



PRNP allelic series from 19 years of prion protein gene sequencing at the MRC Prion Unit

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Key Words:	prion, PRNP, inherited, allele, mutation screening, diagnosis



***PRNP* allelic series from 19 years of prion protein gene sequencing at the MRC Prion Unit**



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Short Title: Diagnostic molecular genetics of *PRNP*

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ABSTRACT: Mutation of the human prion protein gene (*PRNP*) open reading frame (ORF) accounts for almost all reported familial concurrence of prion disease. The more common mutations globally: octapeptide repeat insertions, P102L, D178N, E200K, and V210I have occurred in large multigenerational pedigrees and display autosomal dominant inheritance, however, many rare genetic changes have been reported but are of uncertain pathogenicity. Based on 19 years of *PRNP* sequencing at the MRC Prion Unit, London, and analysis of 3664 samples from patients referred with suspected prion disease and healthy populations, we present novel allele combinations, healthy control population data, results of screening the *PRNP* ORF in DNA from the entire referral series and the CEPH human genome diversity cell line panel. Of the 10 alleles detected in patients for which detailed cases histories are presented, 4 are unreported (G54S, D167N, V209M, Q212PP), two changes are thought to be pathogenic but have not been described in our regions (P105L from the UK, G114V from India and Turkey), and the remainder reported in healthy control populations or in *trans* to known pathogenic mutations suggesting non- or low pathogenicity (G54S, 1-OPRI, G142S, N171S, V209M, E219K). New genotype-phenotype

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correlations and population frequencies presented will help the diagnosis and genetic counseling of those with suspected inherited prion disease.

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KEY WORDS: prion, *PRNP*, inherited, allele, mutation screening, diagnosis

INTRODUCTION

Human prion diseases are invariably fatal neurodegenerative disorders with sporadic, infectious and inherited aetiologies. According to the protein-only hypothesis (Griffith, 1967), the normal cellular isoform of the prion protein (Prusiner, 1982) (PrP^{C}) is misfolded and aggregates into a disease-associated state termed PrP^{Sc} . Sporadic or classical Creutzfeldt-Jakob disease (sCJD), a rapidly progressive dementing illness, accounts for the majority of human cases with a relatively uniform geographical distribution and incidence of 1-2/million/year (Collinge, 2001). Acquired prion diseases, defined as those transmitted from humans or animals, include vCJD, kuru, growth-hormone and dura mater graft associated CJD. The acquired prion diseases are rare and variable in incidence and geography related to the distribution of their exposures. Inherited prion diseases (IPD) comprise around 10-15% of the total and are definitively diagnosed by *PRNP* gene (OMIM *176640) analysis.

PRNP comprises two exons with the ORF entirely contained within the second exon. PrP has 253 amino acids, N and C terminal signal peptides, one disulphide bond and a glycosylphosphatidylinositol anchor. Two domains are recognised with distinct allelic series: an unstructured N-terminal domain containing five repeats consisting of a single nonapeptide and four octapeptides, and a structured C-terminal domain. Over 30 different mutations have been described; these may be grouped into two types: alteration of the number of octapeptide repeats in the N-terminal domain of PrP, or missense mutation resulting in a premature stop codon or amino-acid variant in the C-terminal domain (Mead, 2006).

IPD is remarkably heterogeneous in its clinical presentation and course, and should be considered in all patients with early-onset dementing or ataxic illnesses. Additional to the need for genetic counselling, IPD is notable in that it may have a rapidly progressive clinical course, and therefore is often mistaken for classical CJD, or on occasion for inflammatory or infective brain diseases (Warren *et al.*, 2005). In the latter circumstance, invasive tests such as brain biopsy may be avoided by *PRNP* gene analysis of all patients with suspected prion disease. Second, because many IPDs have been experimentally transmitted to laboratory animals (Mead, 2006) and prions are resistant to conventional sterilization procedures (Bernoulli *et al.*, 1977), precautions should be taken to avoid secondary human transmission of prions on dental or surgical instruments, blood transfusion or organ donation from patients with IPD. Third, for public health purposes, precautions regarding secondary transmission also apply to those family members identified as at risk of inheriting a pathogenic *PRNP* mutation (see http://www.hpa.org.uk/infections/topics_az/cjd/incidents_panel.htm).

Given the importance of diagnostic accuracy in IPD, it is problematic that many of the mutations have been described only in single patients with prion disease or dementing illnesses. Further, there is no validated structural or computational effect or functional assay that distinguishes mutations causative of IPD from the non-pathogenic. It is inevitable that prion disease specialist centres will largely perform *PRNP* gene analysis on patients with suspected prion disease, thus through ascertainment bias, rare genetic variants might be erroneously associated with prion disease. Here we report findings from 15 years of diagnostic *PRNP* neurogenetics, highlighting several positive and negative allelic associations with prion disease. Where applicable and available, the appropriate ethnically matched healthy control population frequencies are also presented. In addition, we present the findings of screening more than 1000 healthy controls from a further 51 world populations with distinct ancestry that represent the CEPH human genome diversity cell line panel. These data may be useful in the interpretation of *PRNP* gene analysis and improve the diagnosis of IPD.

MATERIALS AND METHODS

Subjects

The London referral series includes 1885 subjects referred to the National Prion Clinic at the National Hospital for Neurology and Neurosurgery (NHNN) between 1990 and 2009 with suspected prion disease. As the NHS specialist referral centre for prion disease, the National Prion Clinic reviews all UK patients with prion disease. On occasion, non-UK residents may be assessed at the NHNN, and be referred for *PRNP* analysis. Additionally, international referrals for genotyping are received. The London referral series should therefore be viewed as highly selected, and largely but not exclusively, UK based. Prion disease was diagnosed as probable or definite according to established diagnostic criteria in a minority of cases, however, the majority of cases had unclear or unknown diagnoses and were not assessed or followed up by the National Prion Clinic. Healthy population samples were obtained as follows: Turkish population samples were sent courtesy of Professor Basak, Gujarati controls were made available courtesy of Dr Francesca Capon, and Jamaican controls were sent courtesy of Dr Douglas Higgs. The CEPH (Centre d'Etude du Polymorphisme Humain) human genome diversity cell line panel DNA was obtained from Professor John Hardy (Institute of Neurology, London). Jamaican controls relate to the individual case with [G142S]+[N171S], Gujarati controls relate to the G114V and G54S cases, and the Turkish controls relate to the D167N cases. All other cases were controlled for by examination of allele frequency in the white British controls or CEPH human genome diversity cell line panel controls. All samples were collected with informed consent. All subjects with *PRNP* mutations were assessed by a consultant neurologist, the majority at the National Prion Clinic. Other centers involved in the assessment include the Bogazici University in Istanbul (Turkey) and General University Hospital in Thessaloniki (Greece).

PRNP analysis

Peripheral blood samples were collected in EDTA. Genomic DNA (gDNA) was extracted using the Nucleon BACC2 DNA extraction kit following the supplied protocol. DNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer and diluted to stock at 20ng/μl. PCR primers were used to amplify the entire open reading frame and PCR was performed using MegaMix Blue PCR premix (Microzone). Amplicons were assessed by electrophoresis of 5μl product on a 2% ethidium bromide-stained agarose gel with 5μl HyperLadder IV size standard and visualised using a Biorad Gel Doc 1000 transilluminator and Quantity One 4.5.1 software. Amplicons were cleaned using MicroCLEAN (Microzone) then resuspended in 40μl 18MΩ ddH₂O. Sequencing reactions included BigDye v1.1 (Applied Biosystems) and BetterBuffer (Microzone) and both forward and reverse DNA strands were assessed. Standard run conditions were applied to electrophoresis of sequenced products on an Applied Biosystems 3130xl, using polymer POP7, 50cm arrays and standard run module with sample injection time of 15 seconds. Data analysis was performed using Applied Biosystems Seqscape software v2.5 where analysis filter settings were adjusted to allow assembly of poor data due to insertions or deletions (maximum mixed bases 95%, maximum Ns 95%, minimum clear length bp of 1, and minimum sample score of 1). Poor data or failed reactions were removed from projects by visual inspection of data.

Nucleotide numbering (tables 1 and 2) reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

All samples from affected individuals were assessed for the presence of octapeptide repeat insertion or deletion variants by size fractionation of PCR amplicons, created using primers which amplified the octapeptide repeat region, and assessed by electrophoresis and visualization as described above. Assessment of the presence of insertion or deletion variants in healthy control individuals was performed by sequence electropherogram inspection only.

Confirmation of missense mutations

A second assay was performed to confirm the presence of missense mutations. Confirmatory assays performed comprised: restriction fragment length polymorphism, allele specific oligonucleotide hybridization or re-sequencing analysis depending upon the variation tested (see table 1). Re-sequencing of gDNA utilized alternative primers, 5'-GTGGCCACATGGAGTGACCTGGGCCTC-3' (for) and 5'-GAAAGATGGTGAAAACAGGAAGACC-3' (rev). Full details of PCR, sequencing, and RFLP conditions, including primer details, have been previously published(Wadsworth JD *et al.*, 2008).

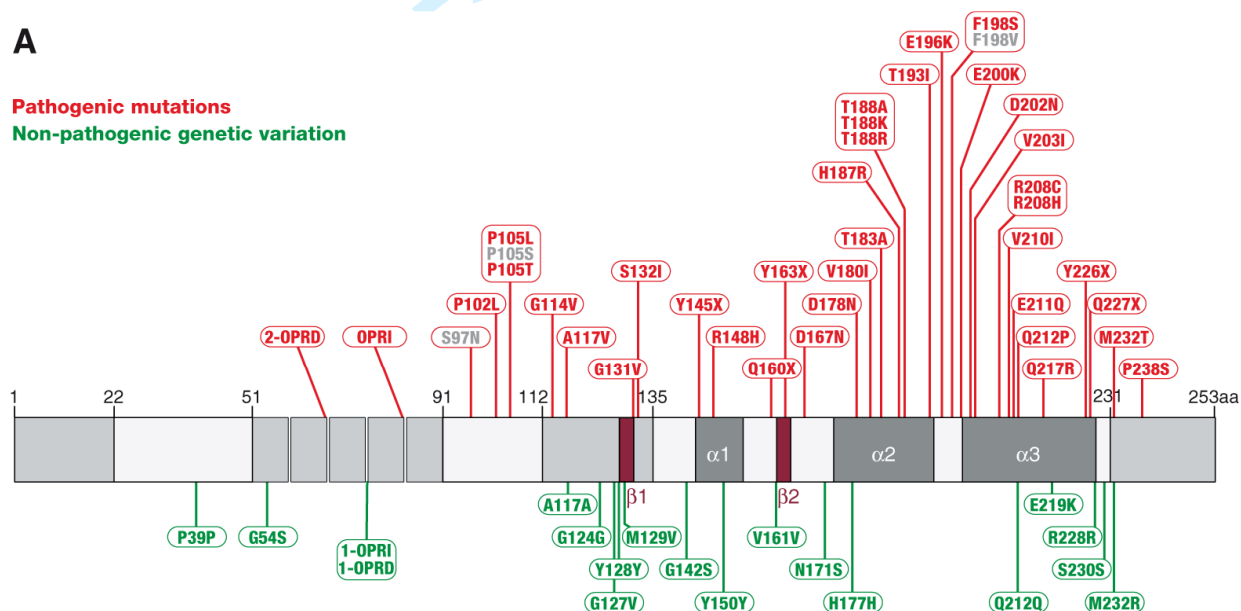
RESULTS

PRNP analysis and population findings

For more commonly occurring definitely pathogenic mutations please see more detailed publications (Mead S *et al.*, 2006; Webb *et al.*, 2008). In patients, novel alleles or genotypes were found at codons 54, 167, 209 and 212. Known rare genetic mutations associated with probable or definite prion disease were found at codons 105, 114, and 212. Previously suspected pathogenic variants found in association with healthy populations were found in the octapeptide repeat region and codon 171 (figure 1).

A

Pathogenic mutations
Non-pathogenic genetic variation



B

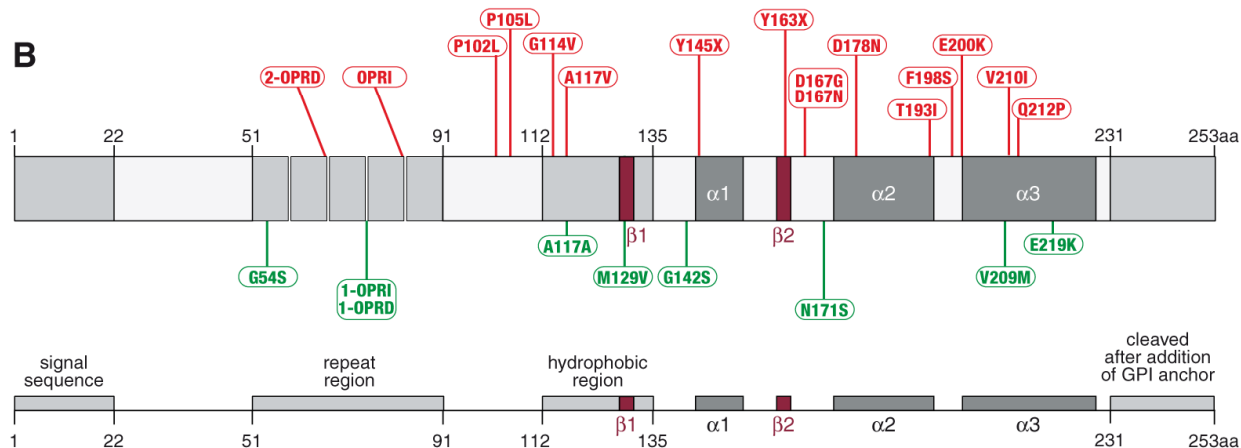


Figure 1A and 1B

Schematic demonstrating *PRNP* with various domains and tertiary protein structures labelled, including pathogenic mutations in red text and non-pathogenic genetic polymorphisms in green text. Variations found in neurodegenerative disease cases, but without a diagnosis of prion disease, are shown in grey text. A) all variations of *PRNP*, including those reported to date and novel variation detected in healthy control individuals in this study. B) mutations and polymorphisms of *PRNP* detected at the MRC Prion Unit in 1885 cases.

In the London referral series a total of 14 pathogenic missense mutations were detected, 5 different octapeptide repeat insertion mutations by length, and one 2 octapeptide repeat deletion. In the same series a total of 6 non-synonymous and 1 synonymous polymorphisms, and deletion or insertion of a single octapeptide repeat unit were detected. The frequency of variations discovered in individuals with suspected prion disease were also assessed in appropriate control populations, white British, Turkish, Gujarati and Jamaican in order to assess the likely pathogenicity of novel variations. These data and the results of screening the *PRNP* ORF are presented in table 1 with 95% confidence intervals shown. .

Variant Description		white British			Turkish			Gujarati			Jamaican			London referral series			
HGV DNA [‡]	HGV AA	p	95% CI [†]		p	95% CI [†]		p	95% CI [†]		p	95% CI [†]		p	#	95% CI [†]	
c.117G>A	p.P39P	0	0.000	0.029	0	0.000	0.036	0.005	0.001	0.030	0	0.000	0.019	0	0	0.000	0.001
c.160G>A	p.G54S	0	0.000	0.029	0	0.000	0.036	0.005	0.001	0.030	0	0.000	0.019	0.0003	1	0.000	0.002
c.202_249del	p.P68_Q83del	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.225_226ins	p.Q75_P76insPHGGGWGQ	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.181_204del	p.H61_P68delPHGGGWGQ	0.004	0.001	0.024	0.03	0.010	0.081	0	0.000	0.021	0.15	0.107	0.206	0.0029	11	0.002	0.005
c.229_252del	p.H77_P84delPHGGGWGQ	0.009	0.001	0.031				0	0.000	0.021				0.0042	16	0.003	0.007
-	2-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0005	2	0.000	0.002
-	4-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0027	10	0.001	0.005
-	5-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0024	9	0.001	0.005
-	6-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0141	53	0.011	0.018
-	7-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
-	9-OPRI*	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.305C>T	p.P102L	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0069	26	0.005	0.010
c.314C>T	p.P105L	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.341G>T	p.G114V	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0005	2	0.000	0.002
c.351A>G	p.A117A	0.01	0.002	0.027	0.04	0.015	0.095	0.01	0.003	0.039	0	0.000	0.019	0.0307	116	0.025	0.000
c.[350C>T;351A>G]	p.A117V	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0053	20	0.026	0.037
c.372C>G	p.G124G	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0005	2	0.001	0.002
c.385A	p.M129	0.65			0.67			0.77			0.68			0.70			
c.385G	p.V129	0.35			0.33			0.23			0.32			0.30			
c.424G>A	p.G142S	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0.03	0.014	0.064	0.0005	2	0.000	0.002
c.435T>G	p.Y145X	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.499G>A	p.D167N	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.500A>G	p.D167G	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.512A>C	p.N171S	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0.05	0.027	0.090	0.0005	2	0.000	0.002
c.532G>A	p.D178N	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0100	38	0.007	0.014
c.578C>T	p.T193I	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.593T>C	p.F198S	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.598G>A	p.E200K	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0117	44	0.009	0.016
c.625G>A	p.V209M	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0003	1	0.000	0.002
c.628G>T	p.V210I	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0008	3	0.000	0.002
c.635A>G	p.Q212P	0	0.000	0.029	0	0.000	0.036	0	0.000	0.021	0	0.000	0.019	0.0013 ‡	5	0.001	0.003
c.655G>A	p.E219K	0	0.000	0.029	0	0.000	0.036	0.04	0.019	0.076	0	0.000	0.019	0.0034	13	0.002	0.007
	Codon 129 genotype (%)																
	MM		43			42			58			46			52		
	MV		43			50			38			44			37		
	VV		14			8			4			10			11		

Table 1

Frequency of genetic variations in *PRNP* and codon 129 genotypes in the London referral series and healthy controls from 4 populations. Three non-white British control populations were chosen to best match the ethnicity of cases with missense variants in the London referral series. Grey bars highlight proposed or known pathogenic mutations. freq=frequency of alleles; †=95% confidence intervals; #=number of cases *=Octapeptide repeat insertion mutations, for clarity and space reasons exact nature of mutations are not given here (available on request); ‡=one homozygous individual; †=HGVS DNA relative to GenBank Reference: NM_000311.3. Nucleotide

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numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

In addition, results of screening the ORF of *PRNP* in the CEPH human genome diversity cell line panel were recorded by continental groups, presented in table 2, and by individual populations, presented graphically in figure 2. Details of allele counts and genotypes for each individual population are available on request. In the 1007 healthy control individuals of the diversity panel a total of 5 non-synonymous and 6 synonymous polymorphisms were detected. In addition, 4 single octapeptide repeat deletions and 2 single octapeptide repeat insertions were detected. These differed either by the nature of the insert or deletion itself, or by the allele on which the variation was found. Allele frequencies are presented with 95% confidence intervals, and the corresponding codon 129 background for each variation.

Variant Description			Subsaharan Africa		Asia		Europe		MiddleEast/N Africa		Oceania		Latin America	
HGV DNA‡	Codon 129 Allele	HGV AA	freq	95% CI†	freq	95% CI†	freq	95% CI†	freq	95% CI†	freq	95% CI†	freq	95% CI†
c.159C>T	M	p.G53G	0	0.000 0.017	0.001	0.000 0.006	0	0.000 0.012	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.160G>A	M	p.G54S	0	0.000 0.017	0.004	0.001 0.010	0	0.000 0.012	0.006	0.001 0.021	0	0.000 0.050	0	0.000 0.017
c.204T>C	M and V	p.P68P	0.014	0.003 0.039	0.002	0.000 0.008	0	0.000 0.012	0	0.000 0.011	0.028	0.003 0.097	0	0.000 0.017
c.181_204del	M	p.H61_P68del	0.055	0.029 0.093	0.002	0.000 0.008	0.003	0.000 0.018	0.009	0.002 0.026	0	0.000 0.050	0	0.000 0.017
c.181_204del	V	p.H61_P68del	0.005	0.000 0.025	0	0.000 0.004	0	0.000 0.012	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.225_226ins	M	p.Q75_P76insPHGGGWGQ	0	0.000 0.017	0	0.000 0.004	0.003	0.000 0.018	0.003	0.000 0.016	0	0.000 0.050	0	0.000 0.017
c.[204T>C]+[229_252del]	V	p.[P68P]+[H77_P84del]	0.014	0.003 0.039	0	0.000 0.004	0	0.000 0.012	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.229_252del	M	p.H77_P84del	0.145	0.102 0.199	0.002	0.000 0.008	0	0.000 0.012	0.015	0.005 0.034	0	0.000 0.050	0	0.000 0.017
c.249_250ins	M	p.Q83_P84insPHGGGWGQ	0	0.000 0.017	0.001	0.000 0.006	0	0.000 0.012	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.351A>G	V	p.A117A	0.045	0.022 0.082	0.010	0.005 0.020	0.019	0.007 0.042	0.035	0.018 0.061	0	0.000 0.050	0	0.000 0.017
c.372C>G	M	p.G124G	0	0.000 0.017	0.001	0.000 0.006	0.003	0.000 0.018	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.424G>A	M	p.G142S	0.059	0.032 0.099	0	0.000 0.004	0	0.000 0.012	0.012	0.003 0.030	0	0.000 0.050	0	0.000 0.017
c.450T>C	M	p.Y150Y	0	0.000 0.017	0	0.000 0.004	0	0.000 0.012	0.003	0.000 0.016	0	0.000 0.050	0	0.000 0.017
c.512A>C	V	p.N171S	0.050	0.025 0.088	0	0.000 0.004	0.003	0.000 0.018	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
c.531C>T	V	p.H177H	0	0.000 0.017	0	0.000 0.004	0	0.000 0.012	0	0.000 0.011	0.028	0.003 0.097	0	0.000 0.017
c.655G>A	M	p.E219K	0	0.000 0.017	0.026	0.016 0.038	0	0.000 0.012	0.006	0.001 0.021	0.056	0.015 0.136	0	0.000 0.017
c.695T>G	M	p.M232R	0	0.000 0.017	0.002	0.000 0.008	0	0.000 0.012	0	0.000 0.011	0	0.000 0.050	0	0.000 0.017
Alleles screened			220		862		310		340		72		210	
Codon 129 Allele Count														
p.M129			128 (58)		730 (85)		218 (70)		235 (69)		55 (76)		75 (36)	
p.V129			92 (42)		132 (15)		92 (30)		105 (31)		17 (24)		135 (64)	
Codon 129 Genotype														
MM			40 (36)		310 (72)		73 (47)		84 (49)		22 (61)		17 (16)	
MV			48 (44)		110 (26)		72 (46)		67 (39)		11 (31)		41 (39)	
VV			22 (20)		11 (2)		10 (7)		19 (12)		3 (8)		47 (45)	

Table 2

A summary of all genetic variations detected in the CEPH human genome diversity cell line panel in the ORF of *PRNP*. Data from 51 world populations are grouped by continental divides. Codon 129 allele counts and genotypes are displayed by absolute numbers and by percentage in parentheses. freq=frequency of alleles; †=95% confidence intervals; ‡=HGVS DNA relative to GenBank Reference: NM_000311.3. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

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HUMAN MUTATION

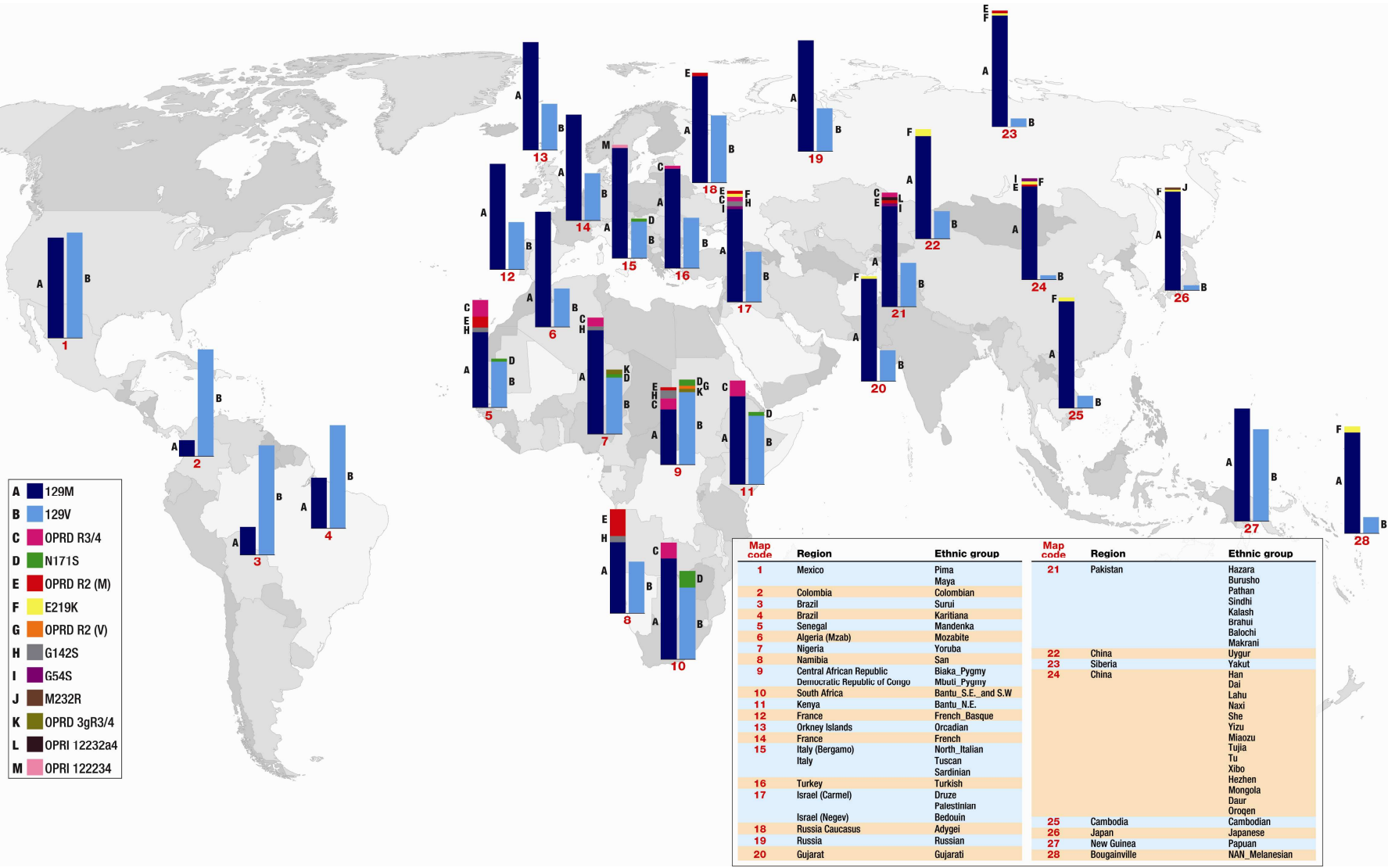


Figure 2
 CEPH human genome diversity cell line panel screening of *PRNP* (genotype details available on request). For each population, or ethnic group, the lefthand bar represents *PRNP* codon 129 methionine alleles, whilst the righthand bar represent valine alleles. The size of the coloured bands, which represent alleles detected, are proportional to the allele frequency detected in a given population.

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MUTATION IN BRIEF

HUMAN MUTATION

Clinical findings in patients with coding variants

P102L + 1-OPRI, p.[P102L]+[Q75_P76insPHGGGWGQ], c.[305c>t]+[225_226inscctcatggtggtgctggggcag]; codon 129MM

We investigated a small pedigree, originally from Sicily that segregated for P102L and a single extra octapeptide repeat (1-OPRI). The proband presented at the age of 56 with a typical history of Gerstmann-Straussler syndrome including a progressive cerebellar ataxia, late cognitive decline and disease duration of 5 years. The proband was found to possess P102L and a normal octapeptide repeat region. The proband's sister, brother and mother were affected with a similar illness, dying at 54, 57 and 60 years of age respectively but *PRNP* analysis was not performed. Two further siblings were unaffected. A healthy descendant of the proband was found to possess the P102L mutation on one chromosome and a 1-OPRI on the other chromosome. The 1-OPRI polymorphism was identified by size fractionation. The octapeptide repeat region comprises a nonapeptide (designated R1) followed by four octapeptides (R2, R2, R3 and R4) which may be distinguished by non-coding nucleotide variants. Within the setting of octapeptide repeat insertions or deletions, further repeats types at the nucleotide level have been described (Goldfarb *et al.*, 1993), for example R2a, where the first codon of eight is R2-like, the central portion identical to either R2 or R3, and the last two codons R3-like. By sequencing this 1-OPRI was characterised as an additional R2 repeat leading to an abnormal R1-R2-R2-R2-R3-R4 repeat region. There was no maternal history of neurological disorders and maternal testing for 1-OPRI variant was not performed. The presence of a 1-OPRI *in trans* to a well established pathogenic mutation supports the conclusion that 1-OPRI may be found as a rare population variant which is supported by our finding of two different 1-OPRI variants, R12234 in individuals from Sardinia and R12232a4 in individuals from the Sindhi linguistic group of Pakistan.

P105L + V209M, p.[P105L]+[V209M], c.[314C>T]+[625G>A]; codon 129VV

Aged 33, a British woman was diagnosed and treated for a psychotic depression. At that time she had clumsiness, balance and gait difficulties and required residential home support. Aged 34 she was investigated for increasing gait disturbances and was noted to have a spastic paraparesis with extrapyramidal features, dyspraxia, urgency of micturition, short term memory difficulties and significant frontal lobe dysfunction. MRI revealed moderate generalised atrophy, an EEG revealed non-specific changes and CSF examination revealed a slightly elevated S100 and absent 14-3-3 protein. The clinical course was one of rapid cognitive decline with psychiatric disturbance, incontinence, leg spasms, and cerebellar dysfunction. At the most recent review this patient, aged 43, was severely ill being bed bound, with a gastrotomy and requiring 24 hour nursing care. On limited formal examination she was mute, had fasciculation in the orbicularis oris, exhibited a pathological glabellar tap reflex, brisk jaw jerk and pout reflex and severe rigidity of the neck muscles. In the limbs she had marked rigidity with contractures, absent lower limb tendon reflexes and mute plantar responses. She was found to have two missense alleles at *PRNP*, P105L and V209M. P105L has been reported to segregate with IPD in Japan, but not previously in Europe. Genotyping of the asymptomatic elderly parents revealed a maternal V209M (codon 129MV) variant only, supporting the conclusion that this allele is a rare non-pathogenic missense change. Molecular analysis confirmed that P105L and V209M were present on different chromosomes

G142S + N171S, p.[G142S]+[N171S], c.[424G>A]+[512A>G]; codon 129MV

This patient presented aged 40 with dizziness, slurred speech, second grade nystagmus, and cerebellar ataxia. She has Jamaican parents. Nerve conduction studies revealed a peripheral neuropathy. Prion disease was suspected and *PRNP* analysis requested. N171S was first reported in *Nature* to be associated with psychiatric disorder in a Brazilian pedigree (Samaia *et al.*, 1997). Our analysis of the healthy Jamaican population revealed the G142S and

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N171S as independently segregating polymorphisms (table 1). We have also shown these changes to be present in the healthy Cameroonian and Yemeni African populations (Mead *et al.*, 2003). Furthermore, our screening of the CEPH human genome diversity cell line panel revealed the presence of G142S on 14% of alleles and N171S on 11% of alleles in the Biaka Pygmies, and G142S ranging between 2-8% of alleles and N171S ranging between 2-14% in additional Subsaharan African populations. G142S was also detected in Israeli populations, and N171S in Sardinian populations. Molecular analyses confirmed that in this patient G142S and N171S were present on different chromosomes.

G114V, p.G114V, c.341G>T; codon 129MV

This South Indian gentleman experienced excessive fatigue, perceived left sided weakness and sensory disturbances of his feet at the age of 75. In the following six months there was a rapid deterioration and he experienced recurrent falls. Examination revealed an asymmetrical akinetic rigid syndrome, broken pursuit eye movements, myoclonic jerks and mild apraxia with well preserved cognition. MRI revealed high signal change in the putamen and caudate nuclei bilaterally but particularly on the right. CSF examination was unremarkable (14-3-3 absent) and EEG revealed left hemisphere slow wave activity. There was no relevant family history of neurological disease. He declined rapidly and died without post-mortem examination, but clinical and investigation findings were strongly supportive of CJD. We have also investigated a further patient with suspected prion disease, aged 34, and found G114V. This female patient was of Turkish origin. G114V is not found in the healthy Gujarati or Turkish populations. These two patients support the pathogenicity of this variant and emphasise a very wide range of age at presentation.

D167N, p.D167N, c.499G>A; codon 129MM

This Turkish gentleman presented aged 33 with forgetfulness, emotional lability and aggressive outbursts. Within a year he was unable to recognise family members and experienced incontinence and a severe non-fluent expressive dysphasia. Examination revealed rigidity, hyperreflexia, extensor plantars, and palmomental and grasp reflexes. An EEG revealed generalised slow activity and MRI revealed moderate frontotemporal cortical atrophy. In the following months his walking deteriorated to a slow and stiff gait, he exhibited an exaggerated startle and he died 2 years after disease onset. There was no suspect family history but, interestingly, his mother, aged 65, is an asymptomatic carrier of the D167N mutation. The pathogenicity of this mutation has not been established.

G54S, p.G54S, c.160G>A; codon 129MM

This patient presented aged 43, with effortful, unclear speech. Other than speech abnormalities – ‘thick and effortful’ initially described as a dysarthria - neurological examination was normal. The patient was born in Uganda and was of Gujarati origin. Her mother died aged 34 of tuberculosis and her father died of old age. Her four siblings were alive and well as were her children with no family history of similar disorders. The speech disorder progressed gradually and by 2004, she had a clear reduction in vocabulary as well as difficulty in word production – non fluent dysphasia being the predominant picture. Examined in 2004, her speech was non-fluent, hypophonic, agrammatical and indistinct. She made both phonemic and semantic errors and there was evidence of apraxia and extrapyramidal signs. By early 2006 (aged 48) she was mute and could not walk. Late in 2006, over the course of three months, she stopped recognising relatives and lost the ability to feed and dress herself. She became incontinent of faeces and urine. She died in December 2006 aged 48. EEG was unremarkable. CSF was 14-3-3 protein negative. CSF total tau was >1200 pg/ml (<325) Amyloid β 1-42 110 pg/ml (>725). MRI brain showed severe cortical atrophy.

Gross examination of the brain was unremarkable. Brain weight was 950g. Sections for histology were taken from the frontal, temporal, parietal and occipital lobes and the cerebellum. GFAP staining revealed patchy astrogliosis of the cerebral cortex and white matter and also within the cerebellar white matter and molecular layer. Immunohistochemistry confirmed widespread distribution of abnormal prion protein in the cerebral and cerebellar cortices, predominantly in a perikaryal distribution. This was associated with little or no histopathological changes on routine haematoxylin and eosin stained sections and was in a pattern unlike that seen thus far in heritable prion diseases.

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G54S was found in a single healthy Gujarati control patient which was screened at the time of the patient referral, suggested this variant was likely to be non-pathogenic, a hypothesis subsequently supported by our screening of the CEPH human genome diversity cell line lane which demonstrated the presence of G54S in 2 further Pakistani linguistic groups and at low levels in China and the Middle East. We have no explanation for the abnormal PrP staining found at autopsy from this patient.

Q212PP, p.[Q212P]+[Q212P],c.[635A>G]+[635A>G] codon 129MM

Aged 36 at disease onset, woman of Irish origin first experienced poor balance, and worsening of her handwriting followed by slurred speech. The unsteadiness caused her to stop driving then give up work 16 months after disease onset. The family history was unremarkable except for a maternal grandmother with dementia in later life. Her paternal and maternal grandparents originated from two neighbouring villages and there was a possibility of consanguinity. The past medical history was unremarkable. On examination 9 months after disease onset she was dysarthric and globally ataxic with some asymmetry and horizontal nystagmus. She was globally hyperreflexic and hypertonic with brisk jaw jerk and bilateral ankle clonus and right extensor plantar response. She had a mini-mental state examination (MMSE) of 27/30 with attentional problems and further cognitive assessment revealed executive dysfunction. Subsequently there has been a steady deterioration in mobility, speech and independence. 2 years after disease onset she developed swallowing difficulties and required assistance with walking. Serial examinations revealed progressive frontal release signs and eye movement disruptions with a remarkably consistent MMSE examination of 26/30. At 4 years after disease onset she was unable to walk and dependent for all activities of daily living with much more severe cognitive impairment, marked spasticity and ataxia.

At the time of the initial assessment, the patient was functioning in the low average range on the verbal scale and in the borderline range of the non verbal scale of the WAIS-R (a standard IQ test). Based on her reading performance, her estimated premorbid level of functioning was likely to have been in the high average range. The early neuropsychology therefore reflected significant deterioration from the level expected on tests of general intelligence. Memory functions were weak and there was some evidence of executive dysfunction. Spelling, calculation, visual perception and visuo-spatial skills were intact. Two years later, the patient completed a reassessment. On tests of intelligence, her performance showed no change since the previous assessment. Memory functions show further deterioration and some difficulties with visuo spatial abilities were noted. There was further evidence of executive dysfunction and speed of processing was reduced. On other focal tests her skills remain preserved.

Detailed investigation for alternative diagnoses proved negative. Sequencing of the *PRNP* open reading frame revealed homozygosity for Q212P mutation. Serial EEGs (over 3 years) were mildly abnormal with diffuse slowing and no periodic sharp wave complexes. MRI brain scan revealed moderate cerebral atrophy only.

DISCUSSION

Our analysis of 3664 *PRNP* sequences has identified a range of novel alleles and genotypes and confirms the pathogenicity of several mutations. The insertion of a small number of octapeptide repeats (<4 extra repeats) has not been described as segregating in families and it is difficult to distinguish whether they are rare polymorphisms or incompletely penetrant mutations. To date there are three reported cases of 1-OPRI associated neurological diseases consisting of additional R2 or R3g octapeptides (Laplanche *et al.*, 1995; Pietrini *et al.*, 2003; Kovacs *et al.*, 2005; Kovacs *et al.*, 2005). All had disease durations of less than 6 months and presented between 58 and 73 years of age with progressive cognitive decline, myoclonus, visual and ataxic gait disturbances. Only one individual had a possible family history. In two cases the 14-3-3 CSF levels were raised and EEG revealed pseudoperiodic triphasic complexes in one case. Taken together with the discovery of 3-OPRI in a healthy Chinese population control, our more recent discovery of 1-OPRI polymorphism in Sardinia and the Sindhi linguistic group of Pakistan, and our coincidental finding of 1-OPRI in a GSS pedigree, supports the non-pathogenicity of these small insertions.

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Thus far, eight P105L patients from five unrelated Japanese families have been reported (Yamada, 2003). To our knowledge our patient with this mutation is the first reported outside of East Asia. Furthermore, all reported cases have been heterozygous at codon 129 with the P105L mutation linked to valine at codon 129 (Yamada *et al.*, 1999). Originally thought to be typified by spastic paraparesis and dementia, significant heterogeneity has been reported in clinical and neuropathological phenotypes among P105L family members (Yamada *et al.*, 1999). Frontal lobe dominant cerebral atrophy on MRI has been a consistent feature of P105L (Kovacs *et al.*, 2002). Interestingly, one patient presented with a 3 year history of sensory and psychiatric symptoms before the onset of neurological symptoms (Shiraishi *et al.*, 2002). Our patient had a 16 year psychiatric history with neurological symptoms at least from the age of 33, which is younger than the documented range of 38 to 50 years. It is unclear whether these differences can be attributed to the additional missense polymorphism valine to methionine at codon 209 (our patient had 129VV unlike previous case reports) or as yet undetermined genetic modifiers of phenotypic presentation. P105L shows similarities with another N-terminal point mutation, P102L, in that they are both histologically characterized by high prevalence of amyloid plaques.

The glycine to valine missense mutation at codon 114 has only recently been reported in a single Uruguayan family with five affected individuals and has not been demonstrated in controls (n=100) (Rodriguez *et al.*, 2005). The mutation was associated with a remarkably early age of onset (range 18-27), mood and psychiatric disturbances followed by dementia, myoclonus, extrapyramidal and pyramidal signs. Interestingly, three asymptomatic family members carrying the mutation were reported, two of whom were in their fifth decade of life, suggesting highly variable penetrance despite the pathogenicity of this mutation in young adult patients. Our two patients considerably extend the clinical range of presentation, world distribution, and confirm pathogenicity.

Only a single patient has been reported previously with Q212P in the heterozygous state, associated with cerebellar ataxia and PrP deposition in the brain and an abnormal PrP immunoblot. Although details were not reported, the disease onset was aged 60 and duration 8 years (Piccardo *et al.*, 1998). The finding of an early onset multisystem neurodegenerative disease associated with 212P homozygosity is evidence for codominance at this codon. The absence of family history of neurodegeneration in an extensively investigated kindred with many elderly relatives, and the existence of three elderly healthy Q212P heterozygotes in pedigree described here, suggests that Q212P in the heterozygous state may have low penetrance in old age. This mutation appears to be extremely rare; however, resulted in a homozygous mutation due to consanguinity in our family. Prion replication is known to proceed faster in the context of homologous protein-protein interactions (Palmer *et al.*, 1991) such that some degree of codominance is expected. An earlier age of onset has been described in patients with homozygosity for 200K, when compared with those heterozygous at this codon (Simon *et al.*, 2000).

Interpreting the genetic basis of suspected inherited prion disease can be difficult when novel variants are discovered in single patients, or alternatively, when known genetic variants are reported where no family history exists or is unclear, or disease segregation with mutations is incomplete (Samaia *et al.*, 1997). The discovery of G54S, G142S and N171S in appropriate control populations strongly indicates that these missense variants are non-pathogenic. The variation at codon 171 has previously been associated with schizophrenia in a Brazilian pedigree, and although our discovery of N171S in a probable case of sCJD is not necessarily contradictory to previous associations (Samaia *et al.*, 1997), the identification of this polymorphism in 5% of normal healthy Jamaicans, and Subsaharan African, Israeli and Sardinian populations does not support pathogenicity. Conversely, absence of G114V, and D167N from all populations screened is suggestive that these are pathogenic mutations, although the penetrance of G114V and D167N may be incomplete. Given the relatively high frequency of G142S and N171S in the Jamaican population these are common polymorphisms. Our patient with sCJD had G142S and N171S on different chromosomes, as confirmed by molecular analysis; modification of phenotype or susceptibility due to these alleles cannot be ruled out. Other compound heterozygotes identified in this study, patients who have inherited P102L and P105L mutations on one chromosome, and 1-OPRI and V209M variations on the other, support the inference that 1-OPRI and V209M are rare polymorphisms that were found coincidentally. Again, molecular analyses confirmed that in both cases that 1-OPRI and V209M were present on wild type chromosome. Indeed, our discovery of 1-OPRI polymorphism in Sardinian and Pakistani healthy individuals strongly supports this hypothesis. The chance of inheriting two different pathogenic mutations on different chromosomes is

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3 extremely unlikely given that IPD has a typical incidence of 1-2/10,000,000/year, however, further work including
4 genotyping of other family members where available would be required for confirmation.
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6 The possibility remains that other missense polymorphisms of *PRNP*, G54S, G142S, N171S, and M232R
7 detected in a CEPH Japanese population, have similar properties to codon 129 in conferring resistance to prion
8 disease in the heterozygous state (Mead *et al.*, 2003). More specifically, resistance to sporadic CJD has long been
9 associated with heterozygosity at codon 129 and codon 219 (Shibuya *et al.*, 1998). Assessment of codon 219 in the
10 CEPH human genome diversity cell line panel revealed 219K allele frequencies of approximately 0.1, up to 0.08
11 and 0.02 in Malanesian, Pakistani and Bedouin populations respectively, however, no association study could be
12 performed.
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14 Our sequencing of the ORF of *PRNP* from this panel revealed several further noteworthy findings. In
15 particular, although not unexpected, common ancestral alleles were detected in Subsaharan Africa and within
16 certain Middle Eastern populations especially the Bedouin group, where the majority of genetic variation known to
17 exist within the *PRNP* ORF, both coding and non-coding, can be seen. Furthermore, it is of interest that
18 Subsaharan African populations including Biaka and Mbuti Pygmy and Nigerian Yoruba groups, demonstrate
19 extremely high levels of a heterozygote state due to coding variations within the *PRNP* ORF. The Pygmy
20 population's figures combined, reveal ~80% individuals (33 of 41) in the heterozygous state. A single octapeptide
21 repeat deletion alone accounts for approximately 7% of heterozygotes detected in this population, and whilst no
22 direct evidence exists in humans of an association of a single octapeptide repeat deletion and susceptibility to prion
23 disease, it remains possible that such deletions offer resistance to certain prion strains in humans.
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25 If any statement about the distribution of pathogenic missense mutations may be made, it is that these cluster in
26 the structured C-terminal domain of PrP. With the exception of the insertional mutations, no missense pathogenic
27 changes are found in the unstructured N-terminal domain. However, the lack of strong clustering of either
28 mutations or polymorphisms within the structure of PrP^C, and the difficulty of well-established computational
29 methods to distinguish pathogenic from neutral or protective genetic variations, does little to help understand the
30 pathogenic mechanism of *PRNP* mutation. Rather, the wide distribution of changes across the structured domain of
31 PrP^C supports a model in which PrP^C is destabilized by mutation in a non-specific way, where missense mutations
32 exert their effect via modulation of PrP:PrP interactions either in the native PrP^C homodimeric state or during
33 conversion and propagation of PrP^{Sc}. This may subsequently allow the protein to refold correctly or misfold and
34 initiate a pathogenic pathway. Effects on the structure and stability of post-translational modified PrP^C cannot be
35 studied using recombinant PrP and therefore cannot be excluded but could be considered improbable.
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