Association of an 11–12 kDa protease-resistant prion protein fragment with subtypes of dura graft-associated Creutzfeldt–Jakob disease and other prion diseases

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Creutzfeldt–Jakob disease can develop in subjects given a cadaveric dura mater graft (dCJD). This disease has a phenotypic heterogeneity despite the lack of genetic variation. Numerous plaque-type prion protein (PrP) deposits are found in the brain of some but not all subjects; hence, there may be two subtypes of this clinical entity. To validate dCJD subtypes further, we carried out a larger-scale clinicopathological analysis and typing of protease-resistant PrP (PrP Sc ) in dCJD cases. Cases with plaque-type PrP deposits (p-dCJD) were shown to be distinct from those without PrP plaques (np-dCJD), from several clinicopathological aspects. Analysis of PrP Sc revealed that, while the major PrP Sc species from both subtypes was of 21 kDa after deglycosylation (type 1 PrP Sc), a C-terminal PrP fragment of 11–12 kDa (fPrP11–12) was associated with np-dCJD but not with p-dCJD. The disease type-specific association of fPrP11–12 was also observed in subjects with other prion diseases. An fPrP11–12-like C-terminal PrP fragment was detected in brain lysates from patients associated with fPrP11–12, but not from patients or normal subjects unassociated with fPrP11–12. Results indicated that fPrP was produced by CJD-associated processes in vivo. The present data provide several lines of evidence that support the need for subtyping of dCJD and contribute to the understanding of the processing of disease-specific PrP species. The unique relationship of fPrP11–12 with CJD phenotype supports the view that the phenotypic heterogeneity of CJD is related to the formation of different types of disease-specific PrP and fragments thereof.

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INTRODUCTION

Creutzfeldt–Jakob disease (CJD) can manifest itself years after surgical implantation of cadaveric dura mater grafts (dura CJD, dCJD). This disease constitutes a major part of iatrogenic CJD (Brown et al., 2000; Thadani et al., 1988). A total of 114 cases have been reported worldwide as of July 2000, including 67 cases in Japan (Brown et al., 2000; Hoshi et al., 2000). The majority of dCJD cases (non-plaque-type dCJD, np-dCJD) take the form of typical CJD (Lang et al., 1998). The affected subjects present with progressive mental deterioration, ataxia, myoclonus and characteristic electroencephalographic findings such as periodic synchronous discharges (PSDs). The pathology is characterized by neuronal loss, spongiform changes and gliosis in atrophic brains. The lesions are associated with synaptic-type diffuse deposits of prion protein (PrP) in the grey matter but not with plaque-type PrP deposits (Kitamoto et al., 1999; Yamada et al., 1994). Some reported cases have deviated, however, from these typical characteristics, despite the lack of variation in the PrP genotype (Kimura et al., 2001; Kopp et al., 1996; Lane et al., 1994; Radbauer et al., 1998; Shimizu et al., 1999; Takahashi et al., 1997; Takashima et al., 1997). These atypical patients have numerous plaque-type PrP deposits in the brain. It has been argued that a subtype of dCJD other than np-dCJD may be present (Shimizu et al., 1999).

In order to examine dCJD subtypes further, we carried out a larger-scale clinicopathological analysis and typing of protease-resistant PrP (PrPSc) from cases of dCJD. Along with differences among subtypes with respect to several clinicopathological aspects, we discovered that np-dCJD was associated with a protease-resistant C-terminal PrP fragment of 11–12 kDa, while dCJD with plaque-type PrP deposits (plaque-type dCJD, p-dCJD) was not. The type (or subtype)-specific association of the PrP fragment was also observed in subjects with other prion diseases, thus arguing for a relationship with the pathogenesis. The present study provides clinical, pathological and biochemical evidence that supports the need for the subtyping of dCJD.

METHODS

Sources of information. Clinical information was obtained from three sources, as follows: (i) the summary of clinical records made by the attending physicians; (ii) physicians’ replies to the inquiry made by the CJD Surveillance Group in Japan; and (iii) descriptions in previous reports (Table 1) (Kimura et al., 2001; Miyashita et al., 1991; Shimizu et al., 1999; Takahashi et al., 1997; Takashima et al., 1997; Yamada et al., 1994, 1997). Statistical analysis of clinical findings and brain weight was carried out using the Student’s t-test.

Genetic analysis. Genomic DNA extracted from peripheral blood leukocytes and frozen brain tissues was used to amplify the open reading frame (ORF) of the PrP gene by PCR (Kitamoto & Tateishi, 1996). Genetic analysis of cerebellum

![Image of Table 1. Clinical profile of dura CJD cases](https://example.com/table1.png)

Table 1. Clinical profile of dura CJD cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Reference</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Duration of CJD (months)</th>
<th>Reason for surgery</th>
<th>Neurological manifestations</th>
<th>Analysis of cerebellum</th>
<th>PrPSc typing</th>
</tr>
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<tbody>
<tr>
<td>Non-plaque type (np-dCJD)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>Yamada et al. (1994)</td>
<td>F</td>
<td>31</td>
<td>27</td>
<td>Pituitary adenoma</td>
<td>A→D→M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>F</td>
<td>55</td>
<td>16</td>
<td>Chiari malformation</td>
<td>A→D/M</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
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<td>F</td>
<td>58</td>
<td>13</td>
<td>Meningioma</td>
<td>D→P→M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>M</td>
<td>69</td>
<td>12</td>
<td>Occipital meningioma</td>
<td>A/D/P→M</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Miyashita et al. (1991)</td>
<td>F</td>
<td>26</td>
<td>20</td>
<td>Hemangioblastoma at cerebellum</td>
<td>A→D→M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Yamada et al. (1997)</td>
<td>M</td>
<td>52</td>
<td>10</td>
<td>Frontal meningioma</td>
<td>V/Dy→A→M</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Plaque type (p-dCJD)</td>
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<td></td>
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<tr>
<td>1</td>
<td>Shimizu et al. (1999)</td>
<td>M</td>
<td>68</td>
<td>8</td>
<td>Left hemifacial spasm</td>
<td>A→D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Shimizu et al. (1999)</td>
<td>F</td>
<td>68</td>
<td>16</td>
<td>Parasagittal meningioma</td>
<td>A→D/V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>F</td>
<td>62</td>
<td>19</td>
<td>MCA aneurysm</td>
<td>A→D/V→M</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>−</td>
<td>F</td>
<td>68</td>
<td>12</td>
<td>Acoustic schwannoma</td>
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<td>M</td>
<td>29</td>
<td>30</td>
<td>Teratoma at pineal gland</td>
<td>P→D/V→A/M</td>
<td></td>
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<tr>
<td>6</td>
<td>Kimura et al. (2001)</td>
<td>M</td>
<td>42</td>
<td>14</td>
<td>Pituitary adenoma</td>
<td>H→Q/D→M</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Takahashi et al. (1997); Takashima et al. (1997)</td>
<td>F</td>
<td>47</td>
<td>18</td>
<td>MCA aneurysm</td>
<td>A/V→D→M</td>
<td></td>
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</tr>
</tbody>
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MCA, middle cerebral artery; A, ataxia; D, dementia; M, myoclonus; P, psychological symptoms; V, visual symptoms; Dy, dysesthesia; H, hemiparesis; Q, quadriplegic.

+, Cases that were subjected to analysis.
obtain the second pellet. Proteins from the first supernatant and in the second pellet were denatured in 1 × Laemmli’s sample buffer, deglycosylated and analysed by Western blot.

Informed consent. Clinical information, peripheral blood and brain tissue from patients and unaffected subjects were obtained with informed consent for research use.

RESULTS

Selection of patients

We examined all dCJD cases in the form of formalin-fixed cerebral sections available for diagnosis and subtyping (Table 1). They included three cases (np-dCJD #1, p-dCJD #1 and 2) that we had reported previously (Shimizu et al., 1999; Yamada et al., 1994) and seven new ones (np-dCJD #2, 3 and 4, and p-dCJD #3, 4, 5 and 6) (Kimura et al., 2001). Only frontal biopsy specimen was available for the p-dCJD #5 subject, who is alive. dCJD cases (np-dCJD #5 and 6, and p-dCJD #7) that had been reported by other authors were also examined when fixed cerebral sections were available (Table 1) (Miyashita et al., 1991; Takahashi et al., 1997; Takashima et al., 1997; Yamada et al., 1997). The total of 13 cases analysed represents nearly two-thirds of the 21 pathologically proven ‘definite’ dCJD cases that have been identified by the CJD Surveillance Group in Japan (Brown et al., 2000; Hoshi et al., 2000; T. Sato & T. Kitamoto, unpublished data).

Cerebral specimens from all 13 cases had the pathological characteristics of CJD as described below. Six cases without plaques were categorized as np-dCJD and the other seven with plaques as p-dCJD (Table 1).

Clinical findings

Clinical profiles are summarized in Tables 1 and 2. There was no significant difference between the two subtypes in the latent period (the period from surgical operation to the onset of CJD) or in the duration of CJD (Table 2). Many np-dCJD and p-dCJD cases presented initially with ataxia as reported (Table 1) (Hoshi et al., 2000; Lang et al., 1998). Mental deterioration such as disorientation or memory disturbance most often followed ataxia. To search for

Table 2. Parameters related to the clinical coarse of dura CJD

<table>
<thead>
<tr>
<th></th>
<th>np-dCJD Mean ± SD (n = 6)</th>
<th>p-dCJD Mean ± SD (n = 7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latent period (years)</td>
<td>9.0 ± 4.9</td>
<td>11.0 ± 2.2</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Duration of CJD (months)</td>
<td>16.3 ± 5.7</td>
<td>16.7 ± 6.4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Period between the onset of CJD and that of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSDs on EEG (months)</td>
<td>3.2 ± 1.3</td>
<td>16.6 ± 6.4</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>myoclonus (months)</td>
<td>4.0 ± 1.5</td>
<td>11.7 ± 4.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>akinetic mutism (months)</td>
<td>3.5 ± 1.6</td>
<td>10.3 ± 3.3</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

*Statistically significant (P < 0.05).
differences among subtypes, we then focused on findings that were well documented and typical of CJD and found that the subtypes differed significantly on three points (Table 2). There was an absence or late occurrence of myoclonus and PSDs on electroencephalography (EEG) in p-dCJD. PSDs never appeared on the EEGs from five subjects with p-dCJD and three p-dCJD cases did not have myoclonus. The third difference was slow progression of neurological dysfunction in p-dCJD, which was represented by a relatively late transition into the state of akinetic mutism.

**Prion protein gene sequence**

Sequence data on the PrP gene were available for all cases except np-dCJD #5. None of the twelve cases examined had mutations in PrP genes. Codon 129 was Met/Met homozygous in all twelve cases examined, while codon 219 was Glu/Glu homozygous in all eleven cases examined (no data was available for np-dCJD #3). These numbers indicated no significant difference from reported data on the Japanese general population [$\chi^2 = 1.09$ (codon 129) and 1.48 (codon 219), $P > 0.1$ for both] (Doh-ura et al., 1991; Kitamoto & Tateishi, 1994).

**Neuropathological findings**

Brain weight was more preserved in p-dCJD than in np-dCJD: 877 ± 102 g (mean ± SD) in np-dCJD ($n = 6$) and 1155 ± 147 g in p-dCJD ($n = 6$) ($P < 0.01$).

Typical CJD pathology was observed in cerebral tissues from all 13 cases. Included were spongiform changes, neuronal loss and synaptic-type diffuse PrP deposits in the grey matter and astrocytic gliosis in the grey and white matter. All seven p-dCJD cases contained plaque-type PrP deposits in the cerebral grey matter (Fig. 1A, B). In addition, unique PrP deposits fringing neuronal cell bodies and processes were found in the cerebral grey matter of five (#1, 2, 3, 4, 7) p-dCJD cases (Fig. 1B). Such perineuronal deposits were not observed in any case of np-dCJD.

We also analysed cerebellar sections from one np-dCJD and five p-dCJD cases (Table 1). Typical CJD pathology was observed in the cerebellum of all six cases and included neuronal loss in the granular layer, atrophy, spongiform changes and gliosis in the molecular layer of the cortex, and gliosis in the white matter. In PrP immunohistochemistry, there were synaptic-type PrP deposits in molecular and granular layers of the cortex in all six cases. There were no plaque-type PrP deposits in the cerebellum of the np-dCJD case, but there were plaque-type deposits in the cortex and white matter in all p-dCJD cases examined (Fig. 1C, D). PrP plaques in the white matter were morphologically unique, either aligning linearly (Fig. 1D, arrow) or surrounding blood vessels (Fig. 1D, arrowheads).

**Typing of PrPSc fragments**

To search for a biochemical marker that would reveal differences among dCJD subtypes, we typed PrPSc from three np-dCJD and five p-dCJD cases as well as 15 sCJD cases (Tables 1 and 3) (Collinge et al., 1996; Parchi et al., 1996). Western blot analysis of PrPSc samples with mAb 3F4 revealed three major PrPSc species of 21–30 kDa from all np-dCJD and p-dCJD cases (Fig. 2A). These PrPSc species had the same molecular masses as those from sCJD cases with the codon 129 Met/Met (sCJD MM1) and were judged to be of Parchi’s PrPSc type 1; they converged into a band at 21 kDa after deglycosylation, as previously reported (Fig. 2A) (Parchi et al., 1996). In constrast, PrPSc from an sCJD case with 129 Val/Val (sCJD VM2) was of 19–28 kDa; it converged into a band at 19 kDa after deglycosylation and was judged to be of Parchi’s PrPSc type 2 (Fig. 2A) (Parchi et al., 1996). This case had plaque-type PrP deposits in the cerebrum (data not shown) (Miyazono et al., 1992; Parchi et al., 1996, 1999; de Silva et al., 1994). Three sCJD cases with 129 Val/Met were split into one case of the PrPSc type 1 (sCJD VM1) and two of the PrPSc type 2 (sCJD VM2) (Fig. 2A). These two subgroups were also pathologically distinct, as previously reported: sCJD VM1 was without PrP plaques, while sCJD VM2 had PrP plaques in the cerebrum.
To characterize PrP\textsuperscript{Sc} from dCJD cases further, PrP\textsuperscript{Sc} samples were probed by mAb #71. This mAb detected three major species of PrP\textsuperscript{Sc} from dCJD and sCJD cases with graded sensitivities to non-, mono- and diglycosylated species, as previously reported (Fig. 2A, B) (Muramoto et al., 2000). In addition, the mAb detected an 11–12 kDa species of PrP fragment (fragmented PrP\textsubscript{11–12}, fPrP\textsubscript{11–12}) in samples from np-dCJD and sCJD (MM1, VM1) cases (Fig. 2B, Table 3), but the fragment was not detected in corresponding fractions from p-dCJD or sCJD (VM2, VV2) cases, or from unaffected subjects (n=8) (Fig. 2B, Table 3).

Deglycosylation did not alter the size of fPrP\textsubscript{11–12} but did intensify the signal (Fig. 3). It was apparent that the fPrP signal on the blot was derived from the non-glycosylated species and that the glycosylated species of fPrP\textsubscript{11–12} also existed and contributed to the fPrP signal but only after deglycosylation. These notions were supported by data on the location of fPrP\textsubscript{11–12} in the entire amino acid sequence of PrP (Fig. 4). Since fPrP\textsubscript{11–12} was detected by mAb #71 but not by mAbs 3F4 or 6H4, it seemed likely that it was the C-terminal fragment that has been processed resulting in the loss of the epitopes for mAbs 3F4 (residues 109–112) (Kascak et al., 1987) and 6H4 (residues 144–152). In accordance with this, fPrP\textsubscript{11–12} was recognized by #2065, another mAb against the C-terminus of PrP. Judging from the location of the epitope for these mAbs, fPrP must encompass residues 171–220. Thus, fPrP\textsubscript{11–12} probably retains the two intrinsic N-glycosylation sites (residues 181 and 197).

The absence of fPrP\textsubscript{11–12} in p-dCJD and sCJD (VM2, VV2) cases and unaffected controls was confirmed in the deglycosylated PrP\textsuperscript{Sc} samples (Fig. 3).

To determine whether other types of prion disease are associated with fPrP\textsubscript{11–12}, we analysed PrP\textsuperscript{Sc} samples from...
various types of prion disease (Table 3). The PrP Sc type determined by mAb 3F4 was type 1 in CJD with the E200K mutation (CJD E200K) and type 2 in the thalamic form of sCJD (sCJD-T) (Martin, 1975) and variant CJD (vCJD), in agreement with previous reports (Fig. 3; data not shown) (Parchi et al., 1997, 1999, 2000). In addition, we discovered that PrP Sc from CJD with the M232R mutation (CJD M232R) was of type 1 (Figs 2A and 3). fPrP11–12 was detected by mAb #71 in CJD E200K, CJD M232R and sCJD-T but not in vCJD (Fig. 3; data not shown). As was the case in dCJD and sCJD, the presence (or absence) of fPrP11–12 in the PrP Sc sample was consistent among cases affected by the same type of disease (Table 3).

To examine whether fPrP11–12 was a product of protease digestion in vitro, we analysed brain lysates from prion disease patients [six cases of sCJD MM1, three of p-dCJD, two of sCJD-T and sCJD VM2, and one each of np-dCJD, sCJD VM2, CJD E200K, CJD M232R (M/M) and vCJD] and unaffected subjects. Intriguingly, the PrP fragment of 11–12 kDa was detected by mAb #71 in brain lysates from the patients (n=11) associated with fPrP11–12, but not from the patients (n=7) or control subjects (n=8) unassociated with fPrP11–12 (Fig. 5A; Table 3). The PrP fragment in the brain lysates (fPrP-in-lysate) was indistinguishable from fPrP11–12 with respect to the immunoreactivity to anti-PrP antibodies (Fig. 5A), molecular mass (Fig. 5B) and solubility: both fPrP11–12 and fPrP-in-lysate were totally insoluble in 10% Sarkosyl.

**DISCUSSION**

In the present study, we have confirmed unique clinical features of p-dCJD that had only been suggested in previous case reports (Kimura et al., 2001; Kopp et al., 1996; Lane et al., 1994; Radbauer et al., 1998; Shimizu et al., 1999; Takahashi et al., 1997; Takashima et al., 1997). These included the absence or late occurrence of myoclonus and PSDs on EEG. In addition, the p-dCJD subjects were slower in reaching the state of akinetic mutism. We have confirmed unique types of PrP deposition in new cases of p-dCJD that had been documented previously (Kimura et al., 2001; Kopp et al., 1996; Lane et al., 1994; Radbauer et al., 1998; Shimizu
et al., 1999; Takahashi et al., 1997; Takashima et al., 1997). These included plaque-type and perineuronal-type deposits in the cerebral cortex and plaque-type deposits in the cerebellar cortex and white matter. Although the difference in the cerebellar pathology will need to be confirmed with more np-dCJD cases, the findings described above mean that dCJD should be classified into two subtypes.

Because np-dCJD and p-dCJD cases showed no differences in the ORF of PrP genes, the most plausible explanation for the origin of subtypes may be differences in the properties of prions in the contaminating dura grafts. Additional information, e.g. the type of the contaminating PrPSc or the PrP genotype of the donor, will be required to answer this question.

In searching for biochemical differences among dCJD subtypes, we found no difference in PrPSc typing by mAb 3F4: both subtypes had the PrPSc type 1 (Parchi et al., 1996). This is consistent with reports of Collinge’s PrPSc type 2 in dCJD cases (Collinge et al., 1996; Parchi et al., 1997). However, our studies with anti-PrP C-terminal antibodies revealed a protease-resistant C-terminal fragment, designated fPrP11–12, that can differentiate between the subtypes: its presence is associated with np-dCJD but not with p-dCJD. fPrP11–12 is distinct from the 3F4-recognizable PrP fragments of 7 or 11 kDa that are major components of amyloid from particular types of Gerstmann–Sträussler–Scheinker disease (GSS) (Tagliavini et al., 1991, 2001) and is also distinct from the 8 kDa PrP fragment with ragged N and C termini that was detected in proteinase K-treated brain lysate fractions from GSS with the P102L mutation (GSS P102L) (Parchi et al., 1998).

The disease-type-specific association of fPrP11–12 suggests a causal relationship with pathological processes that are intrinsic to the disease type, e.g. the structure of PrPSc. In the present study, the coincidence of the formation of PrP plaques and the absence of fPrP11–12 was observed in several types (p-dCJD, sCJD VM2, sCJD VV2 and vCJD) of diseases with the PrPSc type 2 (Table 2). This raises the possibility that the two phenomena are linked. However, this notion is challenged by the fact that the fPrP11–12-like fragment has been associated with a subtype of GSS P102L (Parchi et al., 1998). It is possible that the origin of PrP...
plaques and their relationship with pathogenesis could be heterogeneous between various prion diseases.

The relationship between fPrP11–12 and infectivity/prions remains to be determined. There are no experimental data to show that mutant PrPs with such a large N-terminal truncation as fPrP11–12 can propagate prions by themselves (Fischer et al., 1996; Shmerling et al., 1998; Supattapone et al., 2001). However, preliminary results from our transmission studies that are currently under way suggest a link between transmissibility and fPrP11–12. Brain homogenates from four CJD cases (sCJD MM1, sCJD a link between transmissibility and fPrP11–12. Brain homogenates from four CJD cases (sCJD MM1, sCJD MM1, np-dCJD and CJD M232R) with type 1 PrPSc and homogenates from four CJD cases (sCJD MM1, sCJD...show that mutant PrPs with such a large N-terminal truncation as fPrP11–12 can propagate prions by themselves (Fischer et al., 1996; Shmerling et al., 1998; Supattapone et al., 2001). However, preliminary results from our transmission studies that are currently under way suggest a link between transmissibility and fPrP11–12. Brain homogenates from four CJD cases (sCJD MM1, sCJD MM1, np-dCJD and CJD M232R) with type 1 PrPSc and fPrP11–12 were inoculated into transgenic mice expressing mouse/human chimeric PrP (Kitamoto et al., 2002; T. Kitamoto, S. Mohri & I. Miyoshi, unpublished data). The diseases were transmitted with the incubation period ranging from 140 to 180 days. In contrast, transmissions from three p-dCJD cases with type 1 PrPSc but without fPrP11–12 were unsuccessful for over 600 days after inoculation. These data imply a relationship of fPrP with the infectious properties of prions.

The relationships of fPrP with the phenotype of human prion diseases are distinct from those of other sources of phenotypic variations such as PrPSc types or PrP genotypes and support the view that the phenotypic heterogeneity of the diseases is related, at least in part, to the formation of different types of disease-specific PrP species and/or fragments thereof. The present data may contribute to the understanding of the processing of disease-specific PrP species, a novel aspect in the pathogenesis of human prion diseases.

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