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RESEARCH ARTICLE

Atypical Prion Protein Conformation in Familial Prion Disease with *PRNP* P105T Mutation

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Abstract

Protease-resistant prion protein (PrP^{Sc}) is diagnostic of prion disease, yet its detection is frequently difficult. Here, we describe a patient with a PRNP P105T mutation and typical familial prion disease. Brain PrP^{Sc} was undetectable by conventional Western blotting and barely detectable after phosphotungstate precipitation, where it displayed an atypical pattern suggestive of noncanonical conformation. Therefore, we used a novel misfolded protein assay (MPA) that detects PrP aggregates independently of their protease resistance. The MPA revealed the presence of aggregated PrP in similar amounts as in typical sporadic Creutzfeldt-Jakob disease. These findings suggest that measurements of PrP aggregation with the MPA may be potentially more sensitive than protease-based methodologies.

INTRODUCTION

Human transmissible spongiform encephalopathies (TSE), also termed prion diseases (PrD), encompass a broad spectrum of clinical and biological phenotypes ranging from focal cerebellar or thalamic involvement to panencephalopathic forms with widespread cortical degeneration. While the cause of Creutzfeldt-Jakob disease (CJD) is frequently untraceable, some cases are inherited as late-onset autosomal dominant familial prion diseases (fPrD) co-segregating with mutations in *PRNP*, the gene encoding the cellular prion protein (PrP^{C}). In most populations where this has been studied, fPrD account for 10%–15% of cases (1). The central pathogenetic event in all TSEs is the conversion of PrP^{C} into an orderly aggregated form termed PrP^{Sc}. Both the sporadic and the familial cases of prion diseases are transmissible and have given rise to variant CJD, iatrogenic CJD and Kuru.

The genetic substrate of fPrDs is variable: more than 20 missense mutations, one truncation and several octapeptide insertions of variable length have been reported (1). These mutations are distributed along the entire reading frame of *PRNP* without any obvious hotspots, suggesting that mutant PrP^{C} species may suffer from a loss-of-function, such as, eg, a failure to interact with unidentified protective factors.

Gerstmann-Sträussler-Scheinker disease (GSS) is a hereditary prion disease associated with mutation at codon 102 of *PRNP* (P102L). A neighboring *PRNP* mutation at codon 105 (P105L) has been linked to an atypical variant of GSS (2) and, recently, another mutation on the same codon resulting in a substitution of proline to serine (P105S) was reported to cause a slow-progressing, atypical form of fPrD (3). A third mutation on the same codon (P105T), was reported in a family of East Indian origin with an autosomal dominant neurodegenerative disorder, albeit limited clinical and no biochemical data were reported (4). Here, we report that the *PRNP* P105T mutation leads to fPrD with a unique PrP conformation that is dramatically different from that of other prion diseases and can go undetected by conventional immunochemical diagnostics.

SUBJECT AND METHODS

Case report of the index patient

At the age of 38 years, the index patient (III:4, Figure 2A) was admitted with a history of memory loss that had been progressive over the previous 2 years. The patient had mild cerebellar ataxia, and cognitive testing revealed deficits in all tested memory tasks (learning, recall and verbal and figural recognition). At the time of admission, cerebral magnetic resonance imaging (MRI), electroencephalography (EEG) and CSF examination (cell count, glucose, lactate, protein, isoelectric focusing) were normal and protein 14-3-3 was undetectable, as in healthy controls (5). However, 2[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) revealed hypometabolism in the left hemisphere, particularly in the temporal and parietal lobes and the thalamus, and less pronounced in the frontal lobe (Figure 1A).

Eight months after initial admission, neurological signs had progressed to a pronounced pancerebellar syndrome with saccadic dysmetria as well as ataxia of stance, gait and limb movements. In addition, myoclonic jerks were observed on arms and legs. The EEG was normal and protein 14-3-3 remained undetectable in CSF. Fluid Attenuated Inversion Recovery (FLAIR) and diffusion-weighted MRI images showed hyperintense lesions in the cortical ribbon and thalamus (Figure 1B and 1C).

In the following months, the patient deteriorated further. At the age of 41 years, he became wheelchair-bound, progressed to severe dementia with mutism and was fully dependent in all activities of daily life. At the age of 42 years, he died in a nursing home of aspiration pneumonia.

Family evaluation

A grandfather of the index patient (I:1) died at the age of 50 years with a history of alcohol abuse. The father (II:2) died at the age of 48 years after a 5-year history of progressive dementia with gait disorder. One brother (III:2) of the index patient developed progressive memory problems at the age of 45 years. At the age of 47 years, cognitive testing revealed an amnestic syndrome and frontal deficits. Neurological examination demonstrated a mild pancerebellar syndrome with impaired smooth pursuit eye movements, hypometric saccades, and slight ataxia of stance and gait. No myoclonus was observed. Over the following year, the cognitive deficits gradually progressed. Repeated EEGs revealed focal slowing over the right fronto-temporal areas but no CJD-suggestive alterations such as repetitive discharges. His condition progressively worsened and he died at the age of 48 years.

The two sisters (III:1 & III:3) of the index patient had no neurologic or psychiatric signs or symptoms. One uncle (II:2) had symptoms of depression. No neuropsychiatric symptoms were reported on the other three siblings (Figure 2A).

Sequence of the PRNP open reading frame

All living family members were contacted and offered genetic counseling. The genomic sequence of the *PRNP* gene, which is

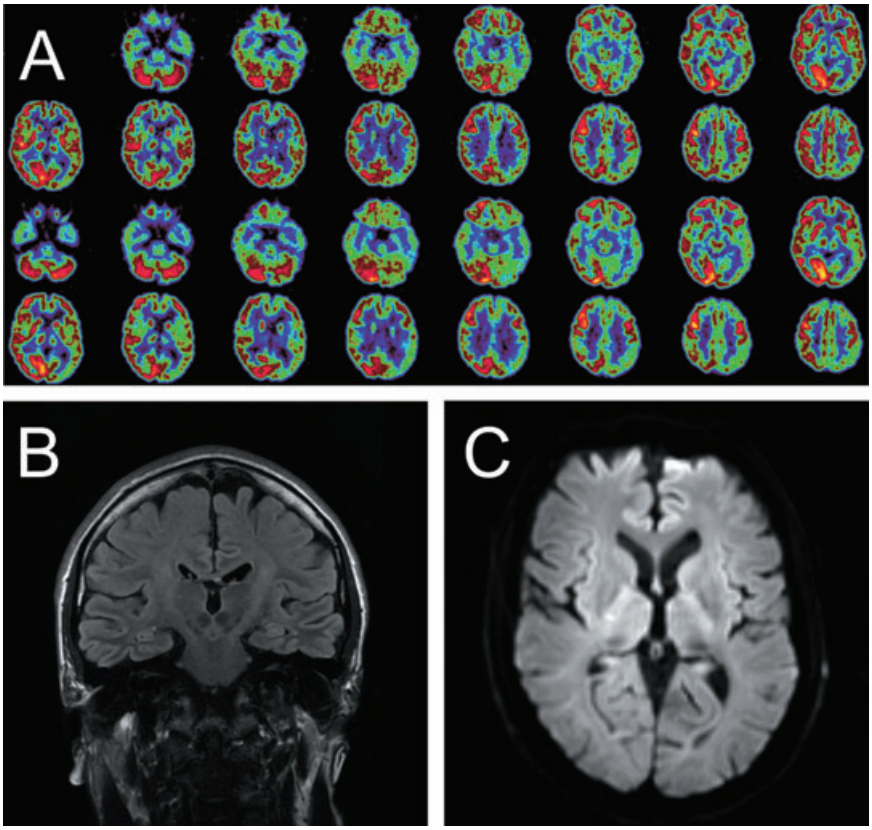

Figure 1. *Patient examination by imaging techniques.* FDG-PET of the index patient **A**. demonstrating reduced FDG uptake in the left hemisphere, particularly in the temporal and parietal lobe as well as thalamus and less pronounced in the frontal lobe. **B**. FLAIR. **C**. Diffusion-weighted MRI images showed hyperintense lesions in the cortical ribbon and thalamus.

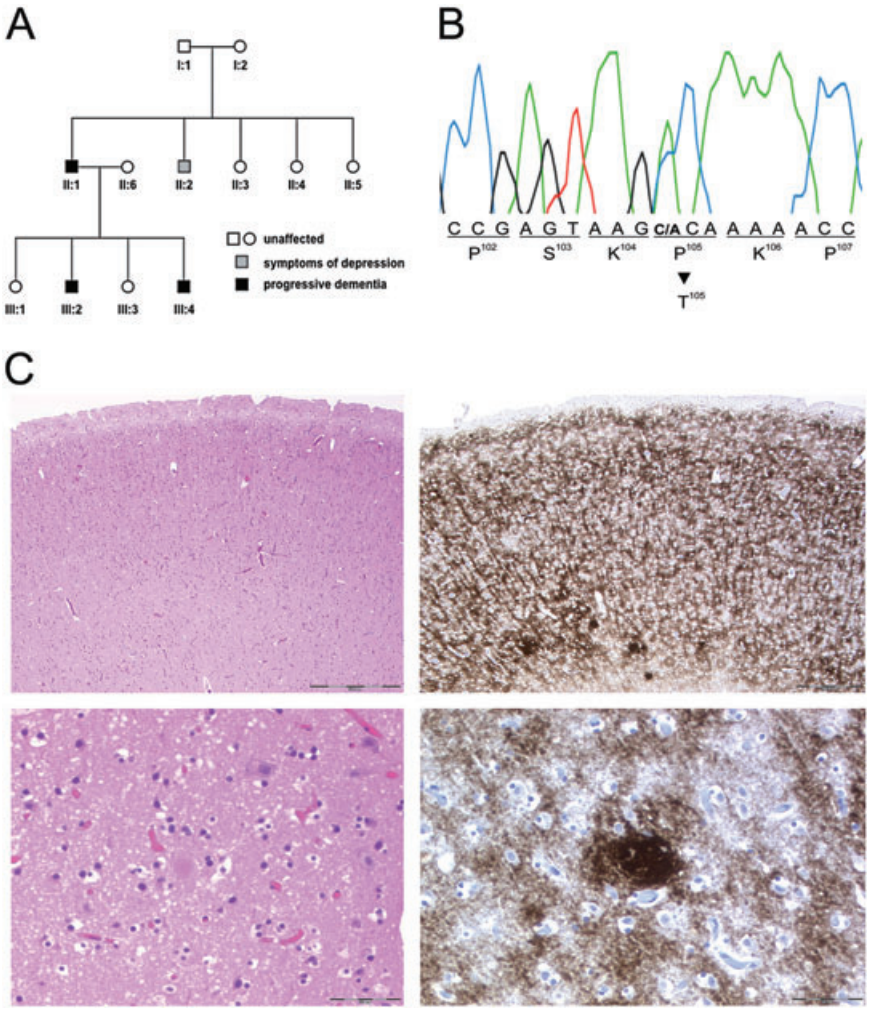

Figure 2. *Pedigree and neuropathology.* **A**. The family tree demonstrating family members with neurological disease (closed symbols) and unaffected individuals (open symbols). **B**. Sequencing analysis of the *PRNP* locus revealed a heterozygous point mutation at nucleotide position 313 [Cytosine or C to Adenine A], resulting in a change of the coding sequence at codon 105 from Proline or P to Threonine or T (P105T). **C**. Hematoxylin and eosin stained sections of cerebral cortex show spongiform change (lower panel, left side). Corresponding sections stained for PrP with antibody 3F4 depict dense synaptic PrP deposits in all cortical layers (upper panel, right side). Some unicentric PrP-plaques are detected in deeper cortical layers (higher magnification in lower panel, right side).

contained in one single coding exon, was determined for all members who, after counseling, gave their consent to the procedure. DNA was extracted using standard protocols (QIAamp, QIAGEN, Hilden, Germany). PCR amplification was done by a universal "touch down" protocol as previously described (6). Amplicons were sequenced by fluorescent dye dideoxy terminators on an ABI PRISM® (Applied Biosystems, Foster City, CA, USA).

Neuropathological Examination

Neuropathological examination was performed 48 h post-mortem at the Swiss National Reference Center for Prion diseases. All tissue specimens were obtained and processed according to established guidelines concerning safety and ethics. Tissue samples for biochemical analysis and tissue blocks for histological analysis were obtained and processed following published protocols (7). Immunohistochemical staining reactions for PrP (3F4, Signet), Glial fibrillary acidic protein (GFAP) (rabbit polyclonal, DAKO), Tau (rabbit polyclonal, DAKO), beta-Amyloid (4G8, Signet), alpha-Synuclein (KM51, Novocastra) and Ubiquitin (rabbit polyclonal, DAKO) were performed after hydrolytic autoclaving.

Biochemical analysis

All procedures were carried out in a biosafety level-3 facility with strict adherence to safety guidelines. The method of sodium phosphotungstic acid (NaPTA) precipitation was adapted from published protocols (8, 9). Briefly, we prepared 10% tissue homogenates in sterile phosphate-buffered saline (PBS) and removed cellular debris by centrifugation. Supernatants corresponding to 500 µg of total protein were adjusted to 150 µL with PBS and were then mixed with equal volumes of 4% sarkosyl in PBS and incubated for 15 minutes at 37°C. Benzonase (Benzon nuclease, Merck) and magnesium chloride were added and the mixture was incubated under constant agitation for 30 minutes at 37°C. Samples were adjusted to a final concentration of 20 µg/mL of PK and incubated at 37°C for 30 minutes. Digestion was terminated with protease inhibitors (Complete protease inhibitor cocktail, Boehringer Mannheim). Samples were adjusted to a final concentration of 0.4% NaPTA, incubated at 37°C for 30 minutes under constant agitation and centrifuged at 15 800 *g* for 30 minutes. Pellets were resuspended in 20 µL of PBS with 0.1% sarkosyl and then 10 µL of loading buffer (NuPAGE 4× lithium dodecyl sulfate sample buffer; Invitrogen) was added. Samples were subjected to standard

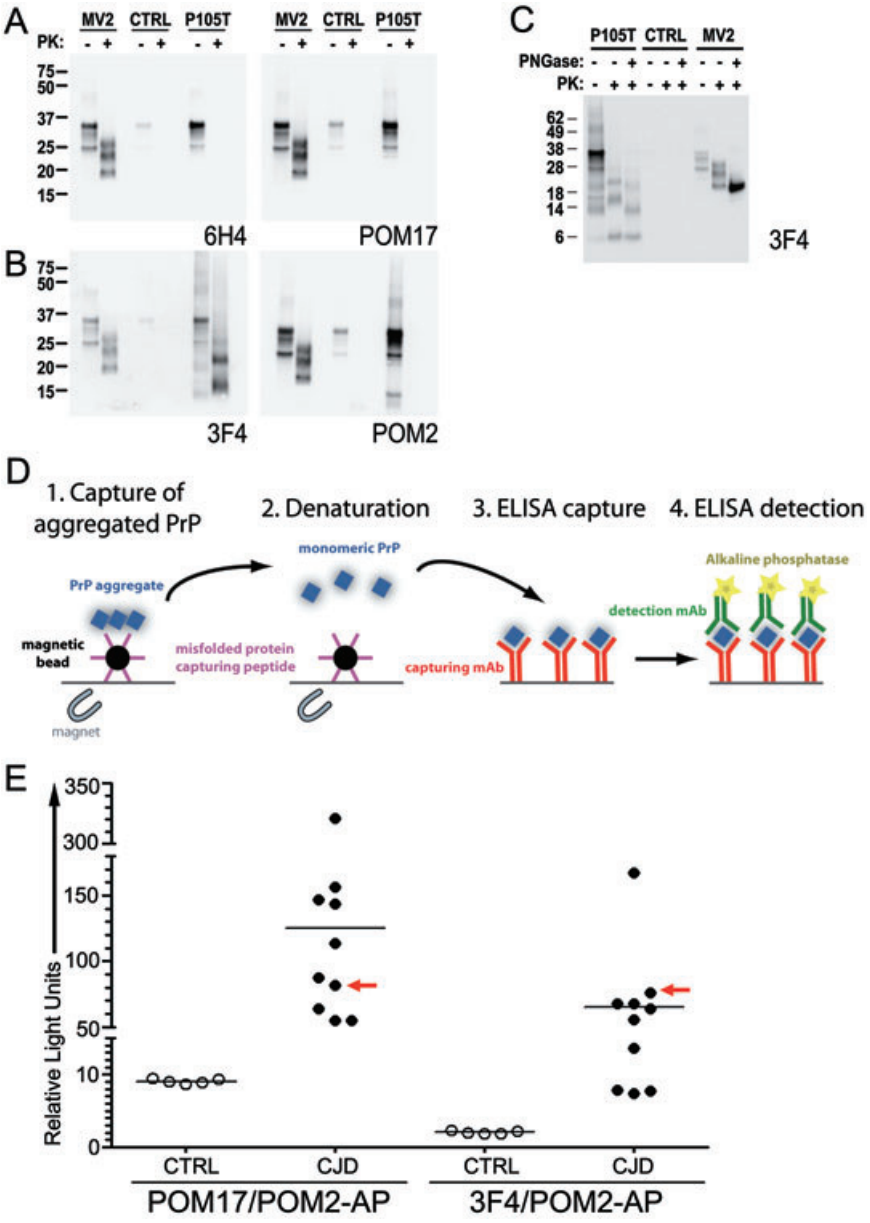

Figure 3. *Biochemical profile of PrP^{Sc}.* Brain homogenates of an MV2 sCJD patient, a non-CJD control or fPrD P105T were subjected to NaPTA precipitation with or without Proteinase K (PK) treatment as noted (A and B). **A.** Replica blots were immunoblotted with 6H4 (left blot) or POM17 (right blot). fPrD P105T seemed devoid or PK-resistant misfolded prion protein. **B.** Blots from (A) were re-blotted—without prior protein stripping—with either 3F4 (left blot, previously 6H4), or the octarepeat-specific POM2 (right blot, previously POM17). While POM2 failed to stain any PK-resistant material in the fPrD case, 3F4 revealed PK-resistant bands of atypical migration pattern. **C.** fPrD P105T, a non-CJD control or MV2 sCJD were subjected to NaPTA precipitation with or without PK and additionally with or without PNGase treatment as noted. **D.** Schematic representation of the principle of the MPA. **E.** MPA assay with two different capture antibodies for detection by sandwich ELISA (POM17 or 3F4), using 100nL/mL brain homogenates from non-CJD control samples (n = 5), sCJD cases (n = 9) and fPrD case P105T (red arrow). Both ELISA detection systems readily identify fPrD P105T.

Western blotting (WB) using monoclonal anti-PrP antibodies: POM1, POM2 or POM17 (10, 11), 6H4 (12) or 3F4 (13).

For deglycosylation, PK-digested and NaPTA-precipitated proteins were boiled in denaturation buffer according to the manufacturer's protocol and incubated with 5000 units of Peptide N-Glycosidase F (PNGase) for 2 h at 37°C (PNGase F, New England Biolabs). Samples were then subjected to WB analysis as previously described.

The misfolded protein assay (MPA) was performed using reagents and methodologies described previously (14, 15) (Figure 3D). Brain homogenate was spiked into buffer (TBSTT) and incubated with peptide-coated beads for 1 h at 37°C with shaking. Beads were washed and bound molecules were eluted, reconditioned and detected by sandwich Enzyme-linked immunosorbent assay (ELISA). After washing Lumiphos Plus substrate (Lumigen, Southfield, MI, USA) was added and results were read on a Luminoskan luminometer (Thermo Electron Corporation, Waltham, MA, USA) by using an end point assay, following the manufacturer's directions.

RESULTS

Molecular genetic analysis

Direct sequencing of the *PRNP* gene in the index patient (III:4) revealed a single heterozygous point mutation at nucleotide position 313 [Cytosine (C) to Adenine (A)], resulting in a change of the coding sequence at codon 105 from Proline (P) to Threonine (T) (P105T; Figure 2B). This mutation was also present in individual III:2 but not in individuals III:1 and III:3. The genotypes at the

polymorphic codon 129 were V/V in the two affected family members (III:2 and III:4), M/V in III:1 and M/M in III:3.

Neuropathological findings

The brain weight was 1260 g. There were no gross pathological changes. On histological examination, spongiform change and neuronal loss were found throughout the cerebral cortex. The cerebellum was only mildly affected. In addition, there was moderate astrogliosis in the cerebral cortex, as well as in the subcortical white matter (GFAP-staining, data not shown). Immunohistochemical staining with antibody 3F4 (anti-PrP) revealed PrP deposits in a dense synaptic pattern in all cortical layers (Figure 2C, upper panel), with some plaque-like deposits and unicentric PrP plaques in deeper cortical layers (Figure 2C, lower panel). Rare PrP plaques were detectable in the molecular and granular cell layer of the cerebellar cortex as well as in the cerebellar white matter. The basal ganglia showed moderate neuronal loss and PrP deposits in a dense synaptic pattern (3F4 staining). Immunohistochemical stains with antibodies against Tau, beta-Amyloid, Ubiquitin and alpha-Synuclein revealed no additional pathology. These findings confirm fPrD with an atypical deposition pattern of misfolded PrP.

Western blotting analysis

We performed Western blotting analysis of brain homogenate from the index patient (fPrD P105T) and compared it to an archival case of sCJD MV2. PK-resistant material with atypical migration pattern was detected after NaPTA (sodium phosphotungstic acid) precipitation (Figure 3A). While the PK-resistant misfolded PrP bands of fPrD P105T were readily detectable by 3F4 (Figure 3B, left blot), they were not immunoreactive with anti-PrP 6H4 and POM17 antibodies with epitopes within the first helix of PrP (Figure 3A, right and left blot, respectively). Similarly, PK-resistant misfolded PrP bands of fPrD P105T were not immunoreactive with the octarepeat-specific POM2 (Figure 3B, right blot). These data suggest that the mutant PrP aggregates acquired anomalous conformations resulting in a novel type of PK-resistant core.

We then characterized the PK-resistant bands of mutant PrP in fPrD P105T using deglycosylation by PNGase. A 6 kDa unglycosylated PK-resistant fragment was identified (Figure 3C). The two bands of higher molecular weight (~15 kDa and ~25 kDa) were probably partially glycosylated, as they appeared with faster migration after PNGase treatment. The pattern shown in Figure 3C was consistent in four independent experiments.

Misfolded protein assay

To obtain independent confirmation of the presence of aggregated, misfolded PrP in fPrD P105T we utilized a newly developed magnetic-bead-based ELISA assay for detection of misfolded PrP, designated MPA. This assay consists of a capturing step that provides selectivity for aggregated PrP and rejects the monomeric cellular form of PrP (PrP^{C}). The captured material is then eluted and measured with a standard PrP sandwich ELISA (Figure 3D). The MPA has the advantage that it does not depend on PK for specificity and thereby significantly gains sensitivity. When we performed the MPA with fPrD P105T, as well as nine sCJD and five non-CJD control samples, we found that the fPrD case was readily identified as positive among the sCJD samples, regardless of the antibody set used in sandwich ELISA (POM17/POM2-AP or 3F4/POM2-AP, Figure 3E). The MPA results, therefore, provide an independent confirmation of the presence of misfolded aggregated PrP in CNS tissue of fPrD P105T.

DISCUSSION

Here, we report a family with autosomal dominant prion disease associated with a *PRNP* mutation at codon 105 (P105T). The histopathological findings were clearly consistent with typical prion disease, and included spongiform encephalopathy with dense synaptic PrP deposits in the cerebral cortex and unicentric PrP plaques in deeper cortical layers. The cognitive and cerebellar phenotype was similar to that of a previously described family carrying the same mutation (4), yet there was no childhood onset and disease progression was slower.

Although all of the previously mentioned findings pointed to a familial prion disease, we encountered unexpected difficulties in demonstrating the presence of proteinase K-resistant prion protein (PrP^{Sc}). Indeed, biochemical analyses could reveal PK-resistant PrP only after NaPTA precipitation step, which concentrates PrP^{Sc} approximately 1000-fold. The paucity of PrP^{Sc} stands in contrast to the abundant immunohistochemical signals, and suggests that the P105T mutation may lead to atypically structured PrP aggregates. Such aggregates may conspicuously accumulate in the CNS, yet they do not appear to acquire much PK resistance.

This explanation is supported by the atypical migration pattern of PK-resistant PrP and the absence of immunoreactivity with antibodies reacting with epitopes in the first helix (6H4 and POM17) or octarepeat-region of PrP (POM2), which suggest a novel PK-resistant core of misfolded aggregated PrP in the index patient. Following deglycosylation with PNGase a small (6 kDa) unglycosylated PK-resistant fragment was identified among the PK-resistant bands. Small PK-resistant fragments of 8 kDa have been reported in GSS linked to P102L mutation (16) and a smaller (<6 kDa) fragment was detected in atypical GSS linked to P105L mutation (2). Moreover, GSS patients carrying the A117V mutation share biochemical features similar to those reported in the present case, including the 6 kDa unglycosylated PK-resistant fragment (17, 18), which was shown to reside in the detergent-insoluble fraction (18). Accordingly, we expect that the short PK-resistant fragments that we detect in the current fPrD case are likely to be insoluble. Recently, a novel subtype of CJD was proposed, showing only a 6-kDa fragment after PK digestion, albeit this 6 kDa fragment is recognized by 6H4 (19), in contrast to the one detected in the present case.

We acquired independent confirmation of the presence of misfolded PrP by the newly developed MPA assay that relies on recognition of an aggregated state of PrP in a PK-independent manner. Despite its novel PK-resistant core, misfolded aggregated PrP in this case of fPrD has repeatedly tested positive among nine sCJD samples. This suggests that PK-sensitive aggregated forms of PrP may contribute to the pathology of the present case, as has been described in GSS cases. Thus, the MPA assay offers a new, highly sensitive diagnostic tool for testing of human material for the presence of misfolded, aggregated PrP. Recently, a novel subtype of sCJD associated with an abnormal isoform of PrP that is

predominantly sensitive to protease digestion, was reported (20). Based on the present data, we fully anticipate that the MPA will diagnose such sCJD cases.

It is generally assumed that the resistance of PrP^{Sc} to PK is a consequence and, therefore, a surrogate marker of PrP aggregation. Because aggregates exclude water from their inner surfaces, they are partially impervious to proteolytic hydrolysis. Consequently, the direct measurement of PrP aggregation—eg, by MPA-based bead capture—should be theoretically superior to protease-based methodologies. The case reported earlier confirms this prediction. The MPA has, therefore, good chances to supplant the demonstration of PrP^{Sc} as the diagnostic method of choice for detecting mammalian prion diseases.

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