Author's response to reviews

Title: Detection of the GPI-anchorless prion protein fragment PrP226* in human brain

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Version: 4 Date: 5 September 2013

Author's response to reviews: see over
Dear Editors of BMC Neurology,

On behalf of all the authors, I'm sending you the revised article *Detection of the GPI-anchorless prion protein fragment PrP226* in human brain.*

We were much honored to receive the report that was given from the Nobel laureate for prion research, Dr. Stanley B. Prusiner. We are thankful to the reviewer for his valuable comments and we are very glad that he finds our article interesting.

Here is the point-by-point replay to his comments.

1.) PK-resistant PrP226* was detected with a highly specific antibody. The authors should add immunoblots of patient tissue using this particular antibody to compare glycosylation patterns and size of PK-resistant PrP226* to the GPI-anchored full-length PrP isoforms usually visualized with antibodies detecting all isoforms.

Answer: We included a new Figure (Figure 1), showing western blots of sCJD brain homogenates and non-CJD controls, on which it is possible to compare glycosylation patterns of PrP226*, detected with mAb V5B2, and full length PrP, detected with mAb 6H4 (Figure 1B). However, we did not use PK for preparation of tissue samples for Figure 1B. As we show in Figure 1C, PrP226* is a PK-sensitive fragment (or at least V5B2 epitope on PrP226* is PK sensitive) and also the PrP226* assay that we describe in the article was developed without the use of PK. We also commented the new figure in the new paragraph of the Results section, which is entitled “The PrP226* C-terminus is degraded by PK”.

2.) Error bars are presented in Figure 1E but not in Figure 1A and C. Please add.

Answer: The reviewer probably meant Figure 2A and C. Results shown in figures 2A and C (now 3A and 3C) are presented as an example only. The reason why we do not show error bars in this two figures is that the results of D and N measurements differ significantly between different experiments (what is usual in ELISA and even more emphasized in DELFIA), while the ratio D/N is quite constant and repeatable. That’s one of the reasons why we use D/N and not D alone. We added this explanation to the Results section in the first paragraph under the title “The PrP226* assay” as well as in a Figure legend of Figure 3.

3.) The authors need to discuss the abnormality in PrP226* DELFIA measurements from healthy patients. I would have expected that the ratio of D/N measurements to be ~1 in a
healthy individual. Do the authors see the same effect with recombinant PrP226* or is this exclusive to PrP226* derived from human tissue?

Answer: The observed abnormalities are mainly the consequence of denaturation of the capture mAb. The denaturant that remains in the sample denatures a certain part of V5B2 paratopes what causes D/N to be less than 1. We discuss this influence in the third paragraph of Discussion and it is shown also in Figure 1B (now Figure 2B). We have made further experiments using recombinant PrP226*, where we removed the denaturant buffer after denaturation so that the final concentration of Gdn-SCN was only 0.2 mM before adding the sample to microtitre plate (i.e. there was approximately 4000x less denaturant added to the plate) and the D/N ratio raised from 0.6 to 0.9. (If the reviewer or Editors find these experiments important, we can add them as Additional files.) However, the procedure of exchanging the buffer is lengthy and therefore not useful for a high throughput assay.

4.) The authors should display the genotype of the GSS patient because the denatured PrP226* DELFIA value is highest from this patient compared to all other examined samples. It would be important to know if the GSS mutation generates this C-terminally truncated PrP without the GPI anchor attached, e.g., the Y226X mutation as reported by Jansen et al.

Answer: The GSS patient included in the study has a classical P102L mutation; however the manifestation of the disease was atypical, similar to a CJD disease. This was described in a case report (Rusina et al., 2012). We included this data in the text of Discussion section, sixth paragraph.

We are also very thankful to the second reviewer, Professor Dr. Clay Goodman.

Here are our point-by-point answers to his comments:

The methodology was adequately described but is daunting particularly when a D/N ratio is employed since there are potentially many confounding contributions from endogenous protease variability, variability in denaturing effects and possible variation in protein aggregate susceptibility to denaturing.

Answer: D/N ratio was already used in prion research and was proved to be a useful formula to obtain better differentiation between CJD positive and negative samples. While D or N data of a certain sample may vary significantly between different experiments, due to slight variations in the protocol, D/N represents a solid piece of information that is characteristic (repeatable) for a certain sample. The use of D/N should not be problematic due to endogenous protease variability, on the contrary, such variations between samples are normalized by dividing D measurement with N measurement, i.e. measurement of the same sample in non-denaturing conditions. Apart from that, we show in Figure 4 and in the Results section under the subtitle “The stability of the fragment PrP226*” that PrP226* levels are relatively resistant to endogenous proteases.
We agree that different prion protein aggregates (arising in different prion strains) need different denaturation conditions (this was proved by Safar et al. in 1998) and therefore some of our samples might have been suboptimally denaturated. However, a certain denaturation procedure had to be chosen that worked best for majority of samples. This is discussed in Discussion, third paragraph.

The sample size is rather small and this is a problem for the specificity of this assay that the anchorless truncated form was detected in non-prion diseased brains. The value of this biomarker is further degraded by the fact there is no correlation with other more conventional markers of disease susceptibility or progression.

Answer: We understand reviewer concerns about a small number of samples, however we must stress that sporadic Creutzfeldt-Jakob disease is extremely rare, occurring with the frequency of 1-2 cases/million people. Therefore in small countries such as Czech Republic and Slovenia it would take many years to collect significantly larger number of samples with written consent from relatives.

DELFIA is a very sensitive immunoassay, therefore the fact that we found a small amount of PrP226* in the healthy patients is not surprising as PrP degradation to some other physiologically important truncated forms has been reported previously. In our article we show that this physiological process is promoted in CJD diseased brain and substantial amounts of PrP226* are packed in prion aggregates. Only these packed molecules that are (specifically) released only after denaturation, may represent the CJD biomarker. PrP226* correlates with PrPres (Figure 5 C and D), which is a widely used biomarker for detection of prion diseases. The advantage of PrP226* biomarker is that is PK independent and therefore not subjected to the variability in PK activity.

The section looking at PK resistant fraction of PrP (PrPres) was significantly weakened significantly by the fact that only 12/24 CJD samples were used.

Answer: The quantity of PrPres was assessed by densitometric evaluations of western blots. As such values may vary significantly between experiments, we had to measure all samples together in the same experiment. For this reason only 12 most outstanding samples (having either very low or very high D/N) were chosen.

The authors did not adequately address the limitations of the study and the conclusions reached are only partially correct. "Using this method we were able to show, that this anchorless PrP fragment is involved in the formation of prion aggregates of CJD/GSS patients. We also show that PrP226* can be detected in some non-CJD brain. The role of PrP226* in healthy brain and the importance of its involvement in the patogenesis of human TSEs remains to be elucidated." The role of PrP226 in health and disease is certainly an area requiring further investigation, but the fact that PrP226 is found in healthy brain draws into question the assertion that PrP226 is involved in the formation of prion aggregates.

Answer: The reviewer is right that the statement “Using this method we were able to show, that this anchorless PrP fragment is involved in the formation of prion aggregates of CJD/GSS patients.” might not be documented well enough as it implies a functional role of PrP226* in the formation of prion aggregates which we did not investigate. We therefore
changed the statement to: “Using this method we were able to show, that this anchorless PrP fragment is a part of prion aggregates in the brain of CJD/GSS patients.”.

Minor issues included the frequent inconsistent spelling of tyrosine as tyrosin and other minor spelling issues. This can be corrected.

Answer: We are thankful to the reviewer for this comment. We corrected spelling.

We hope that our answers and changes that were made in the article sufficiently improved its quality and that the respected Editors will find it suitable for being publish in BMC Neurology.

Sincerely,

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