Inherited prion disease with 4-octapeptide repeat insertion: disease requires the interaction of multiple genetic risk factors

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Genetic factors are implicated in the aetiology of sporadic late-onset neurodegenerative diseases. Whether these genetic variants are predominantly common or rare, and how multiple genetic factors interact with each other to cause disease is poorly understood. Inherited prion diseases are highly heterogeneous and may be clinically mistaken for sporadic Creutzfeldt–Jakob disease because of a negative family history. Here we report our investigation of patients from the UK with four extra octapeptide repeats, which suggest that the risk of clinical disease is increased by a combination of the mutation and a susceptibility haplotype on the wild-type chromosome. The predominant clinical syndrome is a progressive cortical dementia with pyramidal signs, myoclonus and cerebellar abnormalities that closely resemble sporadic Creutzfeldt–Jakob disease. Autopsy shows perpendicular deposits of prion protein in the molecular layer of the cerebellum. Identity testing, PRNP microsatellite haplotyping and genealogical work confirm no cryptic close family relationships and suggests multiple progenitor disease haplotypes. All patients were homozygous for methionine at polymorphic codon 129. In addition, at a single nucleotide polymorphism upstream of PRNP thought to confer susceptibility to sporadic Creutzfeldt–Jakob disease (rs1029273), all patients were homozygous for the risk allele (combined P = 5.9 × 10⁻⁵). The haplotype identified may also be a risk factor in other partially penetrant inherited prion diseases although it does not modify age of onset. Blood expression of PRNP in healthy individuals was modestly higher in carriers of the risk haplotype. These findings may provide a precedent for understanding apparently sporadic neurodegenerative diseases caused by rare high-risk mutations.

Keywords: inherited prion disease; octapeptide repeat insertion; Creutzfeldt–Jakob disease; epistasis

Abbreviations: CJD = Creutzfeldt–Jakob disease; OPRI = octapeptide repeat insertion

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Introduction

Prions are lethal infectious pathogens, largely composed of an abnormally folded host protein (Collinge, 2001). As one of the prototypic protein misfolding disorders, prion diseases share fundamental mechanisms with other neurodegenerative conditions. Human prion diseases may be divided into three categories: acquired, inherited or unexplained—termed sporadic Creutzfeldt-Jakob disease (CJD). Clinically, sporadic CJD and inherited prion disease show remarkable diversity, being associated with several subtypes and eponymous syndromes, and are often misdiagnosed as a result. The aetiology and clinical heterogeneity of sporadic neurodegenerative diseases are poorly understood.

Inherited prion disease accounts for ~15% of human prion diseases, and are always associated with coding mutations in the prion protein gene (PRNP) (Mead, 2006). Two types of pathogenic PRNP mutation exist: point mutations leading to an amino acid substitution in the prion protein or the production of premature stop codons; and alteration of the number of octapeptide repeats. The normal prion protein octapeptide repeat region is composed of a nonapeptide followed by a tandem repeat of four copies of an octapeptide and lies between codons 51 and 91 (Kretzschmar et al., 1986). Insertions of up to nine extra, and deletion of two, octapeptide repeats have been described in patients.

Typically, octapeptide repeat insertion (OPRI)-associated prion disease presents as a syndrome of slowly progressive multifocal dementia accompanied by dyspraxia and cerebellar ataxia. However, the clinicopathological phenotype, both within and between OPRI families shows significant variability. Age of onset varies from adolescence to old age, the clinical course from several months to >15 years and the dominant clinical features may be psychiatric, cognitive, cerebellar or extrapyramidal (Collinge et al., 1992; Laplanche et al., 1999; Moore et al., 2001; Mead et al., 2006; Webb et al., 2008). This is partly due to a coding polymorphism of PRNP codon 129 (Poulter et al., 1992; Mead et al., 2006, 2007). Much of the clinical heterogeneity remains unexplained; however, candidate factors include variation of modifier loci (Lloyd et al., 2001) and heterogeneity of the misfolded prion protein itself (Wadsworth et al., 2006).

In the first patient series of this mutation we report the clinical phenotype, investigative findings, molecular genetic and neuropathological features of all known UK patients with 4-OPRI. We show that the clinical phenotype of 4-OPRI prion disease is distinct from that seen in patients with 5- or 6-OPRI. For a genetically determined disease, we found that a family history of early-onset dementia was remarkably uncommon. To explain this, we provide evidence that the mutation alone appears not to be sufficient to consistently cause the disease: all UK patients were homozygous at two common polymorphisms, in and upstream of PRNP. Our findings provide a precedent for the mechanisms of penetrance and the aetiology of apparently sporadic neurodegenerative diseases.

Materials and methods

Molecular genetics

Genomic DNA was extracted from peripheral blood using the Nucleon Bioscience BACC2 DNA extraction kit. The entire open reading frame of PRNP was assessed by direct sequencing from genomic DNA. The size of insertions was confirmed by fractionation of amplicons from polymerase chain reaction designed to amplify the octapeptide repeat region by agarose gel electrophoresis. The exact nature of additional repeat units was confirmed by a combination of subcloning and by direct sequencing. Sequence data were analysed using allele discrimination assay Seqcape software. rs1029273 upstream of the PRNP open reading frame (Mead et al., 2001) was genotyped using the Taqman 5′ nuclelease allele discrimination assay. For PRNP haplotype analysis, microsatellite markers D20S181, D20S193, D20S473, D20S867, D20S889, D20S116, D20S482, D20S597, D20S895, D20S849, D20S873, D20S595 and D20S194 were amplified. Data were analysed using ABI GeneMapper software v4.0.

For relatedness testing, a subset of the Powerplex16 microsatellite marker set (Promega) was utilized and processed in a similar manner to PRNP microsatellites. This subset included microsatellite markers VWA, D5S818, D13S317, D7S820, D16S539, TH01, TPOX, CSF1PO, PENTA E and PENTA D. Following allele discrimination assay GeneMapper software analysis to provide allele sizes (basepairs), these data were subsequently converted to equivalent Powerplex16 allele sizes (numerical scores) for use in the ML-Relate program (Kalinowski et al., 2006).

Samples

Samples were obtained for 10 patients (Table 1 and Supplementary Material) with informed consent by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh. The clinical, genetic, biochemical and neuropathological studies were approved by the National Prion Clinic, London, or the National CJD Surveillance Unit, Edinburgh.

Statistical analysis

Means, standard deviations (SDs), tests of normality, Mann-Whitney U-test, binomial probability tests, linear regression and χ²-tests were performed using the Statistical Package for the Social Sciences package (SPSS Inc.). Genetic association studies were performed with the use of PLINK (Purcell et al., 2007).

Genealogical investigations

Family lineages were traced back to identify cause of death of parents of affected individuals. Birth and death certificates were obtained from the General Register Office via their website. Where records were not available the original clinical notes, out-patient letters and medical records were used. A family history of neuropsychiatric illness was considered positive when there was early-onset dementia (age <65 years) or a later-onset progressive dementia or behavioural disturbance in the context of abnormal neurological signs leading to death, in keeping with the clinical picture observed in this case series.
Neuropathology

Tissue was fixed in 10% buffered formal saline followed by incubation in 98% formic acid for 1 h. Following post-fixation for 24 h in 10% buffered formal saline, the tissue samples were processed through graded alcohols and paraffin wax embedded.

Paraffin sections were cut at a nominal thickness of 7 μm, boiled in a low ionic strength Tris-ethylenediaminetetraacetic acid buffer, pH 7.8 for 20 min, followed by 15 min in 98% formic acid. Primary antibodies used were anti-glial fibrillary acidic protein rabbit polyclonal anti-serum (Dako), anti-prion protein monoclonal antibodies ICSM 35 (D-Gen Healthcare), anti-prion protein monoclonal antibody 3F4 using high sensitivity enhanced chemiluminescence as described previously (Wadsworth et al., 2006). At least eight blocks were analysed in each case, including frontal, occipital and parietal cortices, temporal cortex with hippocampus, basal ganglia, thalamus, cerebellum and brainstem.

Expression analysis

Whole blood was drawn into ethylenediaminetetraacetic acid and PAXgene™ tubes at a Donor Suite of the National Blood Service with informed consent. DNA was extracted from ethylenediaminetetraacetic acid whole blood using the Nucleon BACC3 kit (GE Healthcare). Samples were genotyped for PRNP codon 129 (rs1799990) and rs1029273 by allelic discrimination using the SDS7500 (Applied Biosystems) system. Primer and probe sequences are available on request. RNA was extracted from PAXgene tubes using the PAXgene™ 96 Blood RNA kit (PreAnalytiX). RNA (1 μg) from each individual homozygous for the M129 allele was reverse transcribed using Omniscript (Qiagen) with random hexamer priming. PRNP transcript levels were measured using the SDS7500 by relative quantification using the delta-delta-Ct method with two different primer limited endogenous controls (β-actin and TATA box binding protein) each duplexed separately with PRNP (assay details on request). The endogenous controls were selected for suitability using geNorm (http://genomebiology.com/2002/3/7/research/0034) to minimize candidate endogenous gene variability in human whole blood. Results of the relative quantities of the PRNP transcript represent the normalization of real-time quantitative polymerase chain reaction data by geometric averaging of relative quantities calculated using the two internal reference genes.

Immunoblotting

Human brain (frontal cortex) was prepared as 10% w/v homogenates in Dulbecco’s sterile phosphate buffered saline lacking Ca2+ and Mg2+ ions and analysed after proteinase K digestion (12.5 or 50 μg/ml final protease concentration, 1 h, 37°C) by immunoblotting with anti-prion protein monoclonal antibody 3F4 using high sensitivity enhanced chemiluminescence as described previously (Wadsworth et al., 2001; Hill et al., 2006).

Results

Clinical findings

The principle clinical features are shown in Table 1 and investigations in Table 2. In summary, the predominant clinical syndrome was a rapidly progressive cortical dementia with myoclonus, motor and cerebellar signs resembling sporadic CJD. A pure cognitive syndrome was apparent in one patient (Patient IV) in which there had been a slowly progressive decline in episodic memory over 7 years in the absence of neurological signs. This patient has a moderate cortical dementia with early defects in episodic memory.
and executive functions, clinically resembling Alzheimer’s disease. Memory deficits and more widespread cognitive decline were the predominant features in Patients I, III, IV, VII, VIII and X. A parental history typical of inherited prion disease was only rarely seen in our patient series.

Age at onset of disease was known for all patients and disease duration was known for nine patients. Both showed considerable variability, the youngest patient presenting in her late thirties and the oldest aged 85 years (or possibly 70 years; see description of living Patient IV in the Supplementary Material). The longest illness duration was 77 months (Patient III) although Patient IV is still alive aged 92 years, with a minimum disease duration of 7 years. The mean (SD) age of onset was 60 (12.97) years (range 39–85 years) with a median disease duration of 414 days (range 59–2319 days).

Biochemical (urea and electrolytes, liver function tests, serum calcium, serum copper, serum angiotensin-converting enzyme, and serum vitamin B12 and folate), haematological (full blood count and erythrocyte sedimentation rate), serological (anti-nuclear antibodies, rheumatoid factor, syphilis serology and anti-cardiolipin antibodies) and thyroid function tests, when performed, were within normal limits. CSF examination was performed in six patients (Table 3). Protein 14-3-3 was consistently positive in those tested (n = 4).

### Genetic analysis

The complete PRNP open reading frame was sequenced in nine patients. DNA was unavailable for Patient IX (the identical twin of Patient X, monozygosity not genetically confirmed). All patients tested were shown to have an additional four R2 repeats, and to be homozygous for methionine at codon 129. No other sequence variations were detected.

We genotyped PRNP-linked and unlinked microsatellites to test: (i) the number of separate ancestral occurrences of the 4-OPRI mutation; and (ii) cryptic close kinship within our series. Relatedness testing at unlinked microsatellites confirmed a mother–son relationship between Patients III and IV ([P] = 6 x 10^{-6}, maximum likelihood of parent offspring 0.57). Other relatedness in this patient series, closer than second degree, was excluded by this analysis. For linked microsatellites, Patients II, VII and X shared a haplotype of 2.5 Mb, differing from the mother/son and each other, at a single 3’ marker (D20S194). Two further patients, Patients I and VI, shared with the above five patients a central core haplotype spanning ~1.2 Mb (D20S889–D20S895). Finally, Patients V and VIII demonstrated a shared 1 Mb haplotype with those described above between D20S97 and D20S895 only, although these two patients shared an extended haplotype of 2 Mb (D20S181–D20S849). Overall our data were consistent with multiple (at least two) ancestral occurrences of 4-OPRI in the UK; however, we cannot exclude a single ancestral occurrence several centuries in the past with time for recombination and mutation events to disrupt the disease haplotype.

**Table 2 Investigations**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Early-onset dementia in parent</th>
<th>EEG</th>
<th>CT</th>
<th>MRI</th>
<th>Neuropathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NA^a</td>
<td>Typical</td>
<td>Gross generalized atrophy</td>
<td>Not done</td>
<td>CJD</td>
</tr>
<tr>
<td>II</td>
<td>No</td>
<td>Typical</td>
<td>Atrophy</td>
<td>Not done</td>
<td>CJD</td>
</tr>
<tr>
<td>III</td>
<td>No^b</td>
<td>Non-specifically abnormal</td>
<td>Normal</td>
<td>Not done</td>
<td>CJD</td>
</tr>
<tr>
<td>IV</td>
<td>No</td>
<td>Not done</td>
<td>Not done</td>
<td>CJD</td>
<td>CJD</td>
</tr>
<tr>
<td>V</td>
<td>Yes</td>
<td>Non-specifically abnormal</td>
<td>Atrophy</td>
<td>High signal in right caudate head and temporal lobe atrophy</td>
<td>NA (patient alive)</td>
</tr>
<tr>
<td>VI</td>
<td>No</td>
<td>Non-specifically abnormal</td>
<td>Not done</td>
<td>Normal</td>
<td>CJD</td>
</tr>
<tr>
<td>VII</td>
<td>No</td>
<td>Suggestive</td>
<td>Atrophy</td>
<td>Not done</td>
<td>CJD</td>
</tr>
<tr>
<td>VIII</td>
<td>No</td>
<td>Non-specifically abnormal</td>
<td>Mild atrophy</td>
<td>Left chronic subdural haematoma</td>
<td>CJD</td>
</tr>
<tr>
<td>IX</td>
<td>No^c</td>
<td>Non-specifically abnormal</td>
<td>Atrophy</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>X</td>
<td>No^c</td>
<td>Normal</td>
<td>Unknown</td>
<td>Atrophy</td>
<td>CJD</td>
</tr>
</tbody>
</table>

A family history was considered positive if there was a history of dementia in a parent with onset prior to the age of 65 years, or a late-onset neurodegenerative disease associated with dementia and neurological signs consistent with inherited prion disease. For EEG, typical refers to classical findings associated with CJD. NA = Not available.

a Family history was censored.

b Son of Patient IV; however, we cannot be certain that Patient IV has inherited prion disease as her clinical phenotype is consistent with Alzheimer’s disease.

c Monozygotic twins, family history refers to parents.
the control UK population (384/1132 UK control haplotypes). The probability that all wild-type haplotypes would be rs1029273C-129M is $P = 5.9 \times 10^{-5}$ (binomial probability). rs1029273C is usually found on a 129M chromosome, but the significance of the association of rs1029273C and 4-OPRI could not be accounted for by an association driven by 129M alone ($P = 0.003$, binomial probability, of finding that all nine wild-type alleles were rs1029273C, with the assumption that all patients were genotype 129MM). With the conservative assumption that Patient IV had Alzheimer’s disease rather than prion disease, these associations remain significant (for the rs1029273C-129M haplotype $P = 1.7 \times 10^{-4}$, for rs1029273C independent of 129M $P = 0.005$).

We also compared the clinical phenotype of 4-OPRI with slightly larger insertional mutations. We did not compare 4-OPRI with smaller insertions as these have been identified in healthy control populations and are not clearly pathogenic (Beck et al., 2010).

OPRI length had a profound effect on mean age of onset with 5- and 6-OPRI having earlier ages of onset. Mean (SD) age at onset in UK patients with 5-OPRI in those with codon 129MM was 42.3 (12.4) years (range 26–63 years, $n = 6$) (Mead et al., 2007). Mean (SD) age of onset in UK patients with 6-OPRI in those with codon 129MM was 31.4 (5.7) years (range 20–49 years, $n = 30$) (Mead et al., 2006); $P = 0.04$ for comparison between 4-OPRI and 5-OPRI, and $P < 0.001$ for 4-OPRI compared with 6-OPRI. The relationship between increasing size of octapeptide repeat insertion, codon 129, and age of clinical onset is shown in Fig. 1. Duration of disease was also significantly shorter ($P < 0.001$) compared with patients with 6-OPRI. However, we found no significant difference between 4- and 6-OPRI for a number of clinical parameters, including myoclonus, cerebellar, extrapyramidal or pyramidal signs.

We went on to consider the frequency and phenotypic effects of rs1029273C-129M in other inherited prion diseases. Here we tested samples from 144 individuals with various inherited prion diseases (5-OPRI, $n = 11$; 6-OPRI, $n = 51$; P102L, $n = 36$; P105L, $n = 1$; A117V, $n = 12$; D178N, $n = 5$; E200K, $n = 26$; and Q212P, $n = 2$). None of the other inherited prion diseases had an absolute association with the upstream risk factor; however, in the entire series, rs1029273C was strongly associated with disease status compared with healthy controls (odds ratio = 1.62, $P = 2.5 \times 10^{-4}$, $\chi^2$-test, 1 degrees of freedom) with an increased frequency of the risk allele in inherited prion diseases. This overall association is potentially confounded by linkage between rs1029273C and certain mutations causing the rs1029273 allele linked to the mutation to be more frequent because of hitch-hiking. It is known that the large UK kindred segregating a 6-OPRI mutation occurs on an rs1029273C-129M haplotype; however, large UK kindreds segregating P102L and A117V occur on rs1029273T-129M and rs1029273T-129V haplotypes. The haplotype background of other mutations is not known with certainty; microsatellite haplotype analysis in P102L, E200K, D178N and 5-OPRI is most consistent with multiple ancestral events. As a result, the mutation haplotype background has little overall impact on the frequency of rs1029273C and adjusting for the most probable background where known, had little effect on the statistical association reported above.

We went on to consider the strength of the association of rs1029273 in different inherited prion diseases, such as those most similar to 4-OPRI on the basis of reduced penetrance and a CJD-like clinical phenotype (e.g. 5-OPRI, E200K, D178N). In this
analysis, we considered only 129MM individuals in cases and controls to exclude the possibility of an association being confounded by hitch-hiking with codon 129. In these three inherited prion diseases there were 47 rs1029273C alleles and 15 rs1029273T alleles, compared with 288/188 in controls, respectively (\( P = 0.019, \chi^2\)-test, 1 degrees of freedom). A similar analysis in the early-onset, highly penetrant inherited prion diseases (e.g. 6-OPRI, P102L, A117V) showed no significant associations independent of the codon 129 genotype. The rs1029273 genotype did not have any effect on the age of onset of inherited prion disease (for example, 6-OPRI, age of onset for rs1029273C = 33 years, rs1029273T = 31 years; \( n = 27 \)), suggesting that in 4-OPRI, and possibly other similar inherited prion diseases, the wild-type chromosome determines the risk of a patient getting disease at any age rather than modifying the age of onset of disease itself.

Electroencephalography

EEGs were performed in nine patients. Two showed characteristic periodic complexes associated with sporadic CJD. One was ‘highly suggestive’ of sporadic CJD and a further five were abnormal but not suggestive of CJD. One was normal. Brief summaries of individual reports are provided in the appropriate patient histories (Supplementary Material).

Neuroimaging

CT scans were performed on seven patients (Patients I, II, III, V, VII, VIII, IX and X) and MRI on five (Patients II, IV, VI, VIII and X). Findings varied from normal appearances to generalized cortical atrophy with cerebellar involvement, with one case having right caudate head and temporal lobe high signal (appearances considered suspicious but not diagnostic of CJD; Case III). In one patient, a left-sided subdural haematoma was seen (Patient VIII). Detailed information about sequences performed for some patients was not available, and were often variable between patients as scans were performed at different stages of magnetic resonance technology.

Neuropathology

Seven patients had post-mortem examinations. Patients II, III and VIII were examined in detail (refer to ‘Materials and methods’ section). A brief summary of salient neuropathological features for other patients with examined post-mortem is included in the patient histories (Patients I, VI, VII and X); however, the three described below are representative of the findings overall.

The macroscopic finding of a subdural haematoma was histologically confirmed in Patient VIII, showing dura mater as well as granulation tissue with extensive capillarization and haemosiderin deposition. In all three cases examined in detail, there was moderate to severe spongiform degeneration in all neocortical areas, often in a patchy distribution. This was associated with a moderate, focally severe neuronal loss with destruction of cortical grey matter (Figs 2A and Supplementary Fig. 1A). In these areas, there was also a severe proliferation of astrocytes, highlighted by glial fibrillary acidic protein immunostaining. Prion protein immunostaining showed a very delicate network of twisted prion protein-positive structures, which were most likely axons and dendrites (Fig. 2B). Typical synaptic prion protein deposits were not seen. Also, thin thread-like structures in the cortex and white matter were labelled, probably corresponding to intra-axonal abnormal prion protein (Fig. 2C and Supplementary Fig. 1B). There was no synaptic pattern and no deposition of plaques anywhere in the cortex. In contrast, the cerebellum showed features that are distinct, and very different from the pathology in the neocortex: there was no cerebellar spongiform degeneration in Case VIII, and only moderate gliosis that was pronounced in the molecular layer. Cases II and III showed moderate microvacuolar spongiform change in the molecular layer (Supplementary Fig. 1C). Prion protein immunostaining showed a very characteristic and unique pattern, where the staining appeared to extend along Purkinje cell processes or was contained within Purkinje cell processes in the molecular layer oriented perpendicularly to the surface. Small rounded deposits were also present in the granular layer in Cases II and III (Fig. 2F and G and Supplementary Fig. 1D). No amyloid plaques were present in the cerebellum. There was also deposition of hyperphosphorylated tau, which varied in shape and extent: while the occipital cortex showed the most intense deposition of hyperphosphorylated tau with formation of neuritip threads and occasional intraneuronal tangles (Fig. 2E), all other cortical areas showed much smaller, stub-like inclusions (Fig. 2D). The cerebellum was almost devoid of hyperphosphorylated tau structures, with only occasional granular inclusions (Fig. 2H). No beta-amyloid was seen in any of these areas.

Expression analysis

We hypothesized that the susceptibility effect of the rs1029273C-129M haplotype was conferred by increased expression of wild-type prion protein. We tested this by measuring PRNP expression in whole blood against selected control genes. Healthy UK blood donors (\( n = 145 \)) were sampled, 51 were homozygous for methionine at codon 129, 14/51 had rs1029273CC genotype, 24/51 were CT heterozygous and 13/51 were TT homozygous. Normalized PRNP expression levels varied from 0.76 to 1.44 with a mean of 1.14 (95% CI 1.02–1.26, \( P = 0.03, \ t\)-test) in CC homozygous individuals relative to TT homozygous individuals, with CT heterozygous individuals having intermediate levels of expression. When individuals were ranked by PRNP expression, the top four individuals with highest levels of relative PRNP expression were rs1029273C-129M homozygous.

Prion protein immunoblotting

Frozen brain was available for analysis from Patients III, VI, VII and VIII. Frontal cortex (grey matter) was prepared as 10% w/v homogenate and analysed after limited digestion with proteinase K by immunoblotting using anti-prion protein monoclonal antibody 3F4. All four patient brain samples showed prominent protease-resistant PrPSc fragments in the molecular mass range of \( \sim 19–30 \) kDa with no evidence for the generation of lower molecular mass prion protein fragments or for the co-existence of different PrPSc types within the same brain sample (Fig. 3).
Previous analysis of brain from 4-OPRI Patients VI and VII together with brain tissue from patients with 2- and 6-OPRI (Hill et al., 2006) showed that the PrPSc types associated with these OPRI mutations are apparently indistinguishable with respect to glycoform ratios and fragment sizes from those observed in classical CJD (Hill et al., 2003; Wadsworth et al., 2008). Consistent with these findings, 4-OPRI Patient III propagated PrPSc with a type 2 fragment size (Fig. 3) that was indistinguishable from type 2 PrPSc seen in 4-OPRI Patient VII (Fig. 3). 4-OPRI Patient VIII propagated PrPSc with a type 3 fragment size (Fig. 3) that we have previously only observed in a single PRNP codon 129 methionine homozygous patient with sporadic CJD (Hill et al., 2003). The clear demonstration of the propagation of different PrPSc types among patients with 4-OPRI provides a molecular basis for phenotypic variability, although the small patient cohort examined here precludes significant analysis. However, in this context, it is interesting to note that Patient VI, who propagated type 1 PrPSc, had a short clinical duration and that this mirrors the association of type 1 PrPSc and short clinical duration seen in sporadic CJD (Wadsworth et al., 1999; Hill et al., 2003).

**Discussion**

We have presented the clinical, neuropathological and molecular genetic analysis of 10 patients with the 4-OPRI mutation of PRNP. All patients were homozygous for methionine at codon 129 and for the previously described sporadic CJD risk allele, rs1029273C. This statistically significant finding suggests that the risk of disease conferred by the 4-OPRI mutation alone may be enhanced by the wild-type chromosome. These additional factors being absent from relatives could account for the reduced penetrance of the mutation indicated by a relatively low frequency of familial concurrence of disease in these pedigrees. While this possibility might have been confirmed by genetic testing of healthy elderly relatives of 4-OPRI probands, there would be major implications for the families and this was not done. The sample of patients analysed was necessarily small and therefore the statistical significance of our genetic findings would not be robust to the addition of several further patients with 4-OPRI with different genotypes at codon 129 and rs1029273.

The effect of heterozygosity of the polymorphic codon 129 of PRNP on susceptibility to and/or incubation time is well
documented in sporadic, acquired (Collinge et al., 1991; Palmer et al. et al., 1991) and some forms of inherited prion disease (Poulter et al. et al., 1992). In 5-OPRI, 6-OPRI, P102L, A117V and F198S, heterozygosity at codon 129 is associated with an older clinical onset compared with homozygosity (Dlouhy et al., 1991; Poulter et al. et al., 1992; Mead et al., 2007; Webb et al., 2008, 2009). Prion disease susceptibility is thought to be conferred on a molecular level by homotypic protein–protein interactions (Palmer et al. et al., 1991) with an important role for the degree to which the wild-type and mutant prion proteins can adopt the same pathogenic conformations (Collinge, 1999; Hill and Collinge, 2003; Collinge and Clarke, 2007). Given that 4-OPRI with 129MM has an older clinical onset than many other inherited prion diseases, it is reasonable to predict that the age of onset of 4-OPRI with 129MV might lie beyond the average human lifespan. This would therefore explain the absence of patients with 129MV in our cohort. While this explanation is plausible, it would also be possible that the presence of the 129V on the wild-type allele prevents the stable generation of prions in 4-OPRI.

Our analyses support the concept of variable penetrance of the 4-OPRI mutation that is dependent upon more than just homology between mutant and wild-type prion protein at codon 129. Additional factors upstream of PRNP presumably act by altered expression of wild-type prion protein. Increased prion protein expression has been known for many years to be a potent susceptibility factor in transgenic mice (Bueeler et al., 1993), and we now report modestly increased expression of PRNP in blood conferred by the risk haplotype. We assumed that the rs1029273C-129M haplotype on the mutant chromosome was shared between all patients because of common ancestry. This conservative assumption was only partially supported by our haplotype analysis, which was in fact most consistent with several different mutational events. In this latter scenario, the observation that all mutant alleles were also associated with the rs1029273C-129M haplotype lends further support to the increased susceptibility conferred by this haplotype.

Four-OPRIs are the smallest insertional mutations of PRNP that are clearly pathogenic. Smaller (1-OPRI and 3-OPRI, but not 2-OPRI) insertions have been found incidentally and in healthy control populations (Beck et al., 2010). Four- through 6-OPRI shows a dramatically earlier clinical onset. Insertional mutations larger than 5-OPRI show similar ages of clinical onset (Mead et al., 2006). Thus, a correlation between insertion size and clinical phenotype is clear over this short range, and not for shorter or longer insertions. These clinical observations may form the basis for an informed correlation between molecular properties of mutant prion proteins and the consequences for the onset and clinical phenotype of the human disease.

Epistasis was initially used by Bateson in 1909 to describe the masking effect of an allele at one locus over a variant at another locus. The term is now more broadly used to describe an interaction between different loci, and has recently received a great deal of interest in complex disease genetics. Epistasis has been implicated in a number of human conditions, such as inflammatory bowel disease (Cummings et al., 2007), colorectal cancer (Felix et al., 2006) and among HLA class II alleles in human immune responses (Lincoln et al., 2009). However, to our knowledge, there are no examples of neurological diseases caused by a mutation associated with a common susceptibility haplotype. Our 4-OPRI inherited prion disease case series may thus provide a useful precedent for understanding how a rare high-risk mutation might typically manifest as an apparently sporadic neurodegenerative disease.

The immunohistochemistry showed a distinct pattern from sporadic CJD. There was very delicate staining of axons and dendrites, rather than synaptic or uncommon plaque-forming deposits that are seen in the sporadic form. The cerebellum in these patients also showed prion protein deposits that seem to follow the dendrites of the Purkinje cells, in that they extend perpendicularly to the surface, giving the cerebellum a ‘tigroid’ appearance. This appearance is similar to previously reported cases with 4-OPRI, and the pattern of cerebellar prion protein deposition is also seen in cases with 5-OPRI (Mead et al., 2007) or 6-OPRI (Capellari et al., 1997; King et al., 2003; Kovacs et al., 2007) but not that of longer (8-OPRI) inserts, which show deposition of frequent amyloid plaques in the cerebellum and the forebrain (Laplanche et al., 1999). In contrast to previous reports, we consistently observed fine, thread-like intra-axonal prion protein deposits in Case VIII and also in cases with 6-OPRI (Reiniger et al., 2010), most likely due to a more sensitive detection method for abnormal prion protein. Remarkably, there was frequent deposition of hyperphosphorylated tau in several cortical areas, even with a very subtle involvement of the cerebellum as reported earlier (Reiniger et al., 2010). Although this tau pathology may have arisen independently from the prion disease, the role of the prion disease in triggering hyperphosphorylation and a secondary tauopathy remains likely, in particular, as no deposition of beta-amyloid was seen in any of the brain regions examined.

![Figure 3](image-url)
The demonstration that the PrPSc types seen in patients with 4-OPRI are apparently indistinguishable from those seen in patients with sporadic CJD of the same codon 129 genotype appears difficult to reconcile with the distinct pattern of prion protein immunohistochemistry seen in 4-OPRI brain. However, in this context, it is important to note that we probe PrPSc conformation by looking at accessibility to scissile bonds cleaved by proteasine K at the N-terminus of the protein. While the sensitivity of the N-terminal third of PrPSc to cleavage by proteasine K does not appear to be altered by a 4-OPRI mutation, this mutation may confer distinct conformational preferences to the N-terminus, which could influence the kinetics of replication or the clearance of mutant PrPSc, thereby accounting for a distinct neuropathological phenotype. Methods other than proteasine digestion will now be required to reveal conformational differences between full-length PrPSc in patients with sporadic CJD and 4-OPRI.

In summary, we report evidence for a susceptibility haplotype that may be an important determinant of penetrance in 4-OPRI and possibly other similar inherited prion diseases. While the molecular basis of the susceptibility allele is unknown, one possibility is that increased risk is conferred by a combination of both homology at codon 129 and higher levels of expression of PRNP. This example may provide a valuable model for understanding other sporadic neurodegenerative diseases.

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Supplementary material

Supplementary material is available at Brain online.

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