Molecular classification of sporadic Creutzfeldt–Jakob disease

Andrew F. Hill,1,4 Susan Joiner,1 Jonathan D. F. Wadsworth,1 Katie C. L. Sidle,1 Jeanne E. Bell,2 Herbert Budka,3 James W. Ironside2 and John Collinge1

1MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, National Hospital for Neurology and Neurosurgery, London, 2National CJD Surveillance Unit, University of Edinburgh, Western General Hospital, Edinburgh, UK and 3Institute of Neurology, University of Vienna, Vienna, Austria

4Present address: Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, Australia

Correspondence to: Professor John Collinge, MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, Queen Square, London WC1N 3BG, UK
E-mail: j.collinge@prion.ucl.ac.uk

Summary
According to the protein-only hypothesis of prion propagation, an abnormal isoform (designated PrPSc) of the cellular prion protein (PrP C) is the principal or sole component of transmissible prions. However, the existence of multiple prion strains has been difficult to accommodate within this hypothesis. We have previously reported the identification of four types of human PrPSc associated with sporadic and acquired human prion diseases. These PrPSc types are distinguished by differing molecular mass of fragments following limited proteinase K digestion and by differing ratios of di-, mono- and unglycosylated PrPSc. That these discrete biochemical features of PrPSc are serially transmissible to human PrP in transgenic mice following experimental transmission suggests that they may be responsible for encoding prion strain diversity. Here we present detailed clinical, pathological and molecular data from a large number of sporadic Creutzfeldt–Jakob disease (CJD) cases. We show that PrPSc types are associated with codon 129 status, duration of illness and neuropathological phenotype. A novel PrPSc type is presented, illustrating further heterogeneity in CJD, and suggesting that further molecular subtypes of CJD may exist at lower frequencies. A molecular classification of sporadic CJD is proposed.

Keywords: Creutzfeldt–Jakob disease, prion disease, prion protein

Abbreviations: BSE = bovine spongiform encephalopathy; CJD = Creutzfeldt–Jakob disease; FFI = fatal familial insomnia; GSS = Gerstmann–Sträussler–Scheinker disease; MM = methionine homozygote; MV = methionine/valine heterozygote; PCR = polymerase chain reaction; PRNP = PrP gene; PrP = prion protein; PrPSc = cellular prion protein; PrPSc = disease associated protease resistant prion protein; vCJD = variant Creutzfeldt–Jakob disease; VV = valine homozygote

Introduction
Prion diseases, or transmissible spongiform encephalopathies, are a group of invariably fatal neurodegenerative disorders affecting both humans and animals. These diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru in humans. Prion diseases are transmissible by inoculation, have long incubation periods and share common histological features. The central feature of prion disease is the conversion of the normal cellular form of the host encoded prion protein (PrP C) to an abnormal isoform designated PrPSc. This conversion occurs posttranslationally and is thought to involve a conformational change rather than a covalent modification. PrPSc may be distinguished from PrP C by its insolubility in detergent and partial resistance to protease degradation. Many data exist to suggest that the principal component of the transmissible agent, or prion, is an abnormal isoform of PrP C, and this forms
the basis of the protein-only hypothesis of prion propagation (Prusiner, 1982). PrP\textsuperscript{Sc}, in contrast to PrP\textsuperscript{C}, is rich in β-structure and is isolated as insoluble aggregates. Prion propagation may involve recruitment of an alternately folded form of PrP\textsuperscript{C}, β-PrP, into such aggregates, the process being driven thermodynamically by intermolecular interactions (Jackson et al., 1999).

Human prion diseases have three distinct aetiologies. They may be inherited in an autosomal dominant fashion through germline mutations in the PrP gene (PRNP), acquired iatrogenically through exposure to contaminated neurosurgical instruments, tissue grafts or human cadaveric pituitary hormones, or arise sporadically. It is now clear that the novel human prion disease, variant CJD (vCJD), first recognized in the UK in 1996 (Will et al., 1996), is an acquired prion disease caused by a BSE-like prion strain (Collinge et al., 1996b; Bruce et al., 1997; Hill et al., 1997). Sporadic CJD makes up ~85% of all recognized human prion disease. Hypothesized causes of sporadic CJD include spontaneous production of PrP\textsuperscript{Sc} via rare stochastic events, somatic mutation of PRNP or unidentified environmental prion exposure. An association with sheep scrapie is not supported by epidemiological studies, which have found a fairly uniform worldwide incidence of sporadic CJD irrespective of scrapie prevalence (Brown et al., 1987). Although spatiotemporal groupings of sporadic CJD have been previously reported (Farmer et al., 1978; Adikari and Farmer 2001), no direct evidence for exposure to a common source of infectious prions has been provided. Indeed, such apparent clustering of cases, while appearing to reach levels of significance when viewed in isolation, can be deemed to be expected by chance alone when analysed within the population as a whole (Collins et al., 2002). However, the lack of such evidence does not exclude the possibility that a fraction of sporadic CJD is caused by environmental exposure to animal or human prions. There is marked genetic susceptibility in sporadic CJD in that most cases occur in homozygotes at codon 129 of PRNP, where either methionine or valine may be encoded. Heterozygotes appear significantly protected against developing sporadic CJD (Collinge et al., 1991; Palmer et al., 1991; Windl et al., 1996). Additionally, a PRNP susceptibility haplotype has been identified, indicating additional genetic susceptibility to sporadic CJD at or near to the PRNP locus (Mead et al., 2001).

The term CJD was introduced by Spielmeyer in 1922, drawing from the case reports of Creutzfeldt in 1920 and Jakob in 1921, and was used in subsequent years to describe a range of neurodegenerative conditions, many of which would not meet modern diagnostic criteria for CJD. The demonstration of experimental transmissibility of CJD, initially to primates (Gibbs et al., 1968), led to the concept of transmissible dementias and allowed diagnostic criteria for CJD to be assessed and refined. Atypical cases could be classified as CJD on the basis of their experimental transmissibility. While the human prion diseases have been classically divided into CJD, GSS and kuru, it is now clear that they represent clinicopathological syndromes within a wider spectrum of prion disease. In particular, kindreds with inherited prion disease have been described with phenotypes of classical CJD and GSS, and also with other neurodegenerative syndromes including FFI (Medori et al., 1992). Some kindreds show remarkable phenotypic variability, which can encompass both CJD- and GSS-like cases, as well as other cases that do not conform to either CJD or GSS phenotypes (Collinge et al., 1992). Cases diagnosed by PrP gene analysis have been reported that are not only clinically atypical, but that lack the classical histological features entirely (Collinge et al., 1990). While the inherited prion diseases can be readily subclassified according to PRNP mutation, subtypes of sporadic CJD have until recently been distinguished only by clinicopathological features.

The core clinical syndrome of classical CJD is of a rapidly progressive multifocal dementia usually with myoclonus. A variety of additional features may be present, including extrapyramidal signs, cerebellar ataxia, pyramidal signs and cortical blindness. The onset is usually in the 45–75 years age group, with peak onset between 60 and 65 years. The clinical progression is typically over weeks, progressing to akinetic mutism and death often in 2–3 months. Around 70% of cases die in <6 months. Raised cerebrospinal fluid 14-3-3 protein, neuronal specific enolase (NSE) and S-100, although not specific for CJD, may be helpful diagnostically in the appropriate clinical context (Jimi et al., 1992; Zerr et al., 1995; Collinge, 1996; Hsic et al., 1996; Otto et al., 1997). The EEG may show characteristic pseudoperiodic sharp wave activity that is helpful in diagnosis, but is present only in ~70% of cases. MRI scanning may show signal changes in the basal ganglia that, although not specific, can be diagnostically helpful (Schröter et al., 2000). Neuropathological confirmation of CJD is by demonstration of spongiform change, neuronal loss and astrocytosis. PrP amyloid plaques are usually not present in CJD, although PrP immunohistochemistry, using appropriate pre-treatments (Budka et al., 1995; Bell et al., 1997), will nearly always be positive.

Atypical forms of sporadic CJD are well recognized. Ten per cent of cases of CJD have a much more prolonged clinical course with a disease duration of over 2 years (Brown et al., 1984). Around 10% of CJD cases present with cerebellar ataxia rather than cognitive impairment, so-called ataxic CJD (Gomori et al., 1973). Heidenhain’s variant of CJD refers to cases in which cortical blindness predominates, with severe involvement of the occipital lobes. The panencephalopathic type of CJD refers to cases with extensive degeneration of the cerebral white matter in addition to spongiform vacuolation of the grey matter, and has been predominantly reported from Japan (Gomori et al., 1973). Amyotrophic variants of CJD have been described with prominent early muscle wasting. However, most cases of dementia with amyotrophy are not experimentally transmissible (Salazar et al., 1983) and their relationship with CJD is unclear.

The marked clinical heterogeneity observed in sporadic CJD is yet to be explained. However, it has been clear for...
many years that distinct isolates, or strains, of prions can be propagated in the same host, and these are biologically recognized by distinctive clinical and pathological features in experimental animals (for a review see Bruce et al., 1992). It is therefore likely that a proportion of clinicopathological heterogeneity in CJD and other human prion diseases relates to the propagation of distinct human prion strains, and their identification would allow an aetiology-based classification of CJD by typing of the infectious agent itself.

The existence of prion strains has been difficult to accommodate within the protein-only model of prion propagation. As the strains can be serially propagated in inbred mice with the same Prnp genotype, they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be re-isolated in mice after passage in intermediate species with different PrP primary structures (Bruce et al., 1994). Distinct strains of conventional pathogen are explained by differences in their nucleic acid genome. However, in the absence of such a scrapie genome, alternative possibilities must be considered. A wealth of experimental evidence now suggests that PrPSc itself may encode strain-specific phenotypic properties. Different subtypes of PrPSc were associated initially with two strains of transmissible mink encephalopathy in hamsters (Bessen and Marsh, 1992, 1994). The different fragment sizes seen on western blots following treatment with proteinase K are attributed to different N-terminal cleavage sites as a result of different conformations of PrPSc. Importantly, these biochemical changes in PrPSc are transmissible to the PrP in a host. This has been demonstrated in studies with CJD isolates, with both PrPSc fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP (Collinge et al., 1996b). These data strongly support the ‘protein-only’ hypothesis of infectivity, and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation.

Recently, several human PrPSc types have been identified that are associated with different phenotypes of CJD (Collinge et al., 1996b; Parchi et al., 1996, 1999). Parchi and colleagues reported two molecular subtypes of PrPSc amongst cases of sporadic CJD, and have proposed a preliminary classification of sporadic CJD based on clinical, pathological and these molecular criteria (Parchi et al., 1996, 1999). We reported the identification of four types of human PrPSc associated with sporadic and acquired human prion diseases (Collinge et al., 1996b; Wadsworth et al., 1999). These PrPSc types are observed on western blots after limited proteinase K digestion of brain homogenates, resulting in PrP fragments of differing molecular mass. These PrPSc types are further characterized by the relative intensities of the three PrP bands, representing di-, mono- and unglycosylated PrPSc. In humans the methionine/valine polymorphism at codon 129 of PRNP is associated with different PrPSc types. PrPSc types 1 and 4 have so far only been detected in methionine homozygotes, type 3 in cases containing a valine allele and type 2 in any PRNP codon 129 genotype (Collinge et al., 1996b). Type 4 is uniquely associated with vCJD and thought to arise as the result of exposure to BSE (Bruce et al., 1997; Hill et al., 1997). This PrPSc type shows a glycoform profile with an abundance of the diglycosylated PrP isoform that is not seen in any of the sporadic PrPSc types. Previous studies on PrPSc typing in classical CJD described only two types of banding pattern (Parchi et al., 1996), and it was argued that PrPSc types 1 and 2 we described corresponded with the type 1 pattern of Parchi and colleagues, with our type 3 pattern represented by their type 2 (Parchi et al., 1997). These authors conceded a degree of heterogeneity in their type 1 samples, which has yet to be formally resolved.

Strain-specific protein conformation can also be influenced by metal ion binding to PrPSc (Wadsworth et al., 1999). Two different human PrPSc types, seen in clinically distinct subtypes of classical CJD can be inter-converted in vitro by altering the metal ion occupancy. Metal ion chelation of these PrPSc types leads to a change in PrPSc conformation and exposure of a new proteolytic cleavage site, resulting in a shift in mobility of the PrPSc proteinase K digestion fragments observed on western blots. The dependence of PrPSc conformation on the binding of copper and zinc represents a novel mechanism for post-translational modification of PrP, and for the generation of multiple prion strains. This and other methodological differences in detection of relatively subtle biochemical differences in PrPSc may be responsible for the discrepancy between these studies.

Molecular classification of human prion disease has important implications for epidemiological studies investigating the aetiology of sporadic CJD, since it is possible that associations may be apparent between environmental variables and individual human prion strains that are lost when epidemiological studies assess sporadic CJD as a whole. Here we present molecular, clinical and neuropathological analysis of a large-scale blinded study of PrPSc types in sporadic CJD and propose a molecular classification system, which includes the analysis of the metal ion-dependent conformation of PrPSc.

Patients and methods

Selection of patients

Cases in this study derived from an unselected series of patients with a neuropathologically proven diagnosis of sporadic CJD where frozen brain material was available for analysis by western blot, and from whom consent to use autopsy material for research purposes had been obtained. Ethical permission for research on autopsy materials stored in the National CJD Surveillance Unit was obtained from Lothian Region Ethics Committee.

The clinical data for each case were summarized to include the age and sex of the patient, the duration of the illness, presenting clinical features and subsequent major clinical signs and symptoms and the EEG findings.
The frozen brain tissue was stored at −80°C and samples were taken from the frontal cortex for DNA analysis and western blot analysis for PrPSc. Repeated freeze–thaw cycles were avoided during storage.

DNA sequencing
DNA was extracted from brain tissue and used to determine the PRNP codon 129 genotype and the presence of any known or novel alterations. The complete PRNP open reading frame was amplified by polymerase chain reaction (PCR) using oligonucleotide primers chosen not to overlay a polymorphism in the 5' untranslated region of the gene, which can lead to non-amplification of certain alleles, as described previously (Palmer et al., 1996). The PCR product was sequenced using both strands on an ABI 373 or 377 automated DNA sequencer using BigDye chemistry (Perkin Elmer, Cambridge, UK).

Western immunoblotting
Brain tissue homogenates (10% w/v) from samples of frontal cortex were prepared in Dulbecco’s phosphate buffered saline (PBS; lacking Ca²⁺ or Mg²⁺) by serial passage through needles of decreasing diameter. The homogenates were cleared of particulate matter by centrifugation at 1000 r.p.m. (80 g) for 1 min in a microfuge (Eppendorf, Cambridge, UK). Samples of supernatant were removed and proteinase K added from a 1 mg/ml stock solution (prepared in water) to give a final concentration in the sample of 50 μg/ml. Following incubation at 37°C for 60 min, samples were centrifuged at 14 000 r.p.m. (15 800 g) for 1 min in a microfuge before termination of the digestion by the addition of an equal volume of 2× SDS sample buffer [125 mM Tris–HCl (pH 6.8), 20% v/v glycerol, 4% w/v sodium dodecyl sulphate, 4% v/v 2-mercaptoethanol, 0.02% (w/v) bromophenol blue] containing 8 mM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF; Pefabloc SC, Roche, Lewes, UK) and immediate transfer to a 100°C heating block for 10 min. Samples were centrifuged at 14 000 r.p.m. (15 800 g) for 1 min in a microfuge prior to electrophoresis in 16% Tris–glycine gels (Novex; Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Gels were electrophoretically transferred onto PVDF membrane (Immobilon-P; Millipore, Watford, UK) and subsequently blocked in PBS containing 0.05% v/v Tween-20 (PBST) and 5% (w/v) non-fat milk powder for 60 min. After washing in PBST, the membranes were incubated with anti-PrP monoclonal antibody 3F4 (Senetek, Maryland Heights, MI, USA) diluted 1 : 5000 in PBST for at least 60 min before washing in PBST (30 min) and incubation with a horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma, Dorset, UK) diluted 1 : 10 000 in PBST for 60 min. Following washing in PBST (30 min), the membranes were developed using a chemiluminescent substrate (Supersignal, West Pico; Pierce, Cheshire, UK) and visualized on Biomax MR film (Kodak, Hemel Hempstead, UK).

EDTA treatment of brain homogenates
Aliquots of 10% brain homogenate were adjusted with a stock solution of EDTA to give a final EDTA concentration of 25 mM in the sample prior to proteinase K digestion as described above. EDTA was prepared as a 250 mM stock in deionized water and titrated to pH 8.0 with NaOH.

Quantitation and analysis of PrP glycoforms
Western blots were performed as described above but were developed using an alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) and a chemiluminescent substrate (AttoPhos; Promega; Southampton, UK). The membranes were scanned on a Storm 840 PhosphorImager (Molecular Dynamics, Little Chalfont, UK). Quantitation of the PrPSc glycoforms was performed using ImagequaNT software (Molecular Dynamics). Statistical analysis was performed using INSTAT (GraphPad Software, San Diego, USA).

Neuropathology
Brain tissue was fixed in 10% formalin for a minimum period of 3 weeks and extensively sampled to allow a thorough neuro-anatomical investigation. Tissue blocks were selected from frontal, temporal, parietal and occipital regions of the cerebral cortex, the hippocampus, basal ganglia (caudate nucleus, putamen and globus pallidus), anterior and posterior thalamus, hypothalamus, midbrain, pons, medulla, cerebellar hemispheres and vermis and spinal cord at multiple levels (where available).

Tissue blocks were immersed in 96% formic acid for 1 h before routine processing into paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin and other conventional tinctorial methods, and by immunocytochemistry for PrP, using an established and validated technique (Bell et al., 1997), and for glial fibrillary acidic protein. Immunocytochemistry for PrP was performed on a minimum of 12 blocks per case in order to allow comparison of patterns of PrP accumulation. These were designated as follows: reticular (syncytial), perivascular, intraneuronal, plaque like (where no amyloid plaques were identified on routine stained sections), kuru-type plaques (when amyloid plaques were also visible on routine stained sections), florid plaques, cluster plaques and amorphous pericellular and perivascular deposits.

Neuropathological assessment was carried out (by J.W.I. and J.E.B.) blind to the codon 129 genotype and PrP biochemistry, and assessed using a standardized proforma. Spongiform change was assessed on a semiquantitative basis, ranging from a scale of 0 (none) to 3 (severe) (Fraser and Dickinson, 1968; Bruce et al., 1991). The nature of the
spongiform change, and the degree of neuronal loss and astrocytosis, were also recorded, along with a topographical description of the pattern of PrP immunoreactivity. Sections were reviewed in the light of PrP analysis with regard to the novel PrP subtypes, and the results were grouped according to codon 129 genotype and PrP subtype.

Results

Molecular analysis of PrP<sup>Sc</sup> types

We observed the presence of four types of PrP<sup>Sc</sup> following limited proteinase K digestion of brain tissue homogenates from patients with neuropathologically confirmed sporadic, iatrogenic or variant CJD (Fig. 1). These types are distinguished by distinct PrP fragment size and ratios of the three principal PrP glycoforms. While in our earlier studies (Collinge et al., 1996b) we could not distinguish the fragment sizes of the types 3 and 4 human PrP<sup>Sc</sup>, following modifications to our western blotting technique we now observe a consistent difference in their fragment sizes, with type 4 having a slightly larger relative molecular mass compared with type 3.

In addition, we also observed a novel PrP<sup>Sc</sup> type (see Fig. 2A), designated type 6, in a single case of sporadic CJD. Type 6 PrP<sup>Sc</sup> is characterized by PrP fragment size alone, whereby the relative molecular mass of these fragments are larger than those observed for type 1. Type 5 human PrP<sup>Sc</sup> was previously observed in vCJD-infected transgenic mice expressing human PrP encoding valine at codon 129, and has not been seen to date in human samples (Hill et al., 1997).

All type 1 cases were found to be codon 129 methionine homozygotes, whereas type 2 PrP<sup>Sc</sup> was observed in individuals of any codon 129 genotype (Table 1). We previously suggested that type 3 PrP<sup>Sc</sup> may be associated with acquired (iatrogenic) forms of prion disease (Collinge et al., 1996b); however, after analysis of a larger number of samples, we have now observed this type in some sporadic CJD cases. Type 3 PrP<sup>Sc</sup> is generally associated with codon 129 genotypes containing a valine allele, with the exception of a single PRNP codon 129 methionine homozygote case. Type 4 PrP<sup>Sc</sup> can be distinguished by both fragment size and degree of glycosylation of the PrP glycoforms, and is unique to cases of vCJD, which, to date, are all methionine homozygotes (Collinge et al., 1996a; Zeidler et al., 1997; and unpublished data).

In line with other published observations, there is an under-representation of methionine/valine heterozygotes present in our series, compared with the codon 129 frequency of the normal population (Palmer et al., 1991; Windl et al., 1996; Alperovitch et al., 1999; Parchi et al., 1999) (Fig. 3).

Using a chemiluminescent substrate and a phosphorimager, we analysed the relative abundance of the three PrP<sup>Sc</sup> bands (representing diglycosylated, monoglycosylated and unglycosylated PrP<sup>Sc</sup>) observed after proteinase K digestion (Fig. 4). Statistical analysis of the differences in glycoform ratios between types 1–3 and type 4 shows a highly significant difference ($P < 0.0005$ for each glycoform band and type) and further demonstrates the highly distinct glycoform pattern of type 4 PrP<sup>Sc</sup>. The glycoform profiles of type 1 and 2 (of any codon 129 genotype) PrP<sup>Sc</sup> are statistically similar. A striking difference was observed when comparing the glycoform profiles of type 3 MV (methionine/valine heterozygote) and
VV (valine homozygote) cases, however (Figs 2B and 4). Here, the relative intensities of both the di- and unglycosylated PrPSc fragments were significantly different (P < 0.02 and P < 0.01, respectively). The glycoform pattern of type 6 appears similar (on multiple scans) to that observed in type 1 and 2 MM (methionine homozygote) PrPSc. Analysis of the glycoform patterns in the samples prior to proteinase K digestion was not informative, as the glycoform pattern of PrPC is often subject to degradation during transit of samples. The glycoform patterns of PrPSc is, however, unaffected by such degradation, with pattern being maintained after tissue has been incubated at room temperature for 48 h (S.J., A.F.H. and J.C., data not shown).

Following chelation of metal ions with EDTA prior to digestion with proteinase K, PrPSc types 1 and 2 MM show a shift in PrPSc fragment size, producing an indistinguishable pattern of digestion products designated type 2− (Fig. 5) (Wadsworth et al., 1999). All type 1 and 2 MM cases that we have examined uniformly produce type 2− digestion products after treatment with EDTA. We have also now observed that type 2 PrPSc from an MV case also produces a type 2− pattern of digestion products following treatment with EDTA prior to proteolysis. Type 6 PrPSc conformation also shows dependency on metal ions and gave a proteinase K digestion pattern closely similar to the type 2− pattern after treatment with EDTA. Interestingly, to date we have not observed a difference in the molecular mass of type 2V V PrPSc when digested in the presence of EDTA, but have now been able to resolve a subtle shift in PrPSc fragment size in type 3 VV PrPSc (Fig. 5). However, this conformational dependency on metal ions was not observed for every type 3 VV case we have examined, and to date we have not observed metal ion dependency of conformation in type 3 MM samples or in the single type 3 MM case. Further work investigating the metal ion dependence of PrPSc in valine homozygous cases and in codon 129 heterozygous type 2 cases is ongoing. As we have reported previously (Wadsworth et al., 1999), proteinase K digestion of type 4 PrPSc is not influenced by the presence of EDTA (Fig. 5).

**Clinical phenotypes**

A statistically significant shorter duration of illness was found in type 1 patients, who are all codon 129 methionine homozygotes (Fig. 6), compared with each of the other groups. Type 3 MV cases have a significantly longer duration

### Table 1

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Codon 129 genotype</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Type 4</th>
<th>Type 6</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic</td>
<td>MM</td>
<td>17</td>
<td>38</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>MV</td>
<td>0</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>VV</td>
<td>0</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Variant</td>
<td>MM</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

The cases of human prion diseases grouped by disease type, codon 129 status and PrPSc type. Codon 129 genotypes for one iatrogenic type 3 case and two sporadic type 2 cases were unable to be identified.
of illness than VV cases of the same type. This may represent a host genotype effect, with codon 129 heterozygosity increasing the duration of illness itself and also influencing the differences observed in the glycoform profiles between type 3 MV and VV cases, but may also suggest that a different prion strain is responsible for the distinct clinicopathological phenotype in these individuals. There may also be an interaction between PrPSc type and codon 129 genotype influencing the duration of illness. We did not observe any significant difference in the age at onset when comparing the data grouped by PrPSc type.

Clinical investigations
Samples for which EEG investigations were available were analysed by grouping into PrPSc type and codon 129 status (Table 2). Type 1 and 2 MM cases show typical EEG readings in >50% of cases examined. This contrasts with type 2 VV and all type 3 cases, which are all atypical for this investigation. While we have only examined a small sample set, our results agree with those of a previous study showing a distinction in EEG findings by PrPSc type and codon 129 genotype (Parchi et al., 1999).

Neuropathological findings
Neuropathological assessment of the cases was performed blind to the PrPSc typing analysis (Tables 3 and 4). Profiles of the degree of spongiosis observed in each of the PrPSc types grouped by codon 129 genotype is illustrated in Fig. 7. Type 1 cases showed widespread spongiform degeneration in the cerebral cortex, with the occipital cortex being the most affected. Type 1 cases were characterized by the lack of
spongiform change observed in the hippocampus, and mild changes in the basal ganglia, cerebellum and brainstem. This is in marked contrast to the changes seen in type 2 codon 129 MM cases, where the basal ganglia show moderate spongiform changes. Type 2 cases, which are codon 129 heterozygotes or valine homozygotes, show extensive spongiform changes in the basal ganglia and mild changes in the cerebellum in addition to changes in the cerebral cortex. There is no significant difference between codon 129 heterozygote or valine homozygote type 2 cases and the type 3 cases with the same genotypes.

The variation in the neuropathology between the type 1 MM and type 2 MM cases was also reflected in immunocytochemical findings, the type 1 MM cases showing a predominantly synaptic pattern of PrP accumulation (which was also a major feature in the type 2 MV cases) and the type 2 MM cases showing mainly a perivacuolar pattern of PrP accumulation in the cerebral cortex (Fig. 8). None of the MM cases exhibited predominant thalamic pathology.

The type 3 MV cases are characterized by the unique presence of kuru-type plaques, particularly in the cerebellar cortex (Fig. 8). However, these cases share some other features with the type 3 VV cases, in particular the presence of linear accumulation of PrP in a perineuronal distribution in the cerebral cortex. All the type 4 MM cases showed the characteristic histological features of vCJD, with numerous florid plaques in the cerebral and cerebellar cortex and widespread accumulation of PrP in the brain. The type 6 MM case showed neuropathological features that were very similar to the type 2 VV cases.

**Discussion**

Classification of sporadic CJD by both molecular and phenotypic parameters, as demonstrated by the present study, and the correlation of these with epidemiological surveillance is important for understanding of the aetiologies responsible for this syndrome. The data presented here...
Table 4  Patterns of PrP immunostaining for types 1–6

<table>
<thead>
<tr>
<th>Codon 129</th>
<th>PrP&lt;sup&gt;sc&lt;/sup&gt; type</th>
<th>Immunostaining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>1</td>
<td>Synaptic and focal perivacuolar deposits in cerebral cortex. Synaptic deposits only in other regions.</td>
</tr>
<tr>
<td>MM</td>
<td>2</td>
<td>Mostly perivacuolar deposits in cerebral cortex. Focal synaptic deposits in cerebellum and synaptic deposits in other regions.</td>
</tr>
<tr>
<td>MV</td>
<td>2</td>
<td>Synaptic deposits in basal ganglia, cerebellum and brainstem and cerebral cortex. Basal ganglia also had focal deposits, brainstem had tract deposits and cerebral cortex has perivacuolar deposits.</td>
</tr>
<tr>
<td>VV</td>
<td>2</td>
<td>Synaptic deposits in basal ganglia, cerebellum and brainstem. Basal ganglia also had focal deposits and brainstem had tract deposits.</td>
</tr>
<tr>
<td>MV</td>
<td>3</td>
<td>Synaptic deposits in all regions except for hippocampus.</td>
</tr>
<tr>
<td>VV</td>
<td>3</td>
<td>As type 2 VV.</td>
</tr>
<tr>
<td>MM</td>
<td>4</td>
<td>Multiple cluster plaques in the cerebral and cerebellar cortex. Perineuronal staining in the basal ganglia; synaptic deposits in the thalamus and brainstem.</td>
</tr>
<tr>
<td>MM</td>
<td>6</td>
<td>Synaptic deposits in cerebellum and brainstem. Perivacuolar deposition in cerebral cortex and basal ganglia.</td>
</tr>
</tbody>
</table>

Summary of PrP immunostaining patterns observed in sporadic CJD cases grouped by PrP<sup>sc</sup> type and codon 129 genotype. Brain regions examined are: cerebral cortex, hippocampus, basal ganglia, cerebellum and brainstem.

Fig. 7  Lesion profiles of PrP<sup>sc</sup> types 1–3 grouped by codon 129 genotype. Brain regions studied were: FC = frontal cortex; PC = parietal cortex; TC = temporal cortex; HC = hippocampus; OC = occipital cortex; SR = striatum; TH = thalamus; CB = cerebellum; BS = brainstem. Values shown are mean ± SEM and derived from a score from 0 to 3 to designate the degree of spongiform change observed in the brain region examined.
strongly support our previous classification, where, in a smaller number of samples, we observed three patterns of PrPSc associated with sporadic (and iatrogenic) CJD (Collinge et al., 1996b).

The type 1 PrPSc pattern observed in our series of cases is associated exclusively with PRNP codon 129 methionine homozygosity. This is at variance with the earlier and simpler classification reported by Parchi et al. (1999), where their ‘Gambetti type 1’ is also observed in other genotypes, albeit at a much lower frequency. Clinically, our type 1 cases had a significantly shorter duration of illness than that seen in patients propagating the other PrPSc types, including type 2 cases with the same codon 129 genotype. This is consistent with the Gambetti type 1 MM cases, which Parchi and colleagues reported to have a shorter clinical duration than their type 1 MV and VV cases, and had values approximating those seen in our type 2 cases.

At the molecular level, we are able to distinguish type 1 PrPSc from the other types by the fragment size shift seen upon metal ion chelation of PrPSc with EDTA in brain tissue homogenates before proteinase K treatment. We have shown previously that conformations of PrPSc can be influenced by bound metal ions and that chelation of metal ions in brain homogenates prior to proteinase K digestion can alter the fragment size of PrPSc types 1 and 2 MM cases to a common fragment size, designated type 2 (Wadsworth et al., 1999).

The ability to inter-convert type 1 and type 2 MM PrPSc conformations in vitro by alteration of metal ion occupancy provides a plausible explanation for the discrepancy between our classification and earlier molecular classification systems.
(Parchi et al., 1997, 1999). Type 1 CJD is a distinct human prion disease with an aggressive clinical course and remarkably short clinical duration (Wadsworth et al., 1999). The recent identification of two physiologically relevant high affinity copper binding sites in the N-terminal region of PrP (Jackson et al., 2001) suggests that coordination of structure through copper binding at either both sites, or at one or other site, may underlie the distinct metal ion-dependent conformations of type 1 and 2 MM PrPSc. Our finding that the electrophoretic mobility of type 2 VV PrPSc is not altered by the presence of EDTA during proteinase K digestion indicates that its N-terminal structure is distinct from that of type 2 MM PrPSc. Importantly, elements of PrPSc structure that are coordinated through metal ion binding may encode phenotypic information that distinguishes prion strains, as possibly exemplified by the distinct clinical and neuropathological features seen in patients propagating either type 1 or type 2 MM PrPSc. In this regard, we are currently investigating the relationship between metal ion occupancy of human PrPSc conformers and neuropathological phenotype in transgenic mice expressing human PrP M129 (Asante et al., 2002). In addition to providing a potential mechanism for prion strain variation, the ability of metal ions to directly influence human PrPSc conformation also provides further evidence to suggest that the neuropathology of prion diseases may be related to abnormalities in copper metabolism, and raises the possibility that drugs which influence copper metabolism may have therapeutic potential in prion disease (Wadsworth et al., 1999; Jackson et al., 2001).

The differences in electrophoretic mobility that distinguish our classification of PrPSc types from that of Gambetti and co-workers (Parchi et al., 1999), although highly reproducible, are technically demanding to resolve. Gel-to-gel variability obviates the ability to define a sufficiently accurate molecular mass for the unglycosylated band that would distinguish each PrPSc type. For this reason we routinely use internal typing controls of defined PrPSc types on each SDS gel when assigning PrPSc type to an unknown sample. We feel it probable that further subclassification of PrPSc may be achieved in the future, although this will require novel methods of investigation. For example, among different codon 129 genotypes we have observed micro-heterogeneity in fragment sizes seen in type 2 PrPSc samples in which the molecular masses of the unglycosylated band do not correspond precisely to each other or to type 1 or type 3 fragment sizes. Further refinements and new methodologies will be required to investigate these differences and facilitate further subclassification of these PrPSc types.

Neuropathologically, type 1 and type 2 MM cases could be readily differentiated. Type 1 cases had focal spongiform changes in the cerebral cortex and little change observed in the hippocampus, basal ganglia and cerebellum. This contrasts markedly with type 2 MM cases, where widespread spongiform change is seen with involvement of the hippocampus and basal ganglia. Type 2 MV and type 2 VV cases can be distinguished from type 2 MM cases by the severe spongiform change in the basal ganglia, involvement of the cerebellum and presence of plaque like deposits. The type 3 MV cases were defined by the presence of kuru-type plaques, which were clearly visible on routinely stained sections, and allowed differentiation from type 3 VV. The type 4 MM cases all showed the characteristic neuropathological features of vCJD. This suggests that the neuropathological profile for each genotype may be limited in nature and not always influenced in a major way by the PrPSc isotype. Further studies are required to establish whether novel PrPSc isotypes always exhibit characteristic clinical and neuropathological phenotypes, both in man and on transmission to transgenic mice with a human PrP gene of defined codon 129 genotype.

In earlier publications, Parchi and colleagues studied the clinical and neuropathological features in a large series of CJD cases in relation to the PrPSc isotype and codon 129 PRNP genotype (Parchi et al., 1996, 1999). In these publications, two major PrPSc isotypes were identified, in comparison with our findings. However, there are striking similarities between many of the CJD subgroups identified in each of these studies. The type 1 MM, type 2 MM, type 3 VV, type 2 MV and type 3 MV groups in this paper are similar in terms of the neuropathology and lesion profiles of spongiform change to the MM1, MM2 (cortical), VV2, MV1 and MV2 groups, respectively, in the earlier publication. There are also similarities between the type 2 VV cases in our series and the VV1 cases in the earlier papers, although a more extensive overlap with the Parchi et al. VV2 cases is noted. We did not identify any case in this series with the predominant thalamic pathology described in the MM2 thalamic cases in the earlier series. Cases of vCJD showed identical neuropathology and clinical features in both series, with the type 4 isotype as reported (Collinge et al., 1996b).

The close similarities in the sporadic CJD subgroups in these independent studies argue for a biological significance in terms of agent strain, which seems able to produce an identifiable and consistent phenotype in a host of defined PRNP genotype. The clarification of the differences in PrPSc isotypes will be aided by further technical refinements and standardization in western blot methodology. However, to firmly demonstrate the existence of distinct human prion strains biologically will require serial passage studies in experimental animals and extensive transmission studies to transgenic mice expressing only human PrP (either 129 M or V) are in progress to isolate human prion strains and further define an aetiological classification of CJD based on defined subtypes of human prions, and to dissect phenotypic effects of prion strain from host genotypes.

There has been some dispute in the literature regarding the classification of sporadic CJD into two or three PrPSc types (Parchi et al., 1997). After our initial proposal of at least three PrPSc types in sporadic and iatrogenic CJD, it was contested that the mobility shifts observed in our study had been incorrectly matched with those of another study, in which only two types of PrPSc were seen. It was proposed that the
type 1 and 2 PrPSc types observed in the classification presented here represents their type 1 with our type 3 cases corresponding with their type 2. In our previous (Collinge et al., 1996b) and current studies, the typing of PrPSc isoforms is performed blind to genotypic and clinical phenotype information. In addition, our analysis of PrPSc types includes the electrophoresis of type 1–4 PrPSc controls on every western blot, given that accurate molecular mass determination using protein standards on the SDS-PAGE gels does not offer sufficient resolution to characterize the PrPSc type by relative molecular mass alone. By using this approach we have identified a novel PrPSc type, designated type 6 (Fig. 9). For there to be additional PrPSc types, albeit in single cases, is perhaps not unexpected in sporadic CJD given the wide phenotypic range of clinical and pathological features.

From this it is difficult to accept that our classification into three PrPSc types is the result of an artefactual separation of Parchi’s type 1 into the types 1 and 2 reported here, given that there is considerable overlap between cases based on codon 129 genotype, PrPSc type and comparative pathology that needs to be clarified. This will require the classification to be based on the many factors (biochemical, genetic, clinical and pathological) that contribute to the heterogeneity observed in sporadic CJD. Identification of the cleavage sites responsible for the different fragment sizes between the PrPSc types may clarify these discrepancies at the molecular level. However, the purification of human PrPSc is constrained by the recognition that metal ions play a key role in determining strain-specific conformations of PrPSc associated with clinically distinct subtypes of sporadic CJD, which places major constraints upon the design of appropriate purification strategies. The failure of a recent study to reveal strain-specific N-terminal cleavage site heterogeneity in PrPSc in sporadic CJD can be attributed to the methods used for purification, which would have resulted in conformational change due to metal ion chelation prior to proteinase K digestion (Parchi et al., 2000).

A further possible complication to molecular classification of sporadic CJD has been the report that two different PrPSc types exist in different regions of the same brain (Puoti et al., 1999). Using the classification of Parchi it was observed that in some PrPSc type 1 cases, type 2 PrPSc could be observed in certain regions of the brain. The regional distribution of different PrPSc types further underlines the need to compare the same region from different brain samples for comparative PrPSc typing. The samples used in our study were predominantly from the frontal cortex, and samples are routinely typed at least twice using PrPSc type 1–4 controls on every western blot to reduce any typing errors and control for novel typing patterns. We have observed rare cases where two PrPSc types may be superimposed resulting in a double unglycosylated band (Fig. 2C). The occurrence of this apparently novel PrPSc type would not appear to correlate with a distinctive clinico-pathological presentation. It is a possibility that these cases represent further heterogeneity within the spectrum of PrPSc types.

The precise aetiology of sporadic CJD remains obscure and, given that this form of prion disease accounts for ~85% of all cases, requires further definition. The spontaneous conversion of PrPC to PrPSc as a rare stochastic event, or somatic mutation of the PrP gene, resulting in expression of a pathogenic PrP mutant (Collinge, 1997), are plausible explanations. However, other causes, including environmental exposure to human or animal prions, is not ruled out by epidemiological studies as a cause of at least some cases (Collins et al., 1999). The data presented here will allow a more precise molecular classification of human prion disease, with important implications for epidemiological studies into the aetiology of sporadic CJD. Re-analysis of epidemiological data using these molecular subtypes may reveal important risk factors that are obscured when sporadic CJD is analysed as a single entity. BSE transmission to mice expressing human PrP (encoding methionine at codon 129) can produce a molecular phenotype indistinguishable from that seen in type 2 sporadic CJD patients, in addition to the type 4 pattern of vCJD (Asante et al., 2002). This, coupled with the significant increase in the incidence of sporadic CJD in Switzerland, underlines the need for more precise definition of the aetiology of sporadic CJD (Glatzel et al., 2002). Of great public health interest will be the continued surveillance for increases in particular subgroups of sporadic CJD that could be attributed to some form of iatrogenic transmission or association with as yet unrecognized sources of prion infection.

Acknowledgements
We wish to thank J. Beck, T. Campbell, M. Desbruslais and A. Dickinson in the MRC Prion Unit for their technical assistance; L. McCardle and the pathology staff of the NCJDSU for their technical work; D. Ritchie and R. Young for preparation of the figures; S. Brandner for critically reviewing the manuscript; and the MRC Brain Bank for supplying some of the tissues used in this study. The cooperation of all the neurologists and neuropathologists in the UK is gratefully acknowledged. This work was funded by the Wellcome Trust (A.F.H. is a Wellcome Trust Prize Travelling Research Fellow), the European Commission, and the Medical Research Council (UK). The NCJDSU is funded by the Department of Health and the Scottish Executive.

References
Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL, et al. BSE prions propagate as either variant CJD-like or...


Palmer MS, Dryden AJ, Hughes JT, Collinge J. Homozygous prion


Accepted January 10, 2003