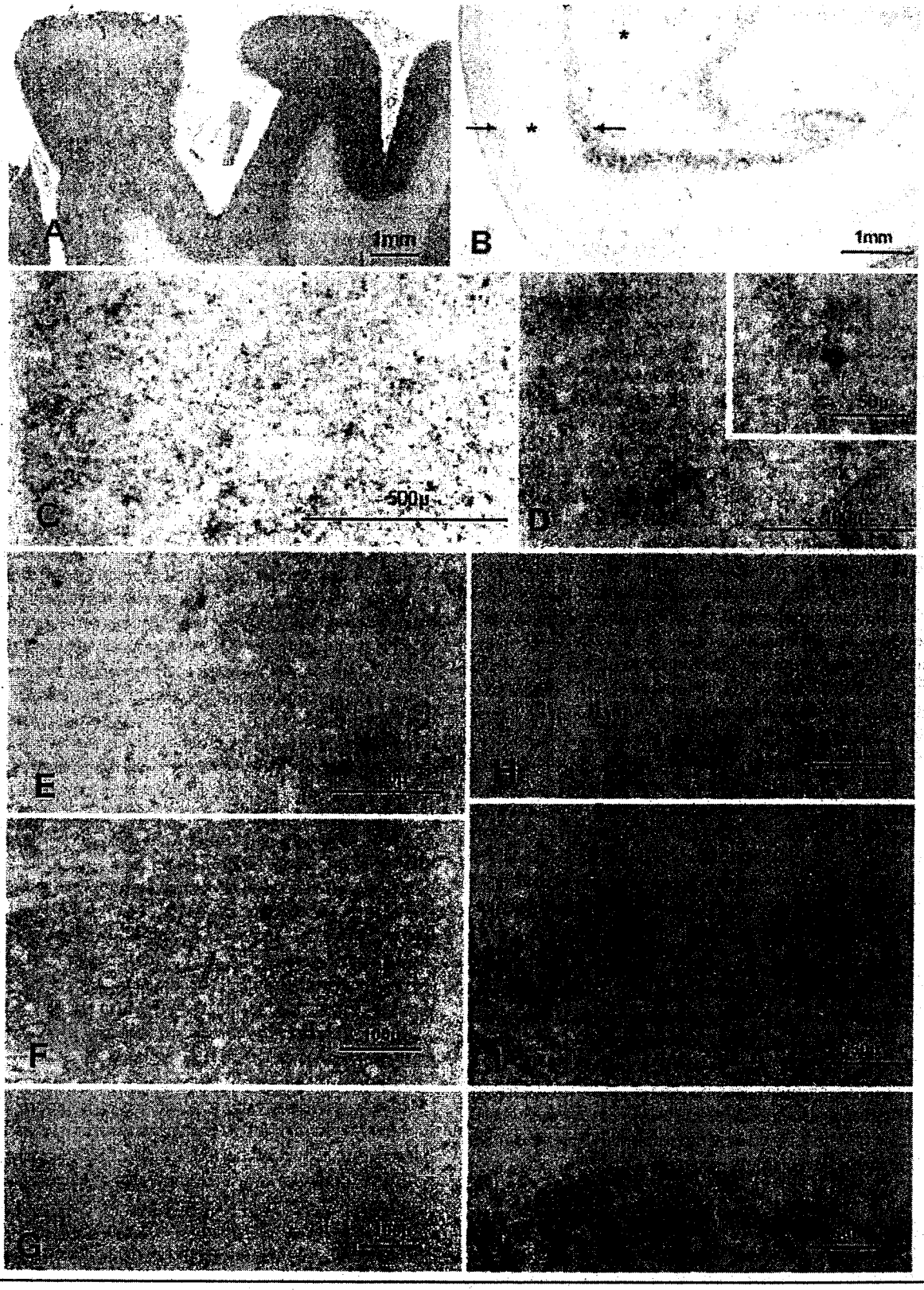

Figure 2.

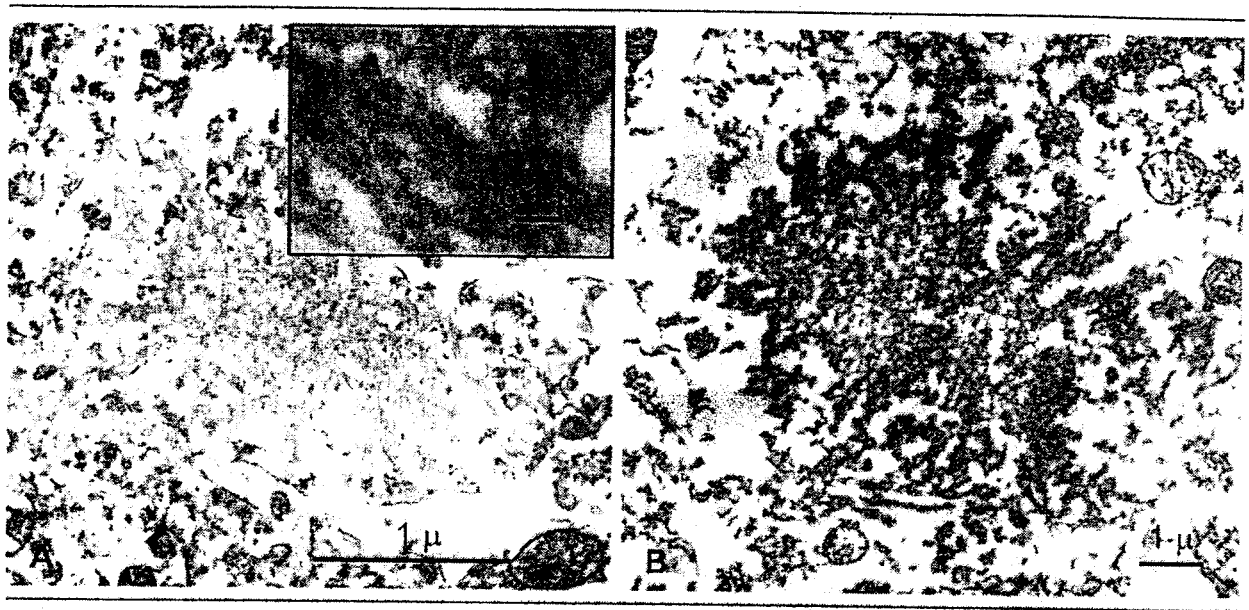

Fig 3. Electron microscopy (EM) of brain microstructures of protease-sensitive prionopathy (PSPr). (A) EM of the eosinophilic microstructures observed at light microscopy (Fig 1D) shows plaquelike formations with fuzzy filamentous appearance (inset). These structures are strongly reactive with antibodies to prion protein (PrP) (B) consistent with PrP microplaques (peroxidase anti-peroxidase with 3F4).

ments from sCJD were clearly detectable with both 3F4 and 1E4 Mab only after treatment with more than 10μg/ml PK, and increased with greater PK concentrations (see Fig 4C; also data not shown). Therefore, a small amount of PrPr detectable with Mab 3F4 is present mostly in the subcortical regions of these subjects. Moreover, most of the PrPr appears to have a different conformation from that of typical PrPr because on PK digestion it generates a unique set of fragments that are detected by 1E4 but not by 3F4.

Fig 2. Prion protein (PrP) immunohistochemistry. (A) Intense and widespread PrP immunostain of the cerebral cortex and (B) distinctive PrP immunostaining pattern in the hippocampal gyrus with staining of the molecular layers (arrows) *but not of the pyramidal cell layer or of the end plate* (asterisk). *(C, D) The cortical staining consists of coarse granules forming loose clusters with larger granules or a tighter aggregate of granules at the center; (D, inset) heavily stained globular structures are also present. (A–D) Protease-sensitive prionopathy (PSPr). (E, F) Immunostaining patterns of the cerebral cortex in sCJDVV2 (E) and sCJDVV1 (F) showing laminar staining and occasional perineuronal staining in sCJDVV2 and weak and fine widespread staining in sCJDVV1. (G) No immunostaining is detectable in the cerebral cortex of a nonprion disease control. (H–J) Cerebellar immunostaining patterns in PSPr (H), sCJDVV2 (I), and sCJDVV1 (J). There is intense and exclusive staining of large granules in the molecular layers in PSPr (H), presumably corresponding to the eosinophilic microstructures surrounded by a pale halo shown in Figure 1D; staining of irregular deposit limited to the granule cell layer in sCJDVV2 (I); no detectable staining in sCJDVV1 (the staining of the granule cell nuclei is nonspecific) (J). (A–I) Monoclonal antibody 3F4.*

Total abnormal PrP and the PrPr conformers were further characterized in abnormal PrP-enriched preparations after the capture of the abnormal PrP with g5p, a single-stranded DNA binding protein with a high affinity for abnormal PrP regardless of its PK resistance.[13,23] The amount of PrP captured by g5p in the PSPr subjects was three times greater than the amount of PrP captured in nonprion disease control subjects (data not shown), but it was nearly 16 times less than the g5p-captured PrP in typical sCJD. As measured by densitometry in arbitrary units, the mean PrP captured by g5p in eight of PSPr subjects was 3.44 ± 2.8% of the total PrP detected by direct gel loading compared with 53.55 ± 24.6% in sCJD ($n = 3$; $p = 0.00015$; Fig 5A). Furthermore, although nearly 90% of the g5p-captured PrP was resistant to PK digestion in sCJD, the PrPr accounted for only 24% of the total abnormal PrP captured in the PSPr subjects (87.59 ± 26.8% in four sCJD cases vs 24.23 ± 14.9% in nine PSPr cases; $p = 0.0001$) (see Fig 5A). The PK-resistant PrP obtained after PrP enrichment from the subjects was distributed in three major bands of approximately 26, 20, and 6kDa, which were detected by both 3F4 and 1E4, and matched the major bands of the immunoblot ladder detected with 1E4 on direct loading (see Fig 5A; also data not shown). A similar PrP banding pattern was obtained after sodium phosphotungstate precipitation, another method of abnormal PrP enrichment.[11,26] It was detected by both 3F4 and 1E4, al-

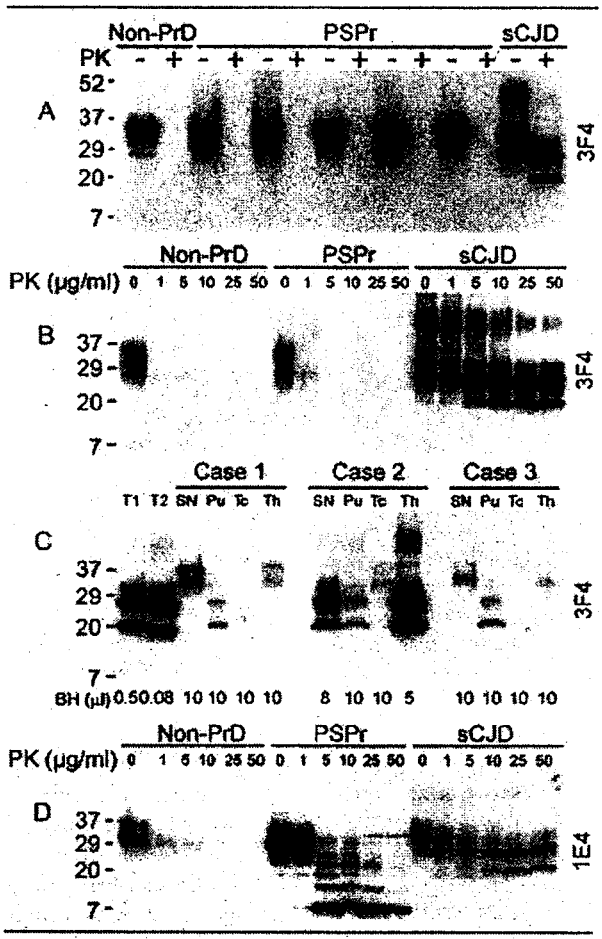

Fig 4. Characterization of prion protein (PrP) in protease-sensitive prionopathy (PSPr). (A) On conventional immunoblots, proteinase K (PK)–resistant PrP is undetectable in nonprion disease control subjects (non-PrD) and PSPr patients, although it is prominent in sporadic Creutzfeldt–Jakob disease (sCJD). (B) PK-resistant PrP from non-PrD and PSPr is not detectable even after treatment with low PK concentrations, but only in sCJD control when probed with the monoclonal antibody 3F4. (C) Subcortical regions of three PSPr cases treated with PK at 50μg/ml before Western blot analysis with 3F4 showed various amounts of PK-resistant PrP in three PSPr cases. Samples from temporal cortex (Tc) were used as controls. (D) When the same samples used in (B) are probed with 1E4, moderately PK-resistant PrP fragments forming a ladder are observed. (A, B, D) Tissues are from the frontal cortex. BH = brain homogenate; Pu = putamen; SN = substantia nigra; T1 = PrPr type 1 control; T2 = PrPr type 2 control; Th = thalamus.

though the bands were much more prominent when probed with 1E4 (see Fig 5B). The abnormal PrP enrichment experiments confirm that, in PSPr patients, there is much less abnormal PrP than in sCJD, and that the proportion of abnormal PrP that is PK resistant is much smaller.

Prion Protein Sedimentation in Sucrose Gradients

After sucrose gradient sedimentation, 30% of the total PrP from the PSPr patients was recovered in fractions 7 to 11 containing large aggregates, whereas these fractions accounted for only 5% of the total PrP in nonprion disease subjects (Figs 6A, B, E). The same fractions contained about 24 and 58% of the total PrP in GSS patients with the A117V mutation and sCJDVV1, respectively (see Figs 6C–E). Also, the percentages of PrP recovered in fractions 2 and 3 differed significantly between PSPr and nonprion disease. PSPr differed from GSS in fractions 7 and 8, and from

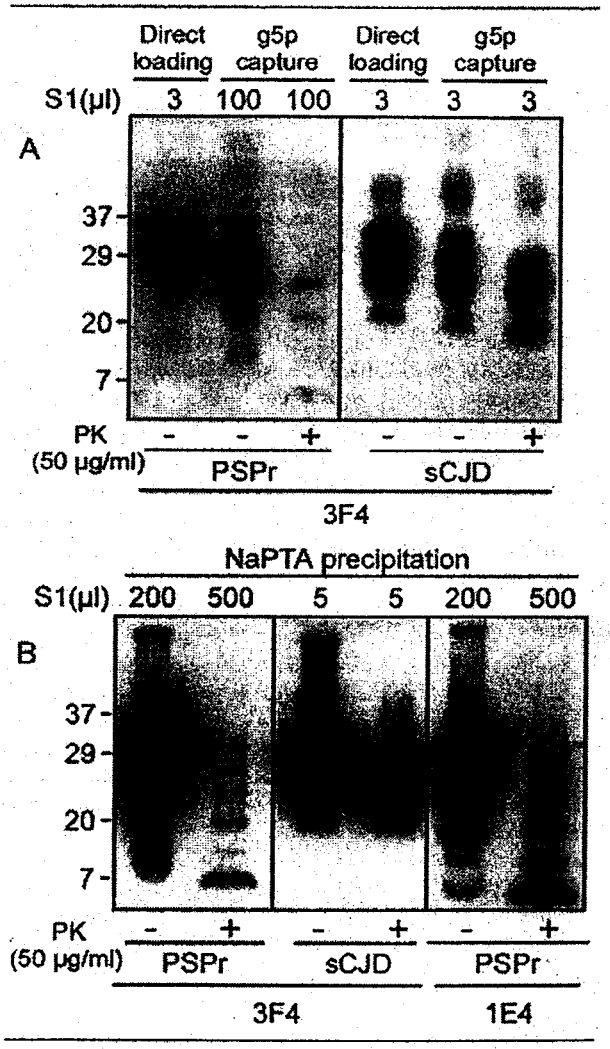

Fig 5. Capture by g5p (A) and sodium phosphotungstate (NaPTA) (B) of prion protein (PrP) from protease-sensitive prionopathy (PSPr) and sCJDMM1 (sporadic Creutzfeldt–Jakob disease). Probing with 3F4 or 1E4 after stripping. The same ladder of proteinase K (PK)–resistant PrP as in Figure 4D is detectable in PSPr preparations after heavy loading of the gel. S1 = supernatant of brain homogenate obtained after low-speed centrifugation (1,000g for 10 minutes).

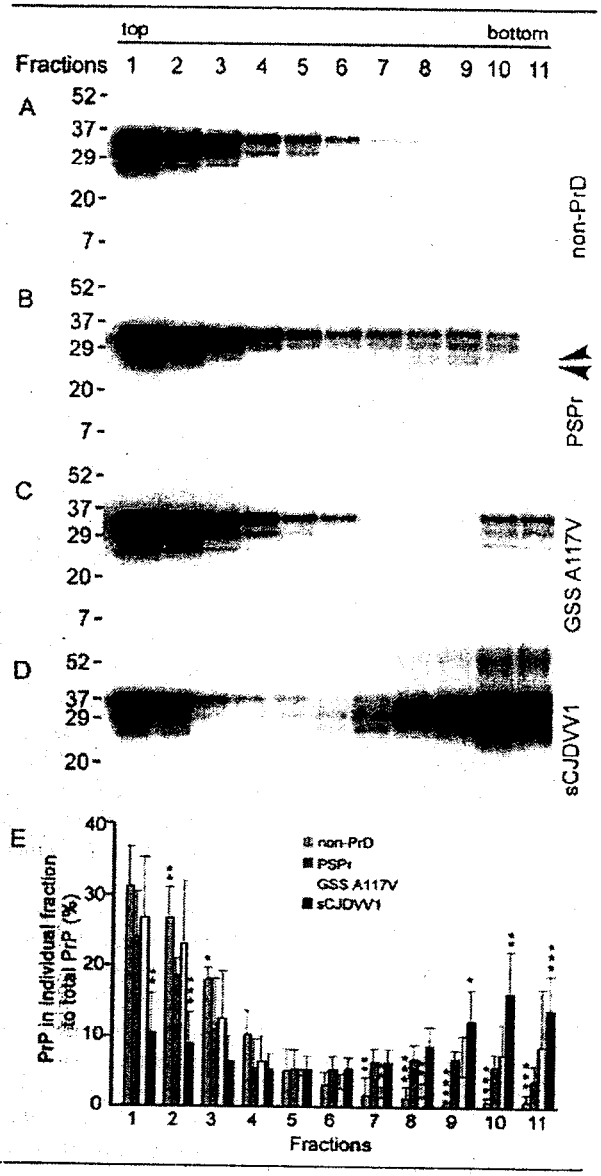

Fig 6. Prion protein (PrP) profiles in sucrose gradient sedimentation. (A) Nonprion disease (non-PrD); (B) protease-sensitive prionopathy (PSPr); (C) Gerstmann–Sträussler–Scheinker disease (GSS) with the A117V mutation (GSSA117V); (D) sCJDVV1; (E) PrP distribution in the fractions plotted as percentages of the total PrP. Although the amounts of PrP from PSPr are similar to those of non-PrD subjects in fractions 1 and 4 to 6, they differ significantly in fractions 2, 3, and 7 to 11, and also clearly differ from GSSA117V in fractions 7 and 8 and sCJDVV1 in fractions 1, 2, and 9 to 11. PSPr fractions 8 to 11 also have a distinctive low double band (B, arrowheads*) not present in the fractions from non-PrD, GSS, and sCJDVV1. n = 6 for non-PrD* (green bars); *n = 6 for PSPr* (red bars); *n = 3 for GSS* (yellow bars); *and n = 7 for sCJDVV1* (blue bars). Vertical bars *refer to standard deviations.* Asterisks *denote PrP fractions from non-PrD, GSS, and sCJDVV1 that by statistical analysis are significantly different from corresponding PSPr fractions.* $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

sCJD in fractions 1, 2, and 9 to 11 (see Fig 6E). In addition to the quantitative differences, the electrophoretic profiles of the high-molecular-weight aggregates from PSPr also differed from those of nonprion disease, GSS, and sCJDVV1 subjects: the lower band was double in PSPr but single in other conditions (see Figs 6A–D). Comparable data were obtained after gel filtration fractionation, which demonstrated that PrP aggregates exceeding 2,000kDa were more abundant in PSPr than in nonprion disease control subjects but much fewer than in sCJD (data not shown).

Discussion

We report 11 patients affected by a disease that involves abnormal PrP and has homogeneous and distinctive features (Table 2). Based on several lines of evidence, we argue that these features allow for the separation of this condition from all known forms of human prion disease. First, the abnormal PrP associated with this disease is predominantly, and in several brain regions almost exclusively, sensitive to protease or PrPs, and the PK-resistant PrP isoform or PrPr has a distinctive electrophoretic profile. The high sensitivity to PK and the distinctive electrophoretic profile of the abnormal PrP clearly distinguish these cases from each of the five subtypes of sCJD and from sporadic fatal insomnia (sFI), the known human sporadic prion diseases.[1] For example, compared with sCJDMM1, the most common and typical sCJD,[2] these cases have 16 times less total abnormal PrP, and the fraction of the total abnormal PrP that is PK resistant is nearly 4 times less. Furthermore, the ladder-like electrophoretic profile of the PrPr associated with this condition has not been observed in either sCJD or sFI, all of which instead are characterized by the presence of the well-known PrPr type 1 or 2.[1] When present, the traditional PrPr, commonly called *PrP27-30,* was located in subcortical regions and was of type 1, another combination not observed in sporadic human prion diseases.[1] Second, these cases are also homogeneous as for the PrP coding genotype because they are all homozygous for valine at codon 129 of the PrP gene, the site of a common methionine/valine polymorphism.[28] Valine homozygosity in white individuals is the rarest 129 genotype, being found only in 12% of people.[28] The sCJD subtypes associated with valine homozygosity, sCJDVV1 and sCJDVV2, have been well characterized and differ from these cases phenotypically and for the characteristics of the abnormal PrP.[1] Third, the pattern of PrP immunostaining and the presence of structures with the features of poorly formed plaques that we observed in the cerebellum are to our knowledge unprecedented. Lastly, the clinical presentation and initial course that prominently features relatively slow cognitive deterioration, occasional gait impairment, and incontinence has evoked the diagnoses of normal pressure hydrocepha-

Table 2. **Summary of Protease-Sensitive Prionopathy Common Features**

Mean Age at Onset (range), yr	Mean Duration (range), mo	Clinical Presentation	Histopathology	PrP IHC	Abnormal PrP	Family History	PrP Genetics
62 (48-71)	20 (10-60)[a]	Cognitive decline (8/11)[b] and mood/behavioral changes (7/11)[b]	Minimal spongiform degeneration with vacuoles larger than typical CJD, and minimal astrogliosis	Intense staining with distinct target pattern in cerebral gray matter; and dot pattern in cerebellar molecular layer	Minimal amount of PK-resistant PrP forming a ladder-like pattern on Western blot	Dementia (8/10)[b] Dementia with age at onset < 61 yr (2/4)[b]	Valine homozygosity at codon 129 No mutation in the PrP gene coding region

[a]One patient alive after 23-month duration and one dead 7 months from onset for other causes excluded. [b]Positive cases/total number of cases. PrP = prion protein; IHC = immunohistochemistry; CJD = Creutzfeldt-Jakob disease; PK = proteinase K.

lus, diffuse Lewy body disease, or frontotemporal dementia, whereas prion disease was suspected only at a later stage based on the relatively short duration.

Although these cases can be easily distinguished from sporadic prion diseases, some of their features such as overrepresentation of PrPs and the multiple PK-resistant PrP fragments, have been reported in GSS.[4] However, all cases of GSS reported to date are associated with a mutation in the coding region of the PrP gene or immediately adjacent to it.[4] None of these cases carried such mutation. Moreover, the ladder-like, PK-resistant, PrP fragments observed in our cases are preferentially detected with 1E4 but not with 3F4, which obviously separates these cases from GSS carrying the multiple PK-resistant PrP fragments. In a recent study, we observed that although 1E4 and 3F4 have adjacent epitopes along human PrP residues 97-112, their accessibility to these epitopes is different because of different neighboring N-terminal residues.[29] It is possible that the 1E4 selectively detected PK-resistant PrP fragments have N-terminal starting sites that are different from those of the well-characterized PrPr types 1 and 2. The earlier evidence clearly indicates that this condition differs from GSS, although the possibility that it represents the long-sought sporadic form of GSS remains to be excluded. Six of the 10 patients with obtainable pedigree had a family history of dementia that cannot be ignored, yet none carried a mutation in the PrP gene ORF. Therefore, at least in some cases, a causative mutation may be located outside the ORF of the PrP gene, a condition never observed in human prion diseases.[1]

All these considerations argue that the 11 patients were affected by a novel condition involving the PrP that cannot be classified within the spectrum of currently known human prion diseases. We suggest the designation of PSPr to emphasize a major distinctive feature (see Table 2).

Compared with other human prion diseases, PSPr is not exceedingly rare, because it accounts for about 3% of all sCJD and 16% of all valine homozygous CJD accessioned by the National Prion Disease Pathology Surveillance Center during the same time period as these 11 patients, making PSPr about as common as some of the well-known sporadic prion diseases (such as sCJDMM2, sFI, and sCJDVV1).[2] Furthermore, because the clinical presentation and the duration of PSPr often do not point to the diagnosis of prion disease, some cases of PSPr may currently be classified within the group of non-Alzheimer's dementias and not be investigated further. Should this be the case, PSPr may be more common than this study suggests.

The small amount of PrPr associated with PSPr and the finding that about 76% of the detectable abnormal PrP is PK sensitive not only hinders the diagnosis but also has implications concerning origin, pathogenicity, infectivity, and classification of PSPr.

The discovery of PrPs has opened a new chapter in prion diseases.[11–15] The demonstration that PrPs forms smaller aggregates than the PrPr counterpart,[16] and that apparently it is competent to convert PrP^C to PrPr in vitro, as well as to seed the polymerization of recombinant PrP into amyloid,[17,18] suggests that PrPs shares defining features with PrPr. However, the pathogenetic mechanisms of PrPs in the absence of PrPr and, therefore, the nature of the prion diseases associated with PrPs currently remain conjectural.

Prion diseases associated with PrPs, in the presence of minimal or no PrPr, have been modeled and studied in detail in a variety of transgenic (Tg) mouse lines carrying mouse homologues of human PrP gene mutants or overexpressing PrP^C.[12,30–33] Two Tg mouse models appear relevant to these cases.

In the first model, Tg mice expressing high levels of mouse PrP carrying the P101L mutation, the mouse equivalent of the human P102L mutation associated with a GSS phenotype,[4,34,35] spontaneously developed a neurodegenerative process characterized by SD and prion plaque formation. After inoculation, they transmitted a disease phenotypically similar to P101L-mutated Tg mice but not to wild-type mice. As in our cases, the affected mice had PrPs but no, or minimal amounts of, PrPr, indicating that PrPs can be associated with a prion disease that is under certain condi-

tions transmissible and has a histopathological phenotype displaying general features of prion diseases.[12]

In the second model, Tg mice carrying the P101L mutation were inoculated with brain homogenate from patients affected by a subtype of GSS P102L characterized by the exclusive presence of an approximately 8kDa PK-resistant fragment reminiscent of the approximately 6kDa fragment observed in small amounts in our cases. The inoculated Tg mice remained largely asymptomatic, but at histological examination, they displayed PrP plaques and had minimal amounts of PrPr.[33] They failed to transmit the disease to wild-type mice, but inoculation to P101L-mutated mice resulted in the formation of PrP plaques in the absence of clinical disease.

These mouse models and now our cases raise issues with the definition of prion diseases. Currently, it is unclear whether PSPr is transmissible because time-consuming transmissibility experiments to different lines of Tg mice and in vitro PrP replication are still ongoing. Should PSPr not be transmissible, the question is whether it is a prion disease. A similar question can be raised for GSS, of which to date only one subtype has been shown to be consistently transmissible.[4] The issue is further compounded by the recent evidence that amyloid β, the pathogenic peptide of Alzheimer's disease, has the propensity to replicate after inoculation into susceptible Tg mice in a conformation-dependent fashion reminiscent of prions.[36] These findings appear to blur the once tight association of prion diseases and transmissibility. It may be more practical to apply the label of prion diseases to all conditions in which the PrP is abnormal and appears to play a central role in the pathology, as in all prion diseases known to date and in PSPr.[37] In contrast, one might reserve the qualification of transmissible to those prion diseases that can be transmitted to recipients expressing relatively normal amounts of wild-type PrP.[36]

The finding that several PSPr patients had first-degree relatives diagnosed with dementia necessitates a search for an underlying genetic cause. In AD, the discovery of mutations outside the gene of the amyloid precursor protein (the central protein in AD, as PrP is in prion diseases) has provided a wealth of information regarding pathogenetic mechanisms of AD.[38] Similarly, the discovery of a mutation outside the PrP gene ORF capable of generating a prion disease may greatly expand our understanding of pathogenetic mechanisms and the role of PrP in prion diseases.

Supported by the NIH grants AG14359 and AG08702, Centers for Disease Control and Prevention (CCU 515004), and the Britton Fund to P.G.; NIH Grant NS049173 to C.S.; and the CJD Foundation to W.Q.Z.

Drs J. McGeehan and G. Kneale kindly provided g5p. Drs J. Hedreen, L. S. Honig, C. S. Calder, L. P. Goldstick, and W. Longstreth helped in obtaining the cases. P. Scalzo and D. Kofskey provided skillful histological and immunohistochemical preparations. B. Chakraborty assisted in the preparation of the manuscript and illustrations.

References

1. Gambetti P, Kong Q, Zou WQ, et al. Sporadic and familial CJD: classification and characterisation. Br Med Bull 2003;66: 213–239.
2. Parchi P, Giese A, Capellari S, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 1999;46:224–233.
3. Parchi P, Castellani R, Capellari S, et al. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. Ann Neurol 1996;39:767–778.
4. Kong Q, Surewicz WK, Peterson RB, et al. Inherited prion diseases. In: Prusiner SB, ed. Prion biology and disease. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2004:673–775.
5. Parchi P, Zou WQ, Wang W, et al. Genetic influence on the structural variations of the abnormal prion protein. Proc Natl Acad Sci USA 2000;97:10168–10172.
6. Parchi P, Chen SG, Brown P, et al. Different patterns of truncated prion protein fragments correlate with distinct phenotypes in P102L Gerstmann-Straussler-Scheinker disease. Proc Natl Acad Sci USA 1998;95:8322–8327.
7. Pattison IH, Millson GC. Scrapie produced experimentally in goats with special reference to the clinical syndrome. J Comp Pathol 1961;71:101–108.
8. Bruce ME, Dickinson AG. Biological evidence that scrapie agent has an independent genome. J Gen Virol 1987;68: 79–89.
9. Kimberlin RH, Cole S, Walker CA. Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. J Gen Virol 1987;68: 1875–1881.
10. Bessen RA, Marsh RF. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J Virol 1994;68:7859–7868.
11. Safar J, Wille H, Itri V, et al. Eight prion strains have PrPSc molecules with different conformations. Nat Med 1998;4: 1157–1165.
12. Tremblay P, Ball HL, Kaneko K, et al. Mutant PrPSc conformers induced by a synthetic peptide and several prion strains. J Virol 2004;78:2088–2099.
13. Zou WQ, Zheng J, Gray DM, et al. Antibody to DNA specifically detects scrapie but not normal prion protein. Proc Natl Acad Sci USA 2004;101:1380–1385.
14. Safar JG, Geschwind MD, Deering C, et al. Diagnosis of human prion disease. Proc Natl Acad Sci USA 2005;102: 3501–3506.
15. Klingeborn M, Wik L, Simonsson M, et al. Characterization of proteinase K-resistant N- and C-terminally truncated PrP in Nor98 atypical scrapie. J Gen Virol 2006;87:1751–1760.
16. Tzaban S, Friedlander G, Schonberger O, et al. Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes. Biochemistry 2002;41:12868–12875.
17. Pastrana MA, Sajnani G, Onisko B, et al. Isolation and characterization of a proteinase K-sensitive PrPSc fraction. Biochemistry 2006;45:15710–15717.
18. Colby DW, Zhang Q, Wang S, et al. Prion detection by an amyloid seeding assay. Proc Natl Acad Sci USA 2007;104: 20914–20919.

19. Gambetti P. Prion strains in human prion diseases. NeuroPrion Network of Excellence, Prion 2006 Abstracts. Available at: http://www.neuroprion.com. Accessed last on March 20, 2008.
20. Pastore M, Chin SS, Bell KL, et al. Creutzfeldt-Jakob Disease (CJD) with a mutation at codon 148 of prion protein gene: relationship with sporadic CJD. Am J Pathol 2005;167: 1729–1738.
21. Kascsak RJ, Rubenstein R, Merz PA, et al. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 1987;61:3688–3693.
22. Kong Q, Huang S, Zou WQ, et al. Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. J Neurosci 2005;25:7944–7949.
23. Yuan J, Xiao X, McGeehan J, et al. Insoluble aggregates and protease-resistant conformers of prion protein in uninfected human brains. J Biol Chem 2006;281:34848–34858.
24. Kim KS, Wisniewski HM, Wen GY. Comparison of four staining methods on the detection of Neuritic plaques. Acta Neuropathol (Berl) 1989;79:22–27.
25. Hunter E. Practical electron microscopy. New York: Cambridge University Press, 1993.
26. Wadsworth JD, Joiner S, Hill AF, et al. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet 2001;358:171–180.
27. Pan,T, Change B, Wong P, et al. An aggregation-specific enzyme-linked immunosorbent assay: detection of conformational differences between recombinant PrP protein dimers and PrPSc aggregates. J Virol 2005;79:12355–12364.
28. Collinge J, Palmer MS, Dryden AJ. Genetic predisposition to iatrogenic Creeutzfeldt-Jakob disease. Lancet 1991;337:1441–1442.
29. Yuan J, Dong Z, Guo JP, et al. Accessibility of a critical prion protein region involved in strain recognition and its implications for the early detection of prions. Cell Mol Life Sci 2008; 65:631–643.
30. Hegde RS, Mastrianni JA, Scott MR, et al. A transmembrane form of the prion protein in neurodegenerative disease. Science 1998;279:827–834.
31. Hegde RS, Tremblay P, Groth D, et al. Transmissible and genetic prion diseases share a common pathway of neurodegeneration. Nature 1999;402:822–826.
32. Westaway D, DeArmond SJ, Cayetano-Canlas J, et al. Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins. Cell 1994;76:117–129.
33. Piccardo P, Manson JC, King D, et al. Accumulation of prion protein in the brain that is not associated with transmissible disease. Proc Natl Acad Sci USA 2007;104:4712–4717.
34. Hsiao K, Baker HF, Crow TJ, et al. Linkage of a prion protein missense variant to Gertmann-Straussler syndrome. Nature 1989;338:342–345.
35. Hsiao KK, Scott M, Foster D, et al. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science 1990;250:1509–1510.
36. Meyer-Luehmann M, Coomaraswamy J, Bolmont T, et al. Exogenous induction of cerebral beta-amyloidogenisis is govered by agent and host. Science 2006;313:1781–1784.
37. Zou WQ. Transmissible spongiform encephalopathy and beyond. Available at: www.sciencemag.org/cgi/eletters/308/5727/1420. September 20, 2007.
38. Selkoe DJ, Podlisny MB. Deciphering the genetic basis of Alzheimer's disease. Annu Rev Genomics Hum Genet 2002;3: 67–99.