Classification of sporadic Creutzfeldt–Jakob disease revisited

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The sporadic form of Creutzfeldt–Jakob disease (sCJD) has been classified on the basis of the molecular mass of the protease-resistant scrapie prion protein (PrPSc), which can be type 1 or type 2, and the genotype at the methionine (M)/valine (V) polymorphic codon 129, which can be MM, MV or VV. In one classification proposed by Parchi et al, [Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, Windl O, Zerr I, Budka H, Kopp N, Piccardo P, Poser S, Rojiani A, Streichemberger N, Julien J, Vital C, Ghetti B, Gambetti P, Kretzschmar H. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 1999; 46: 224–33.] the most common subtype of sCJD, designated sCJDMM1, is viewed as a single entity. Two other classifications proposed by Collinge et al. [Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 1996; 383: 685–90.] and Zanusso et al., [Zanusso G, Farinazzo A, Fiorini M, Gelati M, Castagna A, Righetti PG, Rizzuto N, Monaco S. pH-dependent prion protein conformation in classical Creutzfeldt-Jakob disease. J Biol Chem 2001; 276: 40377–80.] respectively, subdivide sCJDMM1 into two subtypes on the basis of the different molecular mass and phenotypic characteristics, primarily disease duration. To resolve this discrepancy, we divided a group of 22 subjects with confirmed sCJDMM1 according to Parchi et al. into two sub-populations according to whether the disease duration was <5 months (short-duration subjects) or >7 months (long-duration subjects). We then examined the PrPSc molecular mass under the conditions that allowed wide variability of the pH of the PrPSc preparations as well as under stringent pH conditions, using high-resolution gel electrophoresis. We also compared the characteristics of the PrPSc associated with the short- and long-duration subjects using two-dimensional immunoblot, conformational stability immunoassay and sucrose gradient fractionation. Finally, the two sub-populations were also compared with regard to their clinical and pathological features including the lesion profiles. When sample homogenization and protease digestion were performed under stringent pH conditions, the PrPSc molecular mass did not differ between short- and long-duration sCJDMM1 subjects. The conformational characteristics of the protease-resistant PrPSc as well as the clinical and pathological phenotypes were also homogeneous except for the more severe lesions of the long-duration cases. We therefore conclude that the variability of the PrPSc molecular mass underlying the division of sCJDMM1 into two subtypes is largely due to pH variations during tissue preparation, and sCJDMM1 with short and long disease duration have similar phenotypes and PrPSc characteristics. These data indicate that the differentiation of sCJDMM1 into two subgroups is not currently justified.

Keywords: classification, disease duration, prion disease, prion protein, Sporadic Creutzfeldt-Jakob disease

Abbreviations: CSI = conformational stability immunoassay; EDTA = ethylenediamine tetra-acetic acid; GdnHCl = guanidine hydrochloride; mAb = monoclonal antibody; PBS = phosphate-buffered saline; PK = proteinase K; PrPSc = scrapie prion protein; sCJD = sporadic Creutzfeldt–Jakob disease; SDS = sodium dodecyl sulphate; TH = total homogenates

Classification of sCJD revisited

Table 1 Sporadic Creutzfeldt-Jakob disease subtypes according to Parchi and Gambetti, and Collinge.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Onset (Years)</th>
<th>Duration (Months)</th>
<th>Subtype</th>
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<td>MM1c</td>
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<td>1–18</td>
<td>1MM</td>
<td>56–79</td>
<td>1–5</td>
</tr>
<tr>
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<td>51–72</td>
<td>2.5–9</td>
<td>2 MM Long Duration</td>
<td>52–78</td>
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<td>VV1</td>
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<td>14–16</td>
<td>2MV</td>
<td>54–79</td>
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<td>9–36</td>
<td>2VV</td>
<td>41–79</td>
<td>5–9</td>
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<td>MV2</td>
<td>40–81</td>
<td>5–72</td>
<td>Not reported</td>
<td>—</td>
<td>—</td>
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<td>VV2</td>
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<td>3–18</td>
<td>3MV</td>
<td>61–77</td>
<td>7–21</td>
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<td></td>
<td></td>
<td>3VV</td>
<td>46–62</td>
<td>2–11</td>
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Immunoblot profile

<table>
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<th>Type 2</th>
<th>Relative molecular mass not reported</th>
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<tr>
<td></td>
<td></td>
<td>~21 kDa*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~19 kDa*</td>
</tr>
</tbody>
</table>

*Parchi et al., (1996, 1999); †Hill et al., 2003; ‡Share the same disease phenotype; §Refers to the PK-resistant PrPSc fragments; ¶Refers to relative molecular mass of the unglycosylated PrPSc fragment (black bars).

Introduction

Prion diseases or transmissible spongiform encephalopathies are unique among neurodegenerative diseases because they can be sporadic, inherited or acquired by infection (Prusiner and DeArmond, 1994; Gambetti et al., 2003). The form acquired by infection can be transmitted to humans from either other humans or from animals. It therefore poses a serious threat to public health. Accordingly, most developed countries have established surveillance centres to monitor and characterize all prion diseases in order to timely identify human prion diseases acquired by infection as well as animal prion diseases that may be hazardous to humans. However, identification, characterization and classification of human sporadic prion diseases have been hampered by the heterogeneity of the clinical manifestations of these diseases that was observed shortly after their original description. Clinical subtypes of sporadic Creutzfeldt–Jakob disease (sCJD) include the Heidenhain variant, with prominent visual signs (Heidenhain, 1929; Kirschbaum, 1968); myoclonic or typical CJD (Alema` and Bignami, 1959); dyskinetic CJD; thalamic (Stern, 1939; Parchi et al., 1998) and cerebellar (or ataxic) subtypes (Brownell and Oppenheimer, 1965; Jones et al., 1985; Grant et al., 1993). However, the discovery and characterization of the normal cellular prion protein (PrPc) and its abnormal isoform [(scrapie prion protein (PrPSc)] associated with prion diseases, along with the cloning of the PrP gene (PRNP), have now allowed the classification of CJD subtypes on the basis of molecular features rather than clinical or histopathological characteristics. Two predominant forms of PrPSc associated with human prion diseases, which probably reflect two distinct PrPSc conformations, were originally reported by Monari et al. (1994). These two types can be distinguished by their N-termini and by the gel mobility of the PrPSc fragment resistant to digestion with the proteolytic enzyme proteinase K (PK-resistant PrPSc). The PK-resistant PrPSc type 1 has its dominant N-terminus at residue 82 and a gel mobility of the unglycosylated isoform of ~21 kDa, whereas PrPSc type 2 has its dominant N-terminus at residue 97 and an unglycosylated molecular mass of ~19 kDa (Parchi et al., 2000). It has been proposed that sCJD phenotypes are determined by the interaction of these PrPSc types with the host PRNP genotype as determined by the methionine (M)/valine (V) polymorphism at codon 129 (Gambetti et al., 2003). In studies involving over 300 subjects, we proposed a classification of sCJD (here identified as Parchi and Gambetti) that identified six molecular subtypes on the basis of the PRNP codon 129 polymorphism and the PrPSc type (Parchi et al., 1996; 1999). These molecular subtypes matched five distinct phenotypes encompassing those recognized by the aforementioned clinical classification schemes (Table 1) (Heidenhain, 1929; Stern, 1939; Alemà and Bignami, 1959; Brownell and Oppenheimer, 1965). sCJDMM1 and sCJDMMV1 molecular types corresponded with the typical myoclonic sCJD phenotype and the Heidenhain variant, and were by far the most prevalent; on the other hand, sCJDMV2 and sCJDVV2 molecular types, which differed from each other in disease duration and histopathological characteristics, corresponded with the cerebellar/ataxic phenotype, but neither the phenotype of sCJDVV1 characterized by early onset nor that of sCJDMM2 characterized by spongiform degeneration with large vacuoles had previously been described (Stern, 1939; Parchi et al., 1998, 1999). This classification has been practically replicated in a large United Kingdom cohort (Head et al., 2004).

An alternative sCJD classification, proposed by Collinge and co-workers. (here identified as Collinge), utilizes the same molecular features as the Parchi and Gambetti classification (Parchi et al., 1996, 1999), but it is based on three rather than two PrPSc types distinguished by gel mobility (Table 1) (Collinge et al., 1996; Wadsworth et al., 1999;
Hill et al., 2003, 2006). The molecular mass of PrPSc identified as type 1 in the Collinge classification appears to be ~0.5 kDa higher than that of type 1 of Parchi and Gambetti, whereas types 2 and 3 of Collinge appear to match Parchi and Gambetti types 1 and 2, respectively. With the exception of one subject recently reported to be MV heterozygous at codon 129, all the Collinge PrPSc type 1 subjects were MM homozygous at codon 129, and, together with their sCJDMM2, represented their most prevalent sCJD subtype (Collinge et al., 1996; Wadsworth et al., 1999; Hill et al., 2003). It is likely that these two subtypes correspond to the sCJDMM1 subtype of Parchi et al. (1996, 1999). An important feature that distinguishes Collinge PrPSc type 1 and type 2 was that treatment with the metal-ion chelator EDTA resulted in faster gel migration of both Collinge PrPSc types 1 and 2, but the shift was greater in type 1 than in type 2, so that after EDTA treatment the two types co-migrated (Wadsworth et al., 1999; Hill et al., 2003, 2006). These findings imply that the removal of metal ions changes the conformation of both PrPSc types, thereby exposing new proteolytic sites (Wadsworth et al., 1999)—a result that strongly supports the argument that types 1 and 2 are indeed conformationally distinct isoforms of PrPSc. Furthermore, Collinge et al. (1996) consistently reported that their sCJDMM1 was linked to significantly shorter disease duration than their sCJDMM2, although other phenotypic differences were apparently less striking (Hill et al., 2003; Lewis et al., 2005).

A classification of the sCJD similar to that of Collinge, but based on different PrPSc features, has also been proposed by Zanusso et al. (here referred to as Zanusso) (Zanusso et al., 2001). These authors observed that one sub-population of sCJDMM1 subjects (according to Parchi and Gambetti classification) showed pH-dependent PrPSc gel migration, whereas the other sCJDMM1 subjects were insensitive to pH changes, although at neutral pH the gel mobility of the PrPSc was the same in all subjects of both groups (Zanusso et al., 2001). Moreover, the pH-sensitive and pH-insensitive sCJDMM1 subjects had distinct clinical manifestations: the pH-sensitive sCJDMM1 subgroup had a shorter disease duration than that of the pH-insensitive sCJDMM1 subgroup. In sum, the classifications proposed by both Collinge and Zanusso are based on the notion that the sCJDMM1 subtype identified by Parchi and Gambetti is heterogeneous and that it includes two distinct PrPSc isoforms and two phenotypes. The lack of a uniform classification of sCJD has generated confusion, and this impedes the identification and characterization not only of sCJD subtypes but also potentially of forms acquired by infections, as underscored by the finding that the sCJDMM2 subtype of Collinge (but not his sCJDMM1) might also be acquired from bovine spongiform encephalopathy (Asante et al., 2002).

Variability in the gel migration of PrPSc type 1 associated with sCJDMM was recognized previously (Parchi et al., 1996, 1999). However, it was not consistently reproducible and did not correlate with distinct histopathological characteristics. This apparent lack of reproducibility and correlation raised the possibility that varying experimental conditions influenced the gel migration of the PrPSc. More recently, Notari et al. (2004) have shown that the pH of the brain homogenates is variable when the homogenates are prepared in standard buffers, which lack adequate buffering capacity. In turn, the pH of the homogenate affects the gel migration of the PK-resistant PrPSc causing shifts in gel mobility and increasing the number of the bands (Notari et al., 2004). Moreover, according to Notari et al. (2004), the change in pH that accompanied the addition of EDTA, rather than the EDTA per se, appeared to correlate with the gel migration shifts of PrPSc types 1 and 2 of Collinge as reported by Wadsworth et al. (1999).

In an attempt to resolve the discrepancy in sCJDMM1 classification, we have divided the Parchi and Gambetti sCJDMM1 into two groups on the basis of disease duration, the cardinal feature distinguishing sCJDMM1 and sCJDMM2 in the classifications of Collinge and Zanusso. We characterized the PrPSc associated with the two groups (i) by determining PrPSc gel mobility under experimental conditions allowing variability in the pH; and (ii) under more stringent pH conditions; and finally (iii) by performing advanced chemical–physical analyses. The disease phenotype in the two groups, as determined by the clinical and histopathological features, was also comparatively examined.

Material and methods

Reagents and antibodies

Urea, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulphonic acid (CHAPS), DL-dithiothreitol (DTT), iodoacetamide (IAA), tributylphosphine (TBP), ampholine, pH 3–10, immobilized pH gradient (IPG) strips (pH 3–10, 11 cm long) were purchased from Bio-Rad Laboratories (Richmond, CA, USA) and EDTA from Fisher Biotech (Fair Lawn, NJ, USA). Reagents for enhanced chemiluminescence (ECL plus) came from Amersham Biosciences (Piscataway, NJ, USA). The mouse monoclonal antibody (mAb) 3F4 from Signet Laboratories (Dedham, MA, USA) was used to recognize human PrP residues 109–112 (Kascak et al., 1987), 8H4 mAb immunoreacted with human PrP residues 175–185 (Zanusso et al., 1998) and rabbit anti-C-terminal antiserum was immunoreactive to human PrP residues 220–231 (Chen et al., 1995). All other chemicals, including PK, phenylmethylsulphonyl fluoride (PMSF) and guanidine hydrochloride (GdnHCl) were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless specified otherwise.

Subject selection

Subjects with diagnoses of sporadic CJDMM1 according to Parchi et al. (1999) were selected. Their diagnoses were established by histological examination, immunohistochemical staining, immunoblot analysis and PRNP sequencing. All patients lacked pathogenic mutations in PRNP, and there was no familial history of CJD nor known exposure to prions from contaminated food or medical procedures. Subjects were divided into two populations according to whether the disease duration was ≤5 months or >7 months. The first group (short duration) included 12 subjects and the second (long duration), 10 subjects. The longest survival in the second group was 36 months. Duration of disease was measured from the first occurrence of prion-related clinical symptoms and/or signs to death. Clinical...
history and laboratory studies were collected for analysis. When available, both EEG recordings and MRI were also examined.

**Tissue samples**

Frozen brain tissues, obtained at the time of autopsy, were stored at −80°C. Samples were taken from frontal cortex (FC) for western blotting. Tissues were also fixed in formalin, treated with formic acid and used for histopathological and PrP immunohistochemical analyses.

**Molecular genetics**

DNA was extracted from frozen brain tissue, and genotypic analysis of PRNP coding region was performed as described (Parchi et al., 1996, 2000).

**Histopathology and PrP immunohistochemistry**

Semi-quantitative evaluation of spongiosis and gliosis was performed by comparing haematoxylin and eosin-stained sections from all subjects. Lesion profiles of the two populations were carried out as described previously (Pastore et al., 2005). Spongiform degeneration was scored on a 0–4 scale (non-detectable, mild, moderate, severe and status spongiosus), astrogliosis was scored on a 0–3 scale (non-detectable, mild, moderate and severe) and the values for each brain region were averaged. Ten brain areas were examined: FC, parietal cortex (PC), temporal cortex (TC), occipital cortex (OC), hippocampus (HI), basal ganglia (BG), thalamus midbrain (MB), medulla (ME) and cerebellum (CE).

Immunohistochemistry for PrP was carried out on deparaffinized and rehydrated sections immersed in 98% formic acid for 1 h at room temperature (Parchi et al., 1996). Endogenous peroxidase was blocked by immersion in 8% hydrogen peroxide in methanol for 10 min. Sections were completely immersed in 1.5 mM hydrochloric acid and microwave for 10 min. After rinsing, sections were incubated with the mAb 3F4 at 1 : 600, washed and incubated with secondary antibody (goat anti-mouse, Cappel, 1 : 50) followed by incubation with mouse PAP complex (Sternberger; Meyer Immunocytochemicals Inc., Jarrettsville, MD, USA; 1 : 250). Diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivity.

**Preparation of brain homogenates and detergent-insoluble fraction**

Brain tissue homogenates (10% w/v) from sample of FC were prepared in either lysis buffer ‘standard’ (100 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.4) or lysis buffer ‘plus’ (100 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 100 mM Tris, pH 8.0) (Notari et al., 2004). Various brain preparations were used for designated experiments as shown in Fig. 1. The detergent-insoluble fraction (P2) was prepared as described (Zou et al., 2003). In brief, supernatant (S1) from 1000 g centrifugation was subjected to further ultracentrifugation at 100,000 g for 1 h at 4°C (Fig. 1). The new supernatant (S2, detergent-insoluble fraction) was recovered and stored at −80°C. The pellet (P2) was resuspended in lysis buffer. To detect the effect of EDTA on cleavage site of PK in the protein, a 250 mM EDTA stock solution was prepared in ddH2O. Samples were incubated with EDTA, at a final concentration of 25 mM for 10 min at room temperature before PK-digestion (Wadsworth et al., 1999). For PK-digestion of PrP, brain homogenates prepared either in lysis buffer ‘standard’ or lysis buffer ‘plus’ were incubated with 100 μg/ml of PK for 1 h at 37°C. The digestion was stopped by adding PMSF at a final concentration of 3 mM. For deglycosylation of PrP, the proteins denatured by boiling samples in denaturing buffer were incubated with peptide N-glycosidase F (PNGase F) following the product instructions (New England Biolabs Inc., Beverly, MA, USA).

**Western blot**

Samples untreated or treated with PK were boiled in an equal volume of 2x sodium dodecyl sulphate (SDS) sample buffer (6% SDS, 5% β-mercaptoethanol, 20% glycerol, 4 mM EDTA, 125 mM Tris–HCl, pH 6.8) for 10 min. For a set of experiments, samples were deglycosylated with PNGase F as described elsewhere (Monari et al., 1994). Four different types of gels were used for protein separation: 15% or 8–16% Criterion pre-cast Tris–HCl gels 13.3 × 8.7 cm (Bio-Rad), 15% Tris–glycine home-made gels 15 × 15 cm and 8–16% Tris–HCl Protein II xi Ready gel Precast gels 16 × 16 cm (Bio-Rad). Proteins were transferred from gels to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore) at 70 V for 2 h at room temperature and then the membranes were incubated with blocking buffer (5% non-fat milk in Tris-buffered saline Tween-20 (TBS-T)) for 1 h. The membranes were incubated with 3F4 (1 : 40 000) or anti-C, or donkey anti-rabbit at room temperature for 2 h, followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse antibody (1 : 3000) for 1 h. The PrP bands or spots were visualized on Kodak films by the ECL Plus as described by the manufacturer. Immunoblot signals captured with Epi Chemiluminescence were analysed by Image Acquisition and Analysis software. The software has the capability of automatically calculating the mid-point of the PrP band corresponding to the unglycosylated PK-resistant PrP core fragment after introducing the molecular weight values for the standards. Values were statistically analysed by the MATLAB 7.0 software.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was performed as described by the supplier using the Bio-Rad Protein IEF Cell with minor modification (Zou et al., 2003; Pastore et al., 2005). Samples dena-
tured by boiling in SDS sample buffer were incubated with reducing buffer (8 M urea, 2% CHAPS, 5 mM TBP, 20 mM Tris, pH 8.0) for 1 h at room temperature and then incubated with 200 mM IAA for 1 h. Proteins were precipitated with a 5-fold volume of pre-chilled methanol at −20°C for 2 h and centrifuged at 16 000 g for 30 min at 4°C. The pellets were resuspended in 200 µl of rehydration buffer (7 M urea, 2 M thiourea, 1% DTT, 1% CHAPS, 1% Triton X-100, 1% ampholine, pH 3–10, trace amounts of bromophenol blue). The pellets were dissolved in rehydration buffer and subsequently incubated with the IPG strips for 14 h at room temperature, providing gentle shaking. The dehydrated gel strips were transferred onto a focusing tray and focused for ~40 kVh. The focused IPG strips were equilibrated for 15 min in equilibration buffer 1 (6 M urea, 2% SDS, 20% glycerol, 130 mM DTT, 375 mM Tris–HCl, pH 8.8), and then another 15 min in equilibration buffer 2 (6 M urea, 2% SDS, 20% glycerol, 135 mM IAA, 375 mM Tris–HCl, pH 8.8). The equilibrated strips were loaded onto 8–16% Tris–HCl Criterion gels (Bio-Rad). Western blotting was performed as described above.

Conformational stability immunoassay
Aliquots of 10 µl of S1 (10 mg/ml of total proteins) were mixed with 20 µl of GdnHCl stock solution to a final concentration of GdnHCl ranging from 0 to 3 M. After 1 h of incubation at room temperature, samples were diluted with phosphate-buffered saline (PBS) to a final concentration of 0.2 M. After PK treatment, samples were diluted with phosphate-buffered saline (PBS) to a final concentration of 0.05% Tween-20 in 1 M PBS and incubated with 8H4 mAb for 3 h at room temperature (Tzaban et al., 2002; Pan et al., 2005). Eleven fractions were collected from the top of the tube atop a 10–60% step sucrose gradient and centrifuged at 200 000 g for 1 h. Proteins were precipitated with a 5-fold volume of pre-chilled methanol at −20°C for 2 h and centrifuged at 16 000 g for 20 min at 4°C. The pellets were resuspended in 200 µl of rehydration buffer (7 M urea, 2 M thiourea, 1% DTT, 1% CHAPS, 1% Triton X-100, 1% ampholine, pH 3–10, trace amounts of bromophenol blue). The pellets were dissolved in rehydration buffer and subsequently incubated with the IPG strips for 14 h at room temperature, providing gentle shaking. The dehydrated gel strips were transferred onto a focusing tray and focused for ~40 kVh. The focused IPG strips were equilibrated for 15 min in equilibration buffer 1 (6 M urea, 2% SDS, 20% glycerol, 130 mM DTT, 375 mM Tris–HCl, pH 8.8), and then another 15 min in equilibration buffer 2 (6 M urea, 2% SDS, 20% glycerol, 135 mM IAA, 375 mM Tris–HCl, pH 8.8). The equilibrated strips were loaded onto 8–16% Tris–HCl Criterion gels (Bio-Rad). Western blotting was performed as described above.

Velocity sedimentation in sucrose step gradients
The S1 fractions prepared by centrifugation of 20% brain homogenates at 1000 g for 10 min at 4°C were incubated with an equal volume of 2% Sarkosyl for 30 min on ice. The samples were loaded atop a 10–60% step sucrose gradient and centrifuged at 200 000 g in SW55 rotors for 1 h at 4°C (Beckman Coulter, Fullerton, CA, USA) as described with minor modification (Tzaban et al., 2002; Pan et al., 2005). Eleven fractions were collected from the top of the tube for western blot analysis of PrP.

Results
Clinical characteristics
Mean disease durations in the sCJDMM1 short- and long-duration groups were 2.02 ± 0.90 and 14.80 ± 9.31 months (P < 0.01). Patients with long disease duration were slightly but not significantly younger than those with short duration (mean age at onset: 63.6 ± 10.7 versus 72.2 ± 8.9 years). None of the clinical features, including EEG and MRI findings, was predictive of disease duration (Table 2). Dementia, ataxia and periodic sharp waves were more frequent in the short-duration subjects, while focal motor weakness was more common in the patients with long duration, but again the differences were not statistically significant.

Table 2 Clinical findings

<table>
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<th>Long duration</th>
<th>Statistical test</th>
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<td>Age at onset (years)</td>
<td>72.2 ± 8.9</td>
<td>63.6 ± 10.7</td>
<td>NS</td>
</tr>
<tr>
<td>Duration (months)</td>
<td>2.02 ± 0.9</td>
<td>14.8 ± 9.3</td>
<td>P &lt; 0.01</td>
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<tr>
<td>Range</td>
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<td>(7–36)</td>
<td></td>
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<tr>
<td>Dementia</td>
<td>7/11 (64%)</td>
<td>3/6 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ataxia</td>
<td>7/10 (70%)</td>
<td>3/6 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Visual</td>
<td>3/10 (30%)</td>
<td>2/6 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Myoclonus</td>
<td>3/11 (27%)</td>
<td>2/6 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Seizures</td>
<td>2/10 (20%)</td>
<td>1/6 (17%)</td>
<td>NS</td>
</tr>
<tr>
<td>Psychosis</td>
<td>2/10 (20%)</td>
<td>1/6 (17%)</td>
<td>NS</td>
</tr>
<tr>
<td>Focal weakness</td>
<td>1/10 (10%)</td>
<td>2/7 (29%)</td>
<td>NS</td>
</tr>
<tr>
<td>PSWC on EEG</td>
<td>4/7 (57%)</td>
<td>2/5 (40%)</td>
<td>NS</td>
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<td>Positive MRI*</td>
<td>1/10 (10%)</td>
<td>1/5 (20%)</td>
<td>NS</td>
</tr>
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</table>

*aCases with signs/cases examined. There are some variations in the number of cases examined owing to missing clinical data; +Student’s t-test used for analysis of age at onset and duration and Fisher’s t-test used for comparison of frequency of signed between short- and long-duration cases; a as determined with diffusion-weighted MR images.

**Fig. 2** Lesion profiles in long- and short-duration sCJDMM1 subjects. The severity of the spongiform degeneration and astrogliosis was plotted as a function of the brain region. Spongiform degeneration was scored on a 0–4 scale (not detectable, mild, moderate, severe, status spongiosus) and astrogliosis was scored on a 0–3 scale (not detectable, mild, moderate and severe). Individual data points are the mean ± SD of the mean of both lesion scores from 12 short-duration (diamond, pink line) and 8 long-duration (squares, blue line) subjects. Brain regions analysed were frontal cortex (FC), temporal cortex (TC), parietal cortex (PC), occipital cortex (OC), hippocampus (HI), basal ganglia (BG), thalamus (TH), midbrain (MB), medulla (ME) and cerebellum (CE).

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Lesion profiles were similar in both long- and short-duration groups, but as expected, the lesions were significantly more severe in long-duration subjects (mean severity score: 2.16 ± 1.07) than in those with short duration (1.05 ± 0.54) (P < 0.01). In both groups, the cerebral cortices, basal ganglia and thalami were most severely affected, while the hippocampi and brainstem regions were the least affected (Fig. 2). In general, 'status spongiosus', or end-stage neurodegeneration, was encountered only in long-duration subjects. Similarly, moderate (2+) or higher reactive astrogliosis was observed only in long-duration subjects. White matter degenerative changes, including Wallerian degeneration of the corticospinal tracts in the brainstem, were present in two long-duration subjects, but in none of the short-duration subjects. Moreover, marked loss of granular neurons of the cerebellar cortex, with relative sparing of Purkinje cells, was a feature of two long-duration cases but was not present in short-duration subjects. By immunohistochemistry, both groups showed diffuse, fine immunoreactivity that tended to parallel the brain regions affected by spongiosis and astrogliosis, while only the long-duration subjects accumulated deposits with a coarse morphology, presumably reflecting increased PrPsc accumulation (Fig. 2). White matter PrPsc deposits were noted by immunohistochemistry in two long-duration subjects but were not present in any of the short-duration sCJDMM1 patients.

Variations of brain homogenate pH and gel mobility of PK-resistant PrPsc

We observed significant variability in the pH of the total homogenates (TH) when the lysis buffer ‘standard’ was used (Notari et al., 2004). The pH of the TH varied up to 0.72 units with a range spanning from 6.73 to 7.45 in the whole sCJDMM1 subject population. The pH variation in the subjects with short duration was less than that in subjects with long duration (range: 6.85–7.10 in short duration versus 6.73–7.45 in long duration) but the mean pH was similar in the two groups (7.01 ± 0.09 in short versus 7.04 ± 0.24 in long). As expected, the pH heterogeneity of the TH was associated with the variability in gel mobility of the PK-resistant PrPsc bands (Fig. 3A). Using image analysis software that automatically calculates the mid-point of the bands, we observed that the difference in mobility between the fastest and the lowest migrating bands, corresponding to the PK-treated unglycosylated PrPsc isofrom, was 0.75 kDa in long- and short-duration subjects combined. The variability similarly affected both groups (range: 20.95–21.65 kDa in long versus 20.90–21.65 kDa in short), and the means of
the gel mobility were also similar (21.27 ± 0.23 kDa in long versus 21.20 ± 0.18 kDa in short) (Table 3). No significant variation in mobility related to disease duration was detected even when, using the opposite approach, three subgroups were arbitrarily formed according to whether the mobility of the unglycosylated PK-resistant PrPSc fragment was 21.00 kDa or less (fastest migration subjects with mean 20.95 kDa), 21.13 kDa (slowest migration subjects with mean 21.51 kDa) or between these molecular mass values (Fig. 3A), and the disease durations in these three groups were compared. The first two groups, separated by a mean shift of the gel mobility of ~0.56 kDa (comparable with that separating Collinge and Zanussi sCJDMM1 and sCJDMM2 subjects), included both long- and short-duration cases. The long-duration subjects appeared to be slightly better represented in the slowest migrating group (mean molecular mass of 21.51 kDa) indicating a shift opposite to that observed by Collinge et al. (1996) in their longer duration sCJDMM2 subjects. Therefore, in our set of cases, there appears to be no correlation between gel mobility of the PK-resistant PrPSc and the disease duration. Furthermore, more than one unglycosylated PrPSc band was found in some samples, and the presence of multiple bands as well as the gel mobility appeared to correlate inversely with the low pH of the TH (r = −0.76) (Fig. 3A, lanes 1–3).

Much more homogeneous data concerning pH and gel mobility were obtained when the lysis buffer 'plus' was used (Notari et al., 2004). The pH varied between 8.03 and 8.14 with no difference in mean pH between the two subject populations. Only a single band representing the PK-treated PrPSc unglycosylated fragment was present in the gel rather than the multiple bands often observed with the standard buffer (Fig. 3B). The band had almost identical mean molecular mass values—21.04 ± 0.06 kDa in the long-duration and 21.06 ± 0.06 kDa in the short-duration subjects—a difference that was not statistically significant (Fig. 3B and Table 3). Furthermore, the difference in molecular mass between the fastest and the slowest migrating bands in the long- and short-duration groups combined was 0.20 kDa, that is, 0.55 kDa less than that in the TH prepared in standard buffer (0.75 kDa difference with standard buffer versus 0.20 kDa difference with buffer plus).

### Effect of EDTA on PrPSc gel mobility

Treatment of TH preparations with the metal chelator EDTA has been reported to shift the migration of the PK-resistant PrPSc fragments towards lower molecular mass regions of the gel in all Collinge sCJDMM subjects but more prominently in the subjects with short disease duration than in those with long disease duration (Wadsworth et al., 1999). When we added 25 mM EDTA at pH 8.0 (as originally reported) to the TH prepared in standard buffer, we did observe a shift towards the lower molecular mass of the gel across both groups, and it was more marked in the short- than in the long-duration subjects (mean: 0.46 ± 0.15 kDa and range: 0.2–0.78 kDa in the short versus 0.29 ± 0.27 kDa and range: 0.0–0.97 kDa in the long). However, this difference did not reach statistical significance (Fig. 4 and Table 3).

### Table 3 pH of TH and molecular mass of PK-resistant PrPSc

<table>
<thead>
<tr>
<th>sCJDMM1</th>
<th>−EDTA</th>
<th>+EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Mol mass (kDa)</td>
</tr>
<tr>
<td>Standard lysis buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short duration</td>
<td>7.01 ± 0.09</td>
<td>21.20 ± 0.18</td>
</tr>
<tr>
<td>Range (n = 12)</td>
<td>(6.85–7.10)</td>
<td>(20.90–21.65)</td>
</tr>
<tr>
<td>Long duration</td>
<td>7.04 ± 0.24</td>
<td>21.27 ± 0.23</td>
</tr>
<tr>
<td>Range (n = 10)</td>
<td>(6.73–7.45)</td>
<td>(20.95–21.65)</td>
</tr>
<tr>
<td>Lysis buffer plus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short duration</td>
<td>8.085 ± 0.02</td>
<td>21.06 ± 0.06</td>
</tr>
<tr>
<td>Range (n = 12)</td>
<td>(8.04–8.12)</td>
<td>(21.0–21.18)</td>
</tr>
<tr>
<td>Long duration</td>
<td>8.06 ± 0.03</td>
<td>21.04 ± 0.06</td>
</tr>
<tr>
<td>Range (n = 10)</td>
<td>(8.03–8.14)</td>
<td>(21.0–21.20)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD values.
Two of the subjects from the long-duration group did not show any shift after EDTA treatment, while one subject had a shift that exceeded those of all subjects with short duration (Fig. 4). Furthermore, following the addition of EDTA, the pH was increased in each homogenate. However, there was no definite correlation between the extent of the EDTA-associated change in pH and the shift in gel mobility of the unglycosylated PrPSc band.

In order to further investigate whether differences could be detected between the long- and short-duration groups, and secondly, to ascertain whether the shift in gel migration of the PK-resistant PrPSc fragments in our subjects was due chiefly to EDTA, to the change in pH or to a combination of both, we examined the effect of EDTA on TH prepared in lysis buffer plus. The pH and mobility of PK-treated PrPSc were compared both before and after adding 25 mM EDTA (pH 8.0) to TH in buffer plus (pH 8.0). The mean pH in all samples combined did not change significantly following the EDTA addition: 8.076 ± 0.03 before EDTA versus 8.084 ± 0.03 after EDTA. Similar mean pH values were recorded in the short- and long-duration groups—short: 8.085 ± 0.02 before EDTA versus 8.090 ± 0.02 after EDTA; long: 8.06 ± 0.03 − EDTA versus 8.07 ± 0.04 + EDTA) (Table 3). The ranges of pH variation before and after EDTA treatment were identical in both groups. Similarly, no significant migration shift of the PK-treated PrPSc was detected following EDTA treatment in any of the subjects. One case from the short- and one from the long-duration group showed minimal shift of 0.1 and 0.15 kDa, respectively (Table 3). Therefore, the increase in gel mobility of the PK-treated PrPSc following the addition of EDTA occurred only under conditions that permitted a change in pH, which suggest that the two events are related.

Presence of C-terminal PrPSc fragments of 12/13 kDa

In addition to the major PK-resistant PrPSc commonly designated PrP27-30, PK-resistant C-terminal PrPSc fragments of 12/13 kDa (CTF12/13) have been detected in sCJDMM1 as well as in other subtypes of sCJD (Zou et al., 2003). We found equal amounts of CTF12/13 in PrPSc preparations from both long (n = 3) and short (n = 3) duration sCJDMM1 subjects, which indicates that the PrPSc CTF12/13 are inadequate in distinguishing the two groups (data not shown).

Two-dimensional immunoblotting

Two-dimensional (2D) immunoblots of the PK-digested PrPSc showed three sets of spots known to correspond to the diglycosylated, monoglycosylated and unglycosylated PrPSc species that migrated at 30, 27 and 21 kDa within pH ranges of 4.5–7.5, 4.5–8.5 and 6.6–8.7, respectively (Fig. 5A) (Zou et al., 2003; Pastore et al., 2005). The number of spots corresponding to the different PrPSc isoforms and the pattern that these spots formed in the 2D immunoblots were virtually identical in two representative cases from long (n = 3) and short (n = 3) duration groups (Fig. 5A). Following cleavage of the sugar moieties with PNGase F, the number of PrPSc spots was reduced to five, which migrated at 21 kDa, between pH 6.8–8.5 and in apparently identical patterns and ratios in both groups (Fig. 5B). Therefore, 2D immunoblots where proteins are separated on the basis of their electrical charges and relative molecular mass showed great similarity with regard to the number and pattern of the PrPSc spots generated by PK-untreated (data not shown) and PK- and PNGase F-treated preparations from short- and long-duration sCJDMM1 subjects (Fig. 5).

Conformational stability immunoassay

The conformational characteristics of the PK-resistant PrPSc of short- and long-duration sCJDMM1 subjects were comparatively examined with the conformational stability immunoassay (CSI) (Peretz et al., 2001). This test assesses
the stability of the PK-resistant PrPSc as a function of the rate at which it is rendered PK-sensitive by treatment with increasing concentrations of the denaturant GdnHCl. Considerable inter-subject variability was observed especially in the long-duration group; this was probably related to the marked variability of the amount of PrPSc within this group, which also had on average >2-fold more PK-resistant PrPSc than the short-duration group (data not shown). However, following normalization, the two CDI curves failed to show any statistically significant difference (Fig. 6). The GdnHCl concentration required to reduce the PK-resistant PrPSc to half of the original amount, identified as GdnHCl1/2 and used as the measurement of the relative conformational stability of PK-resistant PrPSc, was 2.105 M in long (n = 5) duration and 2.117 M in short (n = 5) duration subjects, respectively (Fig. 6).

**Discussion**

Classifications of prion diseases should be both accurate and practical if they are to assure that individual cases can be easily and consistently identified. To achieve this goal, molecular classifications based on PrP genotype and PrPSc type must constitute the basis for distinct disease phenotypes. Without strong correlation between molecular and phenotypic features, dissimilarities in PrPSc characteristics, although important in understanding prion diseases, have no practical value in disease taxonomy since they are inadequate to identify a distinct strain. On the basis of these guiding principles, Parchi and Gambetti combined two distinct molecular subtypes, sCJDMM1 and sCJDMMV1, into one group; for despite the different PrP genotype as well as some disparities in the N-terminus of the PK-resistant PrPSc, no consistent phenotypic differences could be identified between the two groups (Parchi et al., 1999, 2000).

The major objectives of the present study reflect these principles. We first attempted to determine whether there was sufficient evidence to warrant separating the CJD subtype identified as sCJDMM1 (Parchi et al., 1996, 1999) into two groups (Collinge et al., 1996; Zanusso et al., 2001) on the basis of PrPSc heterogeneity. Secondly, we then assessed whether sCJDMM1 patients with long or short clinical courses (the phenotypic distinction that both Collinge et al. (1996) and Zanusso et al. (2001) found between their sCJDMM1 and
sCJDMM2 subtypes) have other distinguishing clinical or histopathological features, and we searched for a correlation between PrPSc gel mobility and clinical–pathological phenotype. Convincing clinical–pathological and biochemical correlation would support the contention that sCJDMM1 of long and short duration comprise two distinct sCJD subtypes (Collinge et al., 1996; Wadsworth et al., 1999; Hill et al., 2003, 2006; Lewis et al., 2005).

Overall, the variable gel mobility of the PK-resistant PrPSc that we observed, with formation of multiple bands when the standard buffer with low buffering capacity was used in the PK-resistant PrPSc preparations obtained from sCJDMM1 subjects, was comparable with that reported by Notari et al. (2004). The variability in gel mobility was not significantly different in the long- and short-duration cases, even when cases were grouped according to the gel mobility of their PrPSc and disease duration of the PrP Sc mobility groups were compared. Therefore, in spite of this heterogeneity, subjects with long and short disease durations showed no significant difference in gel migration using the standard buffer at pH 7.4.

Similarly, the pH of the brain homogenates fluctuated (with up to 0.72 pH unit difference between lowest and highest pH) when the homogenates were prepared in standard buffer (Notari et al., 2004), but the mean pH was almost identical between the long- and short-duration groups. Furthermore, gel mobility and number of bands appeared to inversely correlate with the pH of the homogenate, which supports the view that variations in gel mobility and the presence of multiple bands in PK-treated PrPSc gel preparations result from conformational changes of PrPSc and efficacy of PK cleaving activity, both of which are pH-dependent (Notari et al., 2004). This pH-dependent variability appeared to be more prominent in long-duration cases, which had a pH range almost three times wider than those of short duration. Brain pH at death has been inversely correlated with age at death as well as with severity of the agonal state, but not with post-mortem interval (under 96 h) or storage (if at −70°C) (Harrison et al., 1995). The wider pH range in long-duration subjects might thus be related to the severity of the histological lesions in these subjects or, less likely, variability of agonal state. It must be mentioned that Lewis et al. (2005) reported detecting no significant pH variability in their PrPSc preparations using a buffer comparable with our standard buffer. Similarly, Polymenidou et al. (2005) observed 'no effect of pH' on PrPSc type 1 of Parchi and Gambetti present in their preparations, although no methodological details were given.

The molecular mass heterogeneity of the PK-treated PrPSc was eliminated by using the buffer plus that with its stronger buffering capacity maintains a constant pH during PK-digestion. Under these conditions, the gel mobility of the PK-resistant PrPSc from the long- and short-duration groups became practically indistinguishable.

Wadsworth et al. (1999) carried out their EDTA study on subjects whom they designated sCJDMM1 and sCJDMM2 according to the Collinge classification, following homogenization of the brain samples in standard PBS buffer. The sCJDMM1 and sCJDMM2 groups (Collinge classification) had mean disease durations of ~2 and 8 months, respectively (Wadsworth et al., 1999; Hill et al., 2003, 2006; Lewis et al., 2005).

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with the buffer plus at pH 8.0. Combined with the data published by Notari et al. (2004), the present findings suggest that EDTA chelation affects gel mobility when it is associated with a change in pH. This pH-related effect, however, does not adequately distinguish sCJDMM1 subjects with long and short disease durations. A small EDTA-related increase in mobility of type 1 and to a lesser extent of type 2 (Parchi and Gambetti classification) inadequate to interconvert the two PrPSc types has been reported by Head et al. (2004) using a buffer similar to our standard buffer.

The finding by Zanusso et al. (2001) that the gel mobility of the PK-resistant PrPSc fragments increased when they raised the pH of their preparations from 7.4 to 8.0 during homogenization and PK digestion is difficult to reconcile with our results. When data from relatively similar experiments are compared, the major discrepancy appears to lie in the fact that the ~1.0 kDa increase in gel mobility observed by Zanusso et al. (2001) occurred only in sCJDMM1 subjects with shorter disease durations. In contrast, under comparable conditions we observed a 0.2 kDa mean increase of the gel mobility in both our long and short disease duration subjects.

Zanusso et al. (2001) used the standard buffer for both of their preparations (at pH 7.4 and pH 8.0), whereas we used the standard buffer at pH 7.4 and the buffer plus (containing 100 mM instead of 10 mM Tris–HCl) at pH 8.0. Since we found that the standard buffer was unable to stabilize the pH of the homogenates, which shifted up to 0.73 pH units, we presume that both preparations by Zanusso et al. (2001) suffered similar pH variability. Notwithstanding the difficulty in comparing these two sets of experiments, we observed pH-related shifts in gel mobility under conditions comparable with those used by Zanusso et al. (2001), but the shifts we observed were similar in both long- and short-duration groups.

Four additional tests based on different analytical principles and designed to demonstrate fine chemical–physical differences between PrPSc species or strains each failed to reveal consistent biochemical differences between our long- and short-duration sCJDMM1 groups. Therefore, none of our comparative data provide evidence that the physicochemical characteristics of the PK-resistant PrPSc are different in our sCJDMM1 subjects of long and short disease durations.

Concerning the clinical and histopathological phenotypes, except for the duration and duration-related histopathological changes, the long- and short-duration subjects showed no significant differences (Table 2). The younger mean age of the long-duration subjects was not statistically significant, but it might explain the longer duration in the younger subjects. The clinical presentation, characterized by dementia and ataxia, was similar in both groups, although the relatively small number of subjects precludes analysis of subtle clinical differences. A more severe histopathological change in subjects with longer disease duration has to be expected. A direct correlation between severity of brain lesions and disease duration has been observed in familial insomnia and sCJDMM2 (Parchi et al., 1995, 1996). However, despite the greater severity corroborated by the intensity of PrP immunostaining, the topography of the lesions was similar in the two groups and matched that described in the original reports on sCJDMM1 (Parchi et al., 1996, 1999).

We arbitrarily selected patients with two distinct and exclusive ranges of disease duration (0.75–4 and 7–36 months for short and long disease duration patients, respectively) with the intent of accentuating possible differences between the two groups. In reality, the duration distribution of the sCJDMM1 patient population is markedly skewed toward short durations (National Prion Disease Pathology Surveillance Center, unpublished data). In 90% of 183 cases the disease duration varied between 0.5 and 5 months (as in our short-duration group) with a sharp peak at 2 months. In contrast, subjects with disease duration of 7–34 months accounted for only ~7% of the total patient population. Therefore, patients with disease durations of >5 months account for a small minority of the sCJDMM1 patient population.

In conclusion, exhaustive study of the PK-resistant PrP fragments and comparative examination of the clinical and neuropathological features reveal no significant differences between subjects with sCJDMM1 of long and short duration. Therefore, sCJDMM1 according to Parchi and Gambetti should retain its status as a single homogeneous subtype of sCJD.

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References
Classification of sCJD revisited


