Regional distribution of anchorless prion protein, PrP226*, in the human brain

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It was shown previously that truncated molecules of prion protein can be found in brains of patients with some types of transmissible spongiform encephalopathy. One such molecule, PrP226*, is a fragment of prion protein, truncated at Tyr226. It was found to be present in aggregates, from which it can be released using chaotropic salts. In this study we investigated the distribution of PrP226* in Creutzfeldt–Jakob disease affected human brain, employing the mAb V5B2, specifically recognizing this fragment. The results show that PrP226* is not evenly distributed among different regions of human brain. Among brain regions analyzed, the fragment was found most likely to be accumulated in the cerebellum. Its distribution correlates with the distribution of PrP[Sc].

Introduction

Prion protein (PrP) is a glycoprotein, bound to the cell membrane through a glycophosphatidylinositol (GPI) anchor, and is expressed in all mammalian cells with the highest levels of expression in neurons.[1] Cellular form of PrP (PrP[C]) can undergo posttranslational modifications that shift secondary structure of the molecule from mostly α-helical to β-sheeted, changing the properties of the protein drastically.[2] Altered molecules, named PrP[Sc], are prone to aggregation and are partially resistant to digestion with proteinase K. The enzyme cleaves off a part of molecule from the N-terminus, leaving the core fragment of 19 or 21 kDa (PrP[res]), whereas PrP[C] molecules are totally degraded.[3] The size of the PrP[res] along with the genotype on polymorph codon 129 is the basis for the molecular classification of sporadic Creutzfeldt-Jakob disease (sCJD).[4]

Prion diseases, also named transmissible spongiform encephalopathies (TSEs), are rare fatal neurodegenerative diseases that affect humans and animals. sCJD is the most common form of human TSE, other forms are much less frequent. A new variant of CJD (vCJD), that first appeared in Great Britain in 1995,[4] was connected to consumption of meat products originating from cattle infected with bovine spongiform encephalopathy.

Besides PrP[Sc], the presence of truncated PrP molecules in brain has been described in sCJD as well as in genetic form of TSE named Gerstmann–Sträussler–Scheinker syndrome.[5-7] Our group reported on the discovery of one such fragment, as well as the production and characterization of a mAb V5B2 specifically recognizing it.[8,9] mAb V5B2 was prepared by immunization of BALB/c mice with a peptide from the sequence of human PrP (amino acid residues 214–226).[8] The mAb was especially interesting, because it discriminated between CJD and non-CJD human brain tissue in dot-blot, immunohistochemistry and western blot.[8,10] Surprisingly, it turned out that the V5B2 does not discriminate between conformational isoforms of PrP[C] and PrP[Sc], but binds exclusively the truncated form of PrP ending with Tyr226.[9] It was shown that it binds with high affinity to recHuPrP(23–226), while no reaction is observed with recHuPrP(23–231).[9] The epitope was determined by alanine scanning and phage display. Substitution of C-terminal Tyr226 as well as the addition of one amino acid C-terminally of Tyr226 completely abolished the binding of V5B2, indicating crucial role of this amino acid residue for binding.[9] The fragment is C-terminally truncated and thus an anchorless PrP molecule that ends with the residue Tyr226 and was therefore named PrP226*.[9] A sandwich immunoassay for detection of PrP226* in brain homogenates using V5B2 was developed,[10] employing denaturation to unpack the PrP226* molecules from the aggregates. This kind of approach was first introduced by Serban et al.,[11] and since then often used for unpacking PrP[Sc] molecules from aggregates. Upon the denaturation with chaotropic salts, PrP[Sc] aggregates are loosened and epitopes, previously unavailable for interaction with certain Ab, are revealed. The ratio between signal of denatured and non-denatured sample is the measure for the epitopes revealed and indicates the amount of detected molecules in the sample.[12] For the purpose of detecting
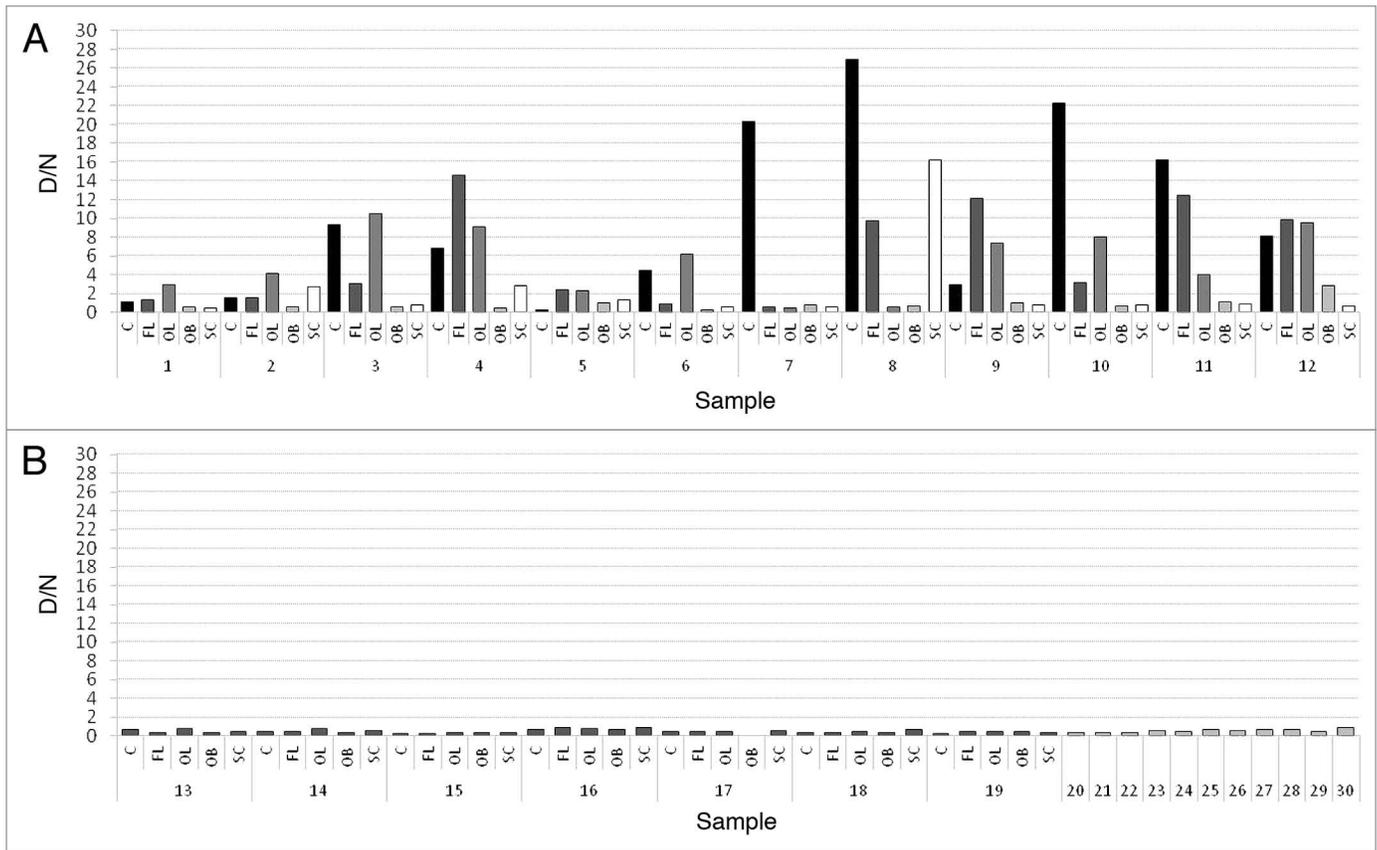
Figure 1. Average D/N ratios for all samples. (**A**) D/N ratios for sCJD samples. C (black); FL (dark-gray); OL (gray); OB (light-gray); SC (white). (**B**) D/N ratios for non-CJD samples. Dark-gray columns represent NC (13–19) and light-gray columns represent NNC (20–30). The data was obtained from PrP226* assay. Sequential numbers of samples are as in **Table 1.** C, cerebellum; FL, frontal lobe; OL, occipital lobe; OB, olfactory bulb; SC, spinal cord.

PrP226*, mAb V5B2 was used instead of an antibody recognizing the whole PrP molecule.

In this study we assessed the presence of PrP226* in five different human brain regions of several sCJD cases and controls. We present for the first time a systematic analysis of distribution of this fragment in sCJD-affected human brain.

Results and Discussion

The amount of PrP226*, represented as D/N ratio, varied considerably between different brain regions of individual cases as well as between the same brain regions of different cases (**Fig. 1A**). There was an obvious difference in amount of PrP226* between cerebellum (C), frontal lobe (FL) and occipital lobe (OL) samples on one hand and olfactory bulb (OB) and spinal cord (SC) samples on the other, the former containing much higher amounts of the fragment. In 5/12 cases the amount of PrP226* was the highest in C, in 4/12 cases in FL and in 3/12 cases in OL. OB and SC were never the regions with the highest amount of PrP226*. We could define as positive 11/12 C samples (92%), 10/12 OL samples (83%), 10/12 FL (83%) whereas only 4/12 SC samples (33%) and 2/12 OB sample (17%) (**Fig. 2A**). For all 12 sCJD cases at least one of five tested regions was positive and at least one was negative (**Fig. 2B**). For all control samples D/N ratio was below 1 (**Fig. 1B**).

We obtained denaturation profiles of samples with high or low D/N ratios to make sure that the ratios correlate with the amount of PrP226* and not with the susceptibility to denaturation (**Fig. 3**). For none of the tested samples did D/N ratio increase with the increasing denaturant concentration, indicating that low D/N ratios are not a consequence of resistance to denaturation.

Measurements of A_{450} of non-denatured samples showed that not all of PrP226* in samples from sCJD cases is entangled in aggregates. Furthermore, free PrP226* was found to be present in minute amounts also in non-CJD brains. Closer examination of the amounts of free PrP226* revealed that samples, defined as positive, contained lower amounts of free PrP226* than sCJD samples, defined as negative, and control samples (**Fig. 4**). The difference in the amounts of free PrP226* between CJD samples, defined as positive, and CJD samples, defined as negative, as well as the difference in the amounts of free PrP226* between CJD samples, defined as positive, and negative controls, was statistically significant ($P < 0.002$ and $P < 0.0001$ respectfully), determined with Kruskal-Wallis test. These data support the hypothesis that free PrP226* is incorporated into the aggregates as they are formed.

The comparison of the results of PrP226* and PrP^{Sc} assay showed that the amount of PrP226* correlates with the amount

of PrP^Sc in the sample as detected in the assay, where mAb 3F4 was used as capturing Ab in 3F4-E12/2 sandwich. Since we systematically analyzed multiple brain regions of each individual case, we additionally found out, that the distribution of PrP226* follows the pattern of PrP^Sc distribution (data not shown). This observation was not surprising in the view of our hypothesis that PrP226* is a part of PrP^Sc aggregates and released from them along with PrP^Sc upon denaturation.

PrP226* is a fragment of PrP, ending with the residue 226. It could therefore be generated from the whole PrP molecule by proteolitic degradation, especially since no protease inhibitors were added into the homogenization buffer. To test the possibility that PrP226* was generated ex-vivo during the homogenization process, multiple CJD and NC samples were homogenized simultaneously in the presence and absence of protease inhibitors. Measurements of A_{450} (**Fig. 5**) revealed slight increase in the amounts of PrP226* when protease inhibitors were used, indicating that PrP226* is degraded rather than generated during homogenization without protease inhibitors. The loss of PrP226* due to the proteolitic activity did not influence D/N ratios crucially. Due to the limited amounts of samples available, we decided not to use protease inhibitors in homogenization buffer, what enabled the usage of the same homogenates also for other experiments, such as proteinase K digestion. This data suggest that PrP226* is a naturally occurring fragment of PrP, generated in healthy brain in minute amount and accumulating during the disease. The fragment could also be further degraded after homogenization. In order to determine whether the amount of PrP226* in the sample changes after multiple defrosting, aliquots of sCJD and non-CJD brain homogenates were subjected to multiple rounds of thawing and freezing cycles. Only minimal changes in measured A_{450} after serial thawing of samples were found, suggesting that the amounts of PrP226* epitopes remained constant during the treatment (data not shown). The conclusion was made, that the PrP226* assay, employing mAb V5B2 and E12/2 is a very robust one. This finding cannot be generalized as different epitopes may undergo different changes.

In this study, we analyzed the distribution of PrP226* in five different regions of human brain. This is the first study addressing the distribution of this fragment in human brain. The fragment was found not to be evenly distributed among different regions of sCJD-affected human brain and no pattern of distribution was found. Cerebellum was identified as the region
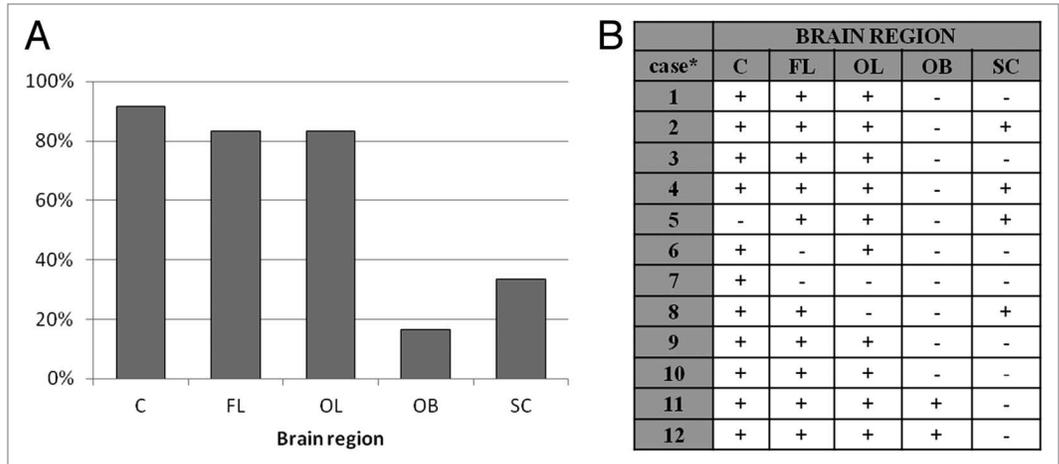
Figure 2. (**A**) Percentage of samples from certain brain region of 12 sCJD cases, which were defined as positive. (**B**) Results of PrP226* assay for all sCJD cases. Sequential numbers of samples are as in **Table 1.** C, cerebellum; FL, frontal lobe; OL, occipital lobe; OB, olfactory bulb; SC, spinal cord; +, sample was defined as positive; -, sample was defined as negative.

	BRAIN REGION				
case*	C	FL	OL	OB	SC
1	+	+	+	-	-
2	+	+	+	-	+
3	+	+	+	-	-
4	+	+	+	-	+
5	-	+	+	-	+
6	+	-	+	-	-
7	+	-	-	-	-
8	+	+	-	-	+
9	+	+	+	-	-
10	+	+	+	-	-
11	+	+	+	+	-
12	+	+	+	+	-
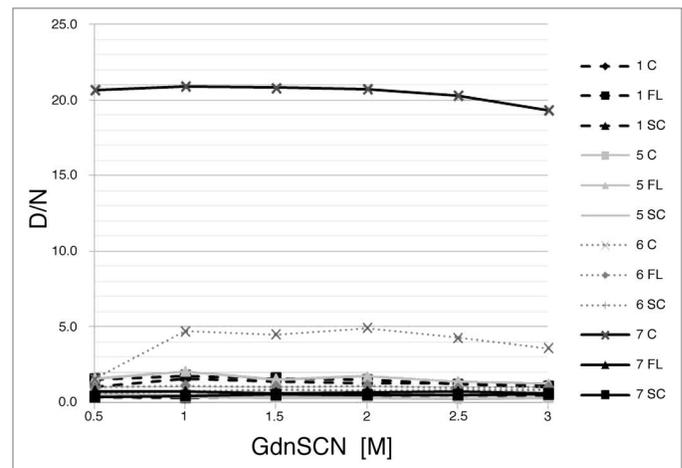
Figure 3. Denaturation profiles. D/N ratios are plotted as the function of the GdnSCN concentration. Sequential numbers of samples are as in **Table 1.** C, cerebellum; FL, frontal lobe; SC, spinal cord.

where PrP226* is most likely to be accumulated, followed by occipital lobe and frontal lobe (**Fig. 2**).

Whereas nothing has been known so far about the distribution of PrP226* in human brain, the regional heterogeneity of PrP^Sc as well as PrP^C in human and animal brain has been addressed in several studies.[13-18] Choi et al.[13] employed conformation dependent immunoassay to investigate the abundance of PrP^Sc in two different brain regions, namely frontal cortex and cerebellum in cases of MM at codon 129 sCJD. They showed that PrP^Sc was most abundant in frontal cortex and less in cerebellum. These findings are in agreement with the former study of Schoch et al.,[17] who analyzed the distribution of PrP^res in nine different brain regions of MM, VV and MV sCJD cases, including frontal cortex, occipital cortex and cerebellum. In MM cases, the most PrP^res was found in cortical regions and less in cerebellum, whereas in VV cases the situation was the opposite, namely
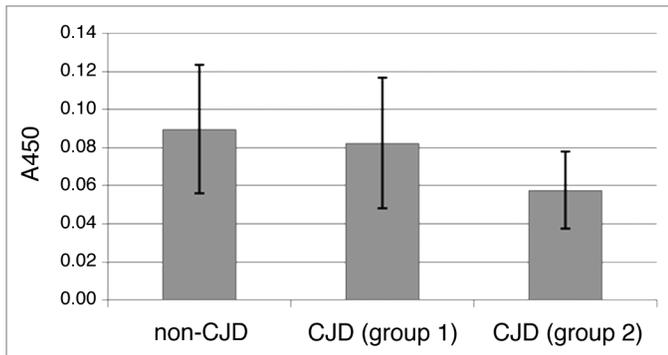
Figure 4. Comparison of A_{450} values of non-denatured samples (all brain regions included). The first bar represents the average value of non-denatured non-CJD samples (NC, NNC). sCJD samples were divided in two groups – samples, that were defined as negative (D/N < 1.2) were included in group 1, samples, that were defined as positive (D/N > 1) were included in group 2. The second bar represents the average value of non-denatured sCJD samples from group 1 and the third bar represents the average value of non-denatured sCJD samples from group 2.

cerebellum was the region with the highest amounts of PrP[res]. It should be taken into consideration that in all mentioned studies, Abs used were directed against epitopes N-terminally of Tyr226. That means that detected molecules could among others also be PrP226*.

Among our studied cases, we had a single VV case in which substantial amounts of PrP[Sc] as well as PrP226* were found only in cerebellum, what is in agreement with before mentioned studies.[13,17] VV type case was also the only analyzed case with such an extreme difference in the amount of PrP226* between brain regions, indicating that genotype may influence not only PrP[Sc], but also PrP226* distribution. For 10 MM cases the results were not uniform, nevertheless, in the majority of cases higher

amounts of PrP[Sc] and PrP226* were found in cortical regions compared with cerebellum, what is in accordance with the findings of Choi et al.[13]

Since PrP226* is a PrP molecule, truncated at the very C-terminus, it lacks the GPI anchor. Until recently it was believed that GPI anchor is an essential feature for conversion of PrP[C] to PrP[Sc], but this assumption was rebutted when it was shown that transgenic mice, expressing only anchorless PrP, can be efficiently infected with scrapie[19,20] and that even spontaneous disease can occur in time.[21] Moreover, Jansen et al.[22] described two unusually presenting GSS cases in which nonsense mutations at one locus at the C-terminus of PrP resulted in the expression of anchorless PrP. In one case the protein ended with Tyr226 what means that abundant amounts of PrP226* were expressed and accumulated in patients brain. With one allele expressing full-length, GPI-anchored PrP, the expression of PrP226* seems to be the cause of the disease. The regional distribution of PrP226* was not addressed and the distribution of PrP[Sc] deposits was assessed according to IHC so the comparison with our results is not possible.

It is not clear at this point how many different PrP fragments, among which PrP226* is quite abundant, can be generated in human brain. They are an important feature in presentation of TSEs but their exact role has not been determined yet. From the information gathered so far on PrP226* it is clear that accumulation of this fragment is characteristic for at least some types of human TSEs (sCJD, vCJD, GSS)[8,22] and also for BSE and scrapie (unpublished data). Since its distribution in sCJD follows that of PrP[Sc] aggregates, it could be considered as surrogate marker for PrP[Sc]. As an anchorless PrP molecule, PrP226* is likely to be present in body fluids such as cerebrospinal fluid, urine and blood to which the development of future diagnostic of human TSE is focused.
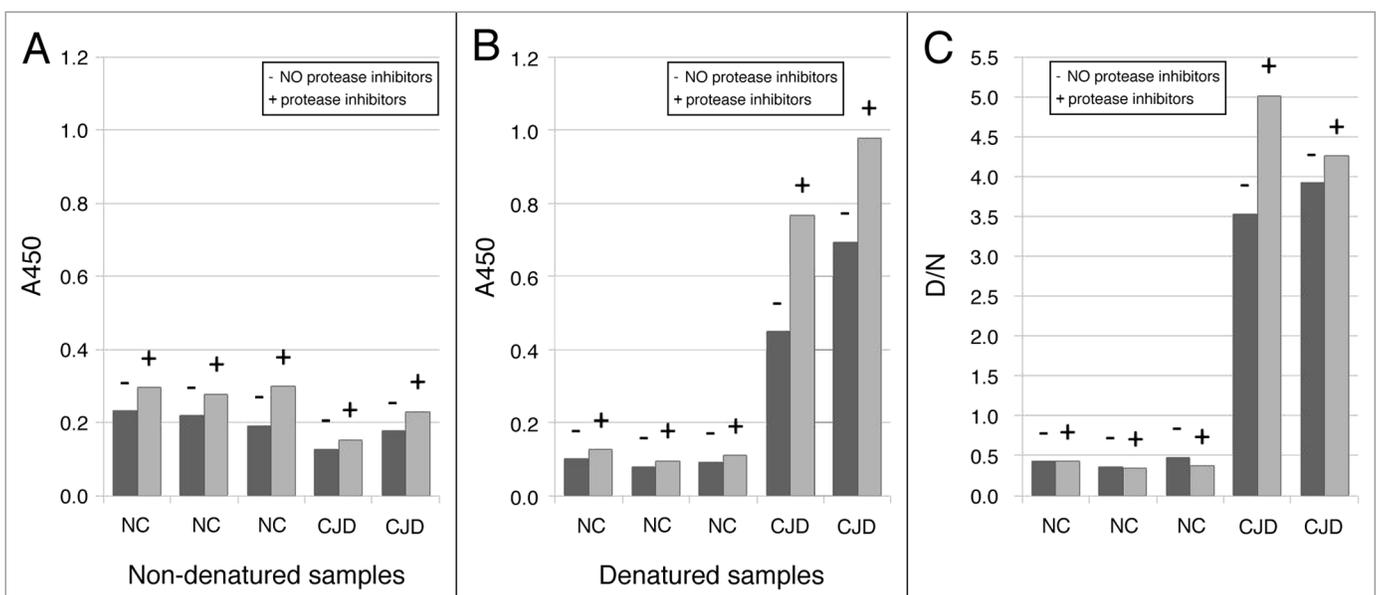
Figure 5. Influence of protease inhibitors in homogenization buffer on the amount of PrP226*. Samples were homogenized in buffer with (+) or without (-) of protease inhibitors. A_{450} values are shown for non-denatured (**A**) and denatured (**B**) samples.

Table 1. Summary of data on patients included in the study

		Age	Gender	Genotype	Pathological diagnosis	Additional pathological diagnosis	Duration of disease (months)	PrP^{Sc} deposition type regarding IHC	Protein 14-3-3	Ataxia	Cerebellar signs	Visual impairment
1	case	75	F	MM	sCJD	AD	1	synaptic	ND	yes	yes	yes
2	case	72	F	MM	sCJD	AD	14	synaptic	-	ND	no	no
3	case	69	F	MM	sCJD	AD	4	synaptic	-	no	yes	yes
4	case	62	M	MM	sCJD	none	3	mixed*	+	no	yes	yes
5	case	71	F	MM	sCJD	AD + PD	18	mixed*	-	no	no	no
6	case	77	M	MM	sCJD	tauopathy	2	synaptic	+	no	no	no
7	case	70	M	VV	sCJD	AD	7	mixed*	ND	yes	yes	ND
8	case	74	F	MM	sCJD	none	5	mixed*	+	no	no	no
9	case	65	M	MM	sCJD	none	2	synaptic	+	yes	yes	no
10	case	81	F	MM	sCJD	AD	3	mixed*	+	yes	yes	yes
11	case	70	M	MM	sCJD	tauopathy	5	synaptic	+	yes	yes	ND
12	case	74	F	MV	sCJD	AD	3	mixed*	+	ND	ND	ND
13	NC	75	F		tauopathy							
14	NC	68	F		AD II-III, diffuse gliosis							
15	NC	69	M		AD III, multiple microinfarcts in cerebral cortex							
16	NC	81	F		AD II, Binswanger disease							
17	NC	60	M		AD VI subependymoma in the third ventricle							
18	NC	68	M		vascular dementia							
19	NC	71	F		AD III, hypoxic encephalopathy							
20	NNC	84	F									
21	NNC	91	F									
22	NNC	77	F									
23	NNC	74	F									
24	NNC	74	M									
25	NNC	81	F									
26§	NNC	51	F		malignant cerebral edema, subarachnoid hemorrhage							
27§	NNC	64	M		cerebral edema, subarachnoid hemorrhage							
28§	NNC	55	F		central nervous system metastases							
29	NNC	69	M									
30	NNC	44	M									

Abbreviations: sCJD, sporadic CJD; NC, neurological control; NNC, non-neurological control; F, female; M, male; AD, Alzheimer's disease; PD, Parkinson's disease, IHC, immunohistochemistry; ND, no data; -, negative; +, positive. *More than one PrP^{Sc}-deposition type was determined by IHC. §These samples were included into the group of non-neurological controls despite the neurological condition, due to the absence of neurodegenerative disease. In all other non-neurological controls, nervous system was not impaired.

Materials and Methods

Consent for using the samples was obtained from National Medical Ethics Committee. Human brain tissue samples of 12 sCJD cases (5 males and 7 females) and 7 clinically suspected sCJD patients, who were disproved after *post mortem* examination by applying immunohistochemistry on PrP^{Sc}, called neurological controls (NC) (3 males and 4 females) were obtained from the Institute of Pathology, Medical Faculty, University of Ljubljana. For each sCJD case and NC, samples from five brain regions (cerebellum (C), frontal lobe (FL), occipital lobe (OL), olfactory bulb (OB) and spinal cord (SC)) were analyzed, except for one NC for which olfactory bulb tissue was not available. In ten cases of sCJD the genotype of the codon 129 was MM, in one case VV and in one case MV. Additionally, 11 cerebellar samples of patients (4 males and 7 females) with no neurodegenerative disease at time of death were analyzed (non-neurological controls, NNC). Detailed information on patients can be found in **Table 1**.

10% (w/v) brain tissue homogenates were prepared in homogenization buffer (10 mM Tris buffered saline, pH 7.5, containing 100 mM NaCl, 10 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate) using zirconium oxide 0.5 mm beads (Next Advance) and Tissue Lyser LT (Qiagen), aliquoted and frozen at -80 °C until use. For the test, each sample was prepared under denaturing and non-denaturing conditions. For denaturation, aliquots of 10% brain homogenates were mixed with equal volume of 3 M GdnSCN and incubated for 15 min at 60 °C with shaking. Nondenatured samples were mixed with equal volume of 50 mM TRIS-HCl instead of denaturant and left at room temperature for 15 min. Before loading to the microtiter plate, sample mixtures were diluted 10 times. For obtaining the denaturation profiles, chosen samples were denatured with increasing concentrations of GdnSCN, ranging from 0.5 M to 3 M GdnSCN in sample mixture. Final concentration of GdnSCN on microtiter plate never exceeded 0.3 M to avoid the negative influence of denaturant on coated Ab.

The immunoassay, used in this study, was based on the assay, developed for the detection of PrP226*,[10] however some important changes were introduced. The DELFIA protocol was substituted with standard ELISA. Unlike in previously described PrP226* assay, here mAb E12/2[23] was used as detecting Ab. Its epitope was located at C-terminal end of helix 1 of human PrP with His155 being crucial for binding.[23] Microtiter plate was coated with mAb V5B2 (5 μg ml^{-1}). Prepared samples were loaded and incubated for 90 min. Plate was then incubated with biotinylated mAb E12/2 and subsequently with HRP-labeled avidin. To develop color reaction, TMB was added and microtiter plate was incubated for 10 min. Reaction was terminated with the addition of 0.1 M H_2SO_4. The absorbance at 450 nm (A_{450}) of denatured samples (D) and of non-denatured samples (N) was measured. The ratios between D and N signals were calculated (D/N ratios). To measure PrP^{Sc}, the microtiter plate was coated with mAb 3F4 (Covance) at concentration of 0.8 μg ml^{-1}. All the other procedures were as in the PrP226* assay.

PrP226* assay was performed at least two times for all samples, except for 5 olfactory bulb samples (2, 5, 13, 14, and 17) and one spinal cord sample (18) that were no longer available after the first measurement. Since the denaturation was used for releasing PrP226* from aggregates, the value of D/N ratio was expected to be high for sCJD samples and 1 or below 1 for non-CJD samples. We defined a sample as positive, if the average D/N ratio was above 1.2 and negative, if the average D/N ratio was below 1.2. The results presented are the average D/N ratios of all the measurements for each sample (**Fig. 1A and B**).

Disclosure of Potential Conflicts of Interest

The authors declare that there are no conflicts of interest or competing financial interests.

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