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PrP genotype in Sarda breed sheep and its relevance to scrapie

Brief Report

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Summary. Several PrP gene polymorphisms modulate sheep scrapie susceptibility. Recently, an increase of scrapie outbreaks has been reported in Italy. A vaccine containing sheep brain homogenate was used in most of the outbreaks. We investigated PrP gene polymorphisms in scrapie-affected and clinically healthy Sarda breed sheep from a flock exposed to the aforementioned vaccine, and in affected Sarda sheep from unexposed flocks. All affected animals were $(Gln/Gln)_{171}$ homozygous. Moreover, we observed no variation for Ala_{136} and a new polymorphism (Lys to Asn) at codon 176. Our findings confirm the correlation between scrapie and $(Gln/Gln)_{171}$ in breeds with no variation for Ala_{136}.

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Scrapie is a fatal neurodegenerative disease of sheep and goats belonging to transmissible spongiform encephalopathies (TSEs), or prion diseases, which include bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease in humans. Similarly to the other TSEs, the main pathogenetic event leading to the disease is thought to be the accumulation, in the brain, of the pathological protease-resistant isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) [18].

The occurrence of scrapie in sheep, as for other human and animal TSEs, is a complex phenomenon that depends upon several factors. Among them, the interaction between strain of the agent and genetic background of the host,

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primarily driven by the amino acid sequence of the prion protein, plays a central role in determining the in-flock incidence and the length of the incubation period of the disease [11, 15].

In sheep, the open reading frame (ORF) of the gene encoding for PrP reveals several polymorphisms, some of which predispose to natural and experimental scrapie. The critical polymorphisms for the development of scrapie depend, at least in part, on the breed of sheep. In some breeds, e.g. in Cheviot, scrapie occurrence is influenced by 2 or 3 different polymorphisms of the PrP gene (at codons 136 Ala or Val, 171 Arg, His or Gln and, possibly, 154 Arg or His) [13], while in others, such as in Suffolk breed, it is influenced only by the polymorphic codon 171 [22]. Since 1996, a marked increase in the number of scrapie outbreaks, notified in sheep and goats, has been reported in Italy. A risk factor represented by a formol-inactivated vaccine prepared with sheep brain homogenate was present in most of the outbreaks [1].

No information is available on the PrP genotype of Italian sheep breeds, nor on the prevalent strains of scrapie in Italy. To surmount these missing informations, we have launched a research project aimed at both sequencing the PrP gene of Italian breeds and determining the critical polymorphisms that are responsible for the development of the disease.

We report here the analysis of the polymorphisms of the PrP coding region in scrapie-affected and clinically healthy sheep of Sarda breed from a flock exposed to the above risk factor, as well as in scrapie-affected Sarda sheep from unexposed flocks.

Our study was carried out on 33 scrapie-affected (group A1) and 79 clinically healthy sheep (group A2), all belonging to a single Sarda breed flock reared in Tuscany and comprising 760 animals. Group A1 was composed by all clinically affected sheep reported by the farmer or by the Local Veterinary Service, from the beginning of the scrapie outbreak up to the stamping-out procedure and whose diagnosis was confirmed by immunohistochemistry. The mean ages at euthanasia or death of clinically affected and clinically healthy animals were 4.7 (1.9 SD, range 1–9 years) and 3.5 years (1.4 SD, range 2–8 years), respectively. In this flock, the above vaccine was administered in 1995.

Moreover, a group of 23 Sarda sheep affected by natural scrapie (mean age 3.0 years, 1.3 SD, range 1–6 years) were also included in the study. The latter animals belonged to 5 flocks reared in Sardinia, which had never been exposed to the vaccine (group B); they were sampled for scrapie diagnosis from 1995 to 1999.

Following euthanasia or death, all scrapie-affected sheep underwent histological examination of the brain. After removal from the skull, a coronal brain portion corresponding to the obex was fixed in 10% formol saline for 12–15 days. After washing in running tapwater, samples were decontaminated with 98% formic acid for 1h and embedded in paraffin. Serial 7 μm thick sections were cut and subsequently stained with haematoxylin and eosin for light microscope examination. For PrP^{Sc} immunohistochemistry, brain tissue sections were mounted on 3-aminoalkyltriethoxysilane-coated slides (Sigma, St. Louis, Mo, U.S.A.).

After pretreatment [23] the sections were immunostained with a rabbit polyclonal antibody (P7/7, 1:500 in PBS), raised against purified PrP^{Sc} from 263 K hamsters scrapie strain [17]. Sections were subsequently incubated with Vectastain Elite ABC (Vector Laboratories Inc., U.S.A.). Immunoreaction was visualised by 3-amino-9-ethylcarbazole, AEC Chromogen Kit (Sigma, St. Louis, Mo, U.S.A.). Sections were finally counterstained with Mayer's haematoxylin.

Preimmune rabbit serum was used to verify the specificity of immunostaining. In each run, control sections of the obex from scrapie-confirmed and scrapie-negative cases in sheep were also included. For genetic analysis, we utilised a previously published technique [20], with slight modifications. We sequenced either the entire ORF of the PrP gene (groups A1 and A2), or the PrP coding region from residue 103 to 198, containing all the non-silent PrP polymorphisms (group B). Overlapping regions of the entire ORF of the PrP gene were amplified with the two sets of primers 5′-TGT AAA ACG ACG GCC AGT TTT ACG TGG GCA TTT GAT GC-3′; 5′-CAG GAA ACA GCT ATG ACC GGT CCT CAT AGT CAT TGC C-3′ and 5′-TGT AAA ACG ACG GCC AGT TGG TGG CTA CAT GCT GGG-3′; 5′-CAG GAA ACA GCT ATG ACC GGC TGC AGG TAG ACA CTC C-3′, while primers 5′-TGT AAA ACG ACG GCC AGT GGT AGC CAC AGT CAG TGG-3′ and 5′-CAG GAA ACA GCT ATG ACC CAG TTT CGG TGA AGT TCT CC-3′ were used to amplify the PrP coding region from residue 103 to 198.

The Tuscany flock under investigation was scrapie-free until 1997. In February 1995, all sheep had been treated by subcutaneous injection with a batch of a vaccine against *Mycoplasma agalactiae*, which has been supposedly related to a number of scrapie outbreaks in Italy [1]. The vaccine was prepared with an homogenate of sheep brain, mammary gland and supramammary lymph nodes obtained from Sarda sheep whose genotypes had not been determined. Unfortunately we could not test the vaccine for presence of scrapie infectivity, due to lack of residual vaccine belonging to the same batch. The first scrapie cases in the Tuscany flock were reported in May 1997. Subsequent cases were then collected from 1997 until October 1998, when compulsory stamping-out was performed on the entire flock. Figure 1 shows the age distribution of sheep died from scrapie in both vaccine-related (group A1) and natural (group B) disease cases. The mean age at death of affected animals in group A1 (4.7 ± 1.9, mean $\pm$ SD) was significantly higher than that of natural scrapie cases in group B (3.0 ± 1.3) (two-tailed unpaired T-test, $P = 0.0006$). In this respect, the mean age of affected animals in group B is in agreement with previous studies investigating the in-flock incidence of natural scrapie in breeds other than Sarda [12]. The age at which sheep will die from scrapie depends upon a number of factors, with special emphasis on the time when they were first exposed to the agent, along with the infectious dose. In natural scrapie, the young age generally reported at onset of disease cases is consistent with an exposure to the scrapie agent during the peri-neonatal period [7]. By contrast, the significantly higher mean age of scrapie-affected sheep in group A1, compared to that of sheep in group B, could suggest an abrupt exposure to the scrapie agent, simultaneously occurring in sheep of all ages within the

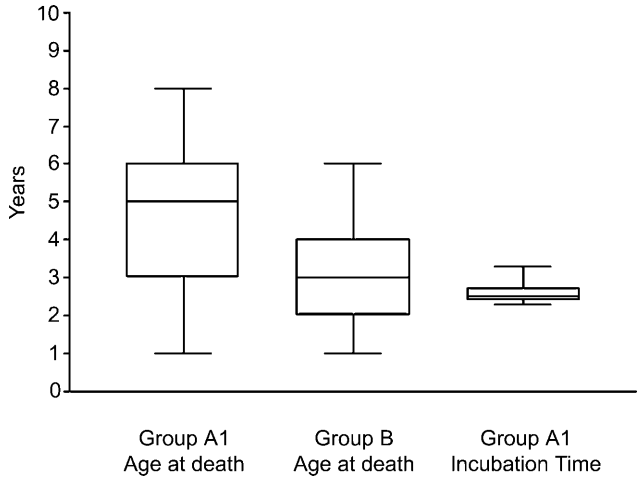

Fig. 1. Age distribution of scrapie cases in groups A1, B along with disease incubation time in group A1. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers extend down to the smallest value and up to the largest

Tuscany flock, as it would be expected after a supposed iatrogenic introduction of the infection via the aforementioned vaccine. Of note is also the finding that two sheep belonging to group A1 were 1-year-old, thus likely representing cases in which a subsequent maternal or horizontal transmission of the infection occurred. However the incubation time, calculated as the time between the vaccination and the death or euthanasia (with the exception of the two one-year-old aforementioned animals), was 2.7 ± 0.22 years (mean $\pm$ SD) (Fig. 1). Interestingly, by comparing the disease incubation time of group A1 sheep and the age at death of group B sheep, the means were not significantly different.

Sequence analysis of the PrP ORF in Sarda breed sheep revealed the presence of non-silent polymorphisms at codons 137 (Met or Thr), 141 (Leu or Phe), 154 (Arg or His) and 171 (Arg or Gln), as well as silent replacements at codons 231 and 237 (Table 1), all previously described in other ovine breeds [2–4, 9, 10]. However, in the Sarda sheep investigated here we also detected two novel polymorphisms of the PrP gene, namely a silent replacement at codon 194 (ACC/ACA) and the other, at codon 176 (AAC/AAA), resulting in the amino acid substitution of lysine to asparagine. The presence of asparagine at PrP codon 176 is well conserved among mammalian species, with the only exception being represented by the horse, in which, interestingly, a substitution with lysine has been reported [24]. In our study, all investigated sheep showed no variation for alanine at codon 136.

The effect of each PrP gene polymorphism on the development of scrapie was assessed in sheep belonging to the flock exposed to the vaccine. Among them, all animals with clinical signs of scrapie (group A1) showed histological evidence of spongiform lesions (Fig. 2), as well as positive PrP^{Sc} immunohistochemistry, in their brain tissue (Fig. 3). All of them were $(Gln/Gln)_{171}$ homozygous. In these animals, no variation at the novel PrP gene polymorphism at codon 176 was detected, with the variability at codons 137, 141 and 154 being too low to point

Table 1. Frequencies of PrP polymorphic codons in examined sheep from the Tuscany flock (groups A1 and A2) and from the 5 Sardinia flocks (group B)

PrP polymorphisms		Group A1	Group A2	Group B
Codon	Amino acid	Scrapie cases (%)	Healthy controls (%)	Natural scrapie cases (%)
137	Met/Met	100	97	100
	Met/Thr	0	3	0
	Thr/Thr	0	0	0
141	Leu/Leu	97	92	96
	Leu/Phe	3	8	4
	Phe/Phe	0	0	0
154	Arg/Arg	91	97	83
	Arg/His	9	3	17
	His/His	0	0	0
171	Gln/Gln	100	23	100
	Gln/Arg	0	57	0
	Arg/Arg	0	20	0
176	Asn/Asn	100	96	100
	Asn/Lys	0	4	0
	Lys/Lys	0	0	0
Number of animals		33	79	23

out any statistical differences between clinically healthy animals (group A2) and scrapie-affected sheep (group A1) (Table 1). On the contrary, the exact Fischer test showed significant differences ($P < 0.001$) in the distribution of $(Gln/Gln)_{171}$ homozygousity and the other genotypes at codon 171 (Table 1). Interestingly, while histidine at codon 154 has been related to scrapie resistance in Cheviot and Texel breed [2, 13] such aminoacid was uncommonly found in this position, in both affected and non-affected Sarda sheep investigated here. This finding, therefore, does not allow us to make any correlation between presence of histidine at codon 154 and scrapie status, as it happens in Suffolk, Romanov and Finn Dorset sheep [6, 8, 14].

The correlation between $(Gln/Gln)_{171}$ homozygousity and scrapie has already been reported in a number of ovine breeds other than Sarda, being usually stronger when no variation for alanine at codon 136 occurs [5, 19, 22]. In Suffolk breed, for instance, the Val_{136} allele is quite rare and there is a strong correlation between $(Gln/Gln)_{171}$ homozygousity and scrapie [15, 22], although rarely heterozygous $(Gln/Arg)_{171}$, or even homozygous $(Arg/Arg)_{171}$ animals (a single case reported) may develop the disease [16]. In our case, however, it should be also pointed out that, due to the compulsory stamping-out of the flock, which was carried out in October 1998, it was not possible to follow all the clinically healthy sheep throughout their entire life span. As a consequence, we cannot rule out the

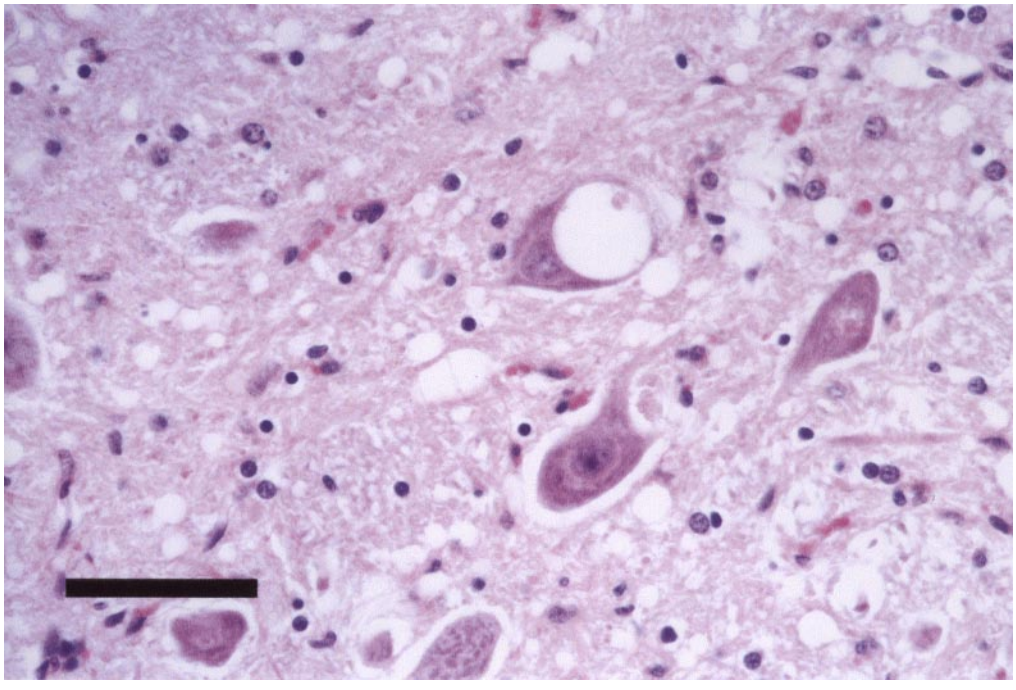

Fig. 2. Sheep scrapie. Brainstem tissue section (dorsal motor nucleus of the vagus nerve) showing typical spongiform changes. Haematoxylin and eosin. Bar: 15 μm

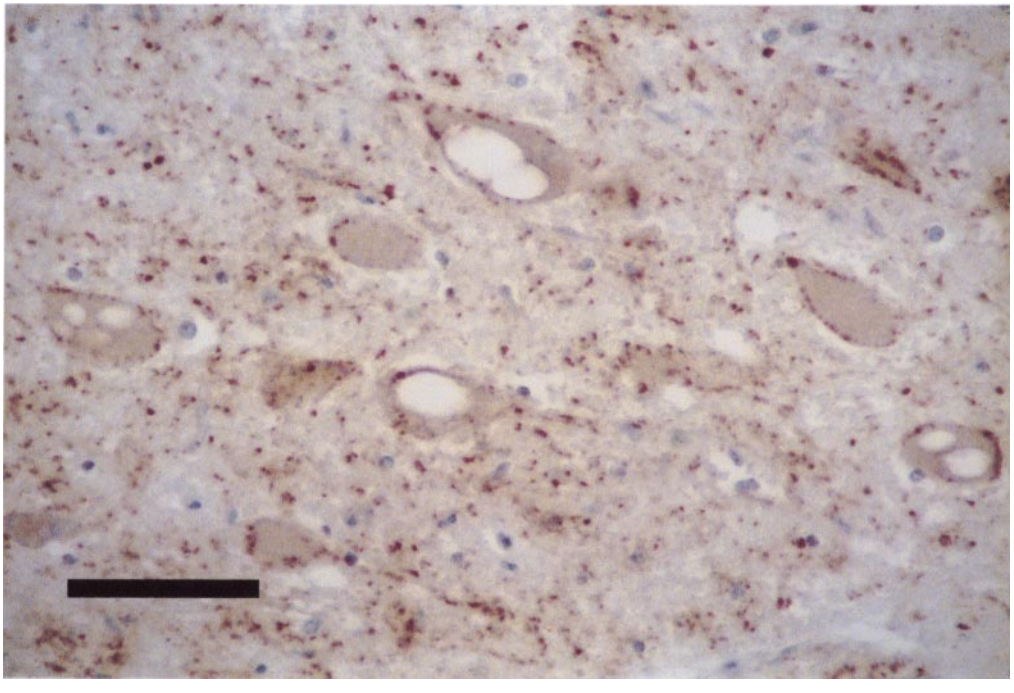

Fig. 3. Sheep scrapie. Brainstem tissue section (dorsal motor nucleus of the vagus nerve) with granular and perineuronal PrP^{Sc} immunolabelling. Avidin-biotin-horseradish-peroxidase complex reaction. Mayer's haematoxylin counterstain. Bar: 15 μm

possibility that some animals, carrying a different genotype, would have become clinically ill later, due to a longer disease incubation time. Interestingly, all the 23 Sarda breed sheep affected by natural scrapie (group B), which were collected during a five years' period (1995–1999), were also $(Gln/Gln)_{171}$ homozygous.

Although no information is available on the biological features of the agent strain(s) involved in the outbreaks investigated here (studies are in progress), we may still assume that $(Gln/Gln)_{171}$ homozygousity plays a crucial role in modulating the genetic susceptibility to scrapie in Sarda breed sheep, with no apparent variation being found, in respect to the different route of infection, between animals affected by natural scrapie and sheep presumably infected by parenteral route through vaccine administration.

In conclusion, the genetic susceptibility to scrapie was investigated, for the first time, in sheep belonging to Sarda breed, the most representative and economically important one within the Italian ovine population. Our future goal is to extend our investigations in greater detail on Sarda breed, aiming with special emphasis at differentiating genetic resistance to the infection from very long incubation time in the least susceptible genotypes, in order to obtain reliable data on the genetic susceptibility of such breed to scrapie. Undoubtedly, all the above data will form the necessary basis for future breeding programmes, focusing at their time on the improvement of genetic resistance of sheep to scrapie, similarly to what is currently underway in other European and extra-European Countries, such as United Kingdom, France, The Netherlands, Norway and USA.

GenBank accession number

The GenBank accession number of the sequence reported in this paper is AF195247.

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References

1. Agrimi U, Ru G, Cardone F, Pocchiari M, Caramelli M (1999) Epidemic of transmissible spongiform encephalopathy in sheep and goats in Italy. Lancet 353: 560–561
2. Belt PBGM, Muileman IH, Schreuder BEC, Ruijter JB, Gielkens, ALJ, Smits, MA (1995) Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. J Gen Virol 76: 509–517
3. Belt PBGM, Bossers A, Schreuder BEC, Smits MA (1996) PrP allelic variants associated with natural scrapie. In: Gibbs Jr CJ (ed) Bovine spongiform encephalopathy: The BSE dilemma. Springer, New York, pp 294–305
4. Bossers A, Schreuder BEC, Muileman IH, Belt PBGM, Smits MA (1996) PrP genotype contributes to determining survival times of sheep with natural scrapie J Gen Virol 77: 2669–2673

5. Clouscard C, Beaudry P, Elsen JM, Milan D, Dussaucy M, Bounneau C, Schelder F, Chatelain J, Launay JM, Laplance JL (1995) Different allelic effects of the codon 136 and 171 of the prion protein gene in sheep with natural scrapie. J Gen Virol 76: 2097–2101
6. Dawson M, Hoinville LJ, Hosie BD, Hunter N (1998) Guidance on the use of PrP genotyping as an aid to the control of clinical scrapie. Scrapie Information Group. Vet Rec 142: 623–625
7. Dickinson AG, Young, GB, Stamp, JT, Renwick, CC (1964) A note of the distribution of scrapie in sheep of different ages. Anim Prod 6: 375–377
8. Elsen JM, Amigues Y, Schelcher F, Ducrocq V, Andreoletti O, Eychenne F, Khang JV, Poivey JP, Lantier F, Laplanche JL (1999) Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. Arch Virol 144: 431–445
9. Goldmann W, Hunter N, Foster JD, Salbaum JM, Beyreuther K, Hope J (1990) Two alleles of a neural protein gene linked to scrapie in sheep. Proc Natl Acad Sci USA 87: 2476–2480
10. Goldmann W, Hunter N, Benson G, Foster JD, Hope J (1991) Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene. J Gen Virol 72: 201–204
11. Goldmann W, Hunter N, Smith G, Foster J, Hope J (1994) PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. J Gen Virol 75: 989–995
12. Hunter N, Foster JD, Hope J (1992) Natural scrapie in British sheep: breeds, ages and PrP gene polymorphisms. Vet Rec 130: 389–392
13. Hunter N, Foster JD, Goldmann W, Stear MJ, Hope J, Bostock C (1996) Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. Arch Virol 141: 809–824
14. Hunter N, Moore L, Hosie BD, Dingwall WS, Greig A (1997) Association between natural scrapie and PrP genotype in a flock of Suffolk sheep in Scotland. Vet Rec 140: 59–63
15. Hunter N (1997) PrP genetics in sheep and the implications for scrapie and BSE. Trends Microbiol 5: 331–334
16. Ikeda T, Horiuchi M, Ishiguro N, Muramatsu Y, Kai-uwe GD, Shinagawa M (1995) Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. J Gen Virol 76: 2577–2581
17. Oberdieck U, Xi YG, Pocchiari M, Diringer H (1994) Characterisation of antisera raised against species-specific peptide sequences from scrapie-associated fibril protein and their application for post-mortem immunodiagnosis of spongiform encephalopathies. Arch Virol 136: 99–110
18. Oesch B, Westaway D, Walchi M, McKinley MP, Kent SBH, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissman C (1985) A cellular gene encodes scrapie PrP 27–30 protein. Cell 40: 735–746
19. O'Rourke KI, Holyoak GR, Clark WW, Mickelson JR, Wang S, Melco RP, Besser TE, Foote WC (1997) PrP genotypes and experimental scrapie in orally inoculated Suffolk sheep in the United States. J Gen Virol 78: 975–978
20. Petraroli R, Vaccari G, Pocchiari M (2000) A rapid and efficient method for the detection of point mutations of the human prion protein gene (PRNP) by direct sequencing. J Neurosci Methods 99: 59–63
21. Sambrook J, Fritsch EF, Maniatis T (1985) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York, pp 17–19

22. Westaway D, Zuliani V, Cooper CM, Dacosta M, Neuman S, Jenny AL, Detwiler L, Prusiner SB (1994) Homozygousity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. Genes Dev 8: 959–969
23. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG (1996) A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347: 921–925
24. Wopfner F, Weidenhofer G, Schneider R, von Brunn A, Gilch S, Schwarz TF, Werner T, Schatzl HM (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. J Mol Biol 289: 1163–1178

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