively high type 2 PrP<sup>res</sup> contents showed unique PrP deposition, namely the patchy plaque-type deposition. Here we demonstrate that the co-occurrence of types 1 and 2 PrP<sup>res</sup> within a single sCJD-MM1 patient is a universal phenomenon.

The trace amounts of type 2 PrP<sup>res</sup> in the sCJD-MM1 brains might result from the involvement of 97-PrP<sup>C</sup> fragments into the MM1 PrP<sup>res</sup> aggregates. Despite the lack of perivacuolar PrP deposition that is characteristic of sCJD-MM2 prions, trace amounts of type 2 PrP<sup>res</sup> were detected in all sCJD-MM1 patients examined. In addition, type 2 PrP<sup>res</sup> could be detected not only in the cerebriums but also in the cerebellums where sCJD-MM2 prions rarely accumulate. These findings support the view of the co-occurrence of multiple PrP<sup>res</sup> fragments within MM1 prions rather than the co-occurrence of multiple prion strains within the same individual. One possible explanation for the co-occurrence of multiple PrP<sup>res</sup> fragments within a single prion strain is the involvement of multiple PrP<sup>C</sup> fragments into the PrP<sup>res</sup> aggregates. There is accumulating evidence that various N-terminally truncated PrP<sup>C</sup> molecules exist in human brains.<sup>23,24</sup> Moreover, T2-reactive 97-PrP<sup>C</sup> fragments existed in normal human brains in the present study. These 97-PrP<sup>C</sup> fragments might be incorporated into the PrP<sup>res</sup> aggregates with full-length PrP<sup>Sc</sup> and might acquire protease resistance as reported in deletion mutant PrPs or green fluorescent protein-tagged PrP.<sup>25,26</sup> These findings lead us to speculate that the minority type 1 PrP<sup>res</sup> detected in the brains from CJD patients classified as type 2<sup>11,12</sup> might also be caused by the involvement of 82-PrP<sup>C</sup> fragments. Because the type 1 PrP<sup>res</sup>-specific antibodies used in these

reports recognize an epitope between residues 82 and 96 of human PrP and can react with full-length PrP<sup>C</sup>, it remains to be determined whether 82-PrP<sup>C</sup> fragments can be generated in human brains. If an antibody that can specifically detect the N-terminal cleavage site at residue 82 is generated, 82-PrP<sup>C</sup> fragments might also be detected in human brains. To examine the possibility of the co-occurrence of multiple prion strains within one individual, whether transmissibilities and pathological phenotypes can be split into two groups in the transmission studies using the limiting dilution of these CJD brains should be addressed in the future.

The analysis of the type 2/total PrP<sup>res</sup> ratio (ie, the T2/3F4 ratio) using the T2 antibody may have significance in classifications of sCJD. In the present study, two of 23 sCJD-MM1 patients showed high T2/3F4 ratios and the unique PrP deposition designated as the patchy plaque-type. Because the patchy plaque-type deposition was similar to the coarse PrP deposition in the sCJD-MM1+2 patients, these deposits might be precursors of the perivacuolar PrP deposition or variant deposition patterns of MM2 prions. The finding of the patchy plaque-type deposition in the sCJD patients who have been neuropathologically and biochemically classified into MM1 raises the possibility that the co-occurrence of MM1 and MM2 prions within the same brain might be underestimated in the conventional classification. Hereafter, sCJD-MM1 patients need to be examined by the T2 antibody, and the focal accumulation of MM2 prions must be tested carefully in patients showing high T2/3F4 ratios compared with the mean value of the sCJD-MM1 patients (5.1%). On the other hand, although the mean T2/3F4 ratio of the sCJD-MM1+2 patients was significantly higher than that of the sCJD-MM1 patients, some of the sCJD-MM1+2 patients showed low T2/3F4 ratios indistinguishable from those of the sCJD-MM1 patients. These results suggest that examination using the T2 antibody is insufficient to discriminate sCJD-MM1+2 from sCJD-MM1 when the amount of type 2 PrP<sup>res</sup> is marginal and the examined brain region is limited. To avoid missing the focal accumulation of MM2 prions, multiple brain regions need to be examined by the T2 antibody, as suggested when using the conventional Western blot analysis.<sup>9</sup>

Unfortunately, immunohistochemical analysis of type 2 PrP<sup>res</sup> using the T2 antibody was unavailable in the present study. To eliminate the infectivity of sCJD prions, all brain sections had to be treated with formic acid, and infectious conformers were denatured. Therefore, the PrP<sup>res</sup> type-specific cleavage by PK treatment was impossible in tissue sections. We also attempted to detect the endogenous protease-cleaved type 2 PrP<sup>res</sup> without PK treatment. In this preliminary experiment, the patchy plaque-type PrP deposition in the sCJD-MM1 patients with the high T2/3F4 ratios showed the T2-immunoreactivities, whereas the synaptic-type deposition was not immunolabeled with the T2 antibody (A. Kobayashi and T. Kitamoto, unpublished). However, as 97-PrP<sup>C</sup> fragments also exist in human brains, we cannot conclude whether the T2 immunoreactivities observed in the sCJD

brains without PK treatment represent the existence of type 2 PrP<sup>res</sup>.

In conclusion, the present study, together with evidence from other groups,<sup>11,12</sup> suggests that the co-occurrence of multiple PrP<sup>res</sup> fragments within a single sCJD patient is a universal phenomenon. These findings show that the conventional typing of PrP<sup>res</sup> merely represents the predominant PrP<sup>res</sup> subpopulation among multiple co-existing PrP<sup>res</sup> fragments. Besides the general co-occurrence of multiple PrP<sup>res</sup> fragments, the condition of PK digestion easily affects the size of PrP<sup>res</sup>.<sup>27,28</sup> Indeed, insufficient PK digestion can generate type 1 PrP<sup>res</sup>-specific antibody-reactive fragments in the sCJD patients classified as type 2.<sup>29</sup> Furthermore, it is possible that the conventional Western blot analysis fails to detect type 2 PrP<sup>res</sup> in sCJD-MM1+2 cases showing very focal perivacuolar PrP deposition in the brain.<sup>9</sup> These confusing aspects of PrP<sup>res</sup> typing question the validity of the conventional molecular typing system. For a precise classification, it may be appropriate that the neuropathological phenotyping [synaptic (SY), perivacuolar (PV), plaque (PL), or patchy plaque (PP)] be combined with the molecular typing [eg, sCJD-MM1(+2)/SY+PV] the sCJD-MM patient showing synaptic + perivacuolar PrP deposition, but not type 2 PrP<sup>res</sup> in the conventional Western blot analysis.

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