Screening optimization methods for prion disease biomarker diagnostics

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Abstract

Prion diseases are progressive neurodegenerative disease caused by the misfolding of a normal host protein. The long pre-clinical phase of the disease is followed by a rapid clinical course. Highly specific and sensitive diagnostics are lacking. The necessary discovery of peripheral biomarkers will aid in the identification, prevention, and treatment of the disease. Mass spectrometry analysis determined a list of 10 upregulated proteins in Creutzfeldt-Jakob disease (CJD) infected rats. As a first step in validating the results western blot analysis in uninfected rats was used to test for antibody efficiency. Antibodies for 14-3-3γ, neuron specific enolase (NSE), and ribonuclease T2 (RNaseT2) detected the proteins in the cerebrospinal fluid (CSF) of uninfected rats. Neural cell adhesion molecule (NCAM), a protein whose levels were constant in both infected and uninfected was proposed as a control. It was not detectable in CSF, however. Identifying peripheral tissue biomarkers for preclinical prion disease will lead to understanding the biology of prion disease, which may ultimately lead to possible management and prevention strategies.
Introduction

Prion diseases are a class of transmissible rapidly progressive neurodegenerative diseases with no treatment, cure or preclinical/clinical ante-mortem diagnosis. They are characterized by the accumulation and aggregation of a misfolded conformer of a normal prion protein (PrP\(^c\) to PrP\(^Sc\))\(^1\). There are genetic, sporadic and acquired forms, all of which have differences in pathology and PrP\(^Sc\) accumulation/deposition. In humans, prion disease includes genetic CJD, familial fatal insomnia, Gerstmann-Straussler-Scheinker syndrome, sporadic CJD (sCJD), iatrogenic CJD, kuru as well as variant CJD (vCJD)\(^2\). VCJD poses the highest risk for the population, as it is transmissible within and across species\(^2\). It is estimated that 1/2000 people in the UK are positive in their appendices for vCJD, and there is growing concern that preclinical carriers will iatrogenically spread the infection\(^3\). To avoid this, diagnostics and preventative medicines require further investigation.

Current diagnosis relies on a combination of tests including magnetic resonance imaging (MRI), electroencephalogram (EEG), CSF and blood biomarker tests, as well as clinical symptoms. More recent studies have investigated the peripheral tissues and CSF for diagnostic purposes, as these methods use less invasive techniques. For example, nasal swabs allow detection of PrP\(^Sc\) in nasal epithelium in sCJD patients\(^4\). Definitive confirmation of the disease is a post mortem examination, brain or tonsil biopsy in the case of vCJD\(^1\). PrP\(^Sc\) deposition in the tonsil only occurs in vCJD\(^2\), which limits diagnostic testing using peripheral tissues. CSF contains PrP\(^Sc\) in infected patients for all prion diseases but at low concentrations difficult to detect by most methods. Other proteins are easier to detect in the CSF of infected patients, as they are more abundant
than PrP\textsuperscript{Sc}. Proteins such as the 14-3-3 family\textsuperscript{5} and NSE\textsuperscript{6} have been previously shown to be upregulated in human prion disease, but are not specific as they are also upregulated in other neurodegenerative diseases\textsuperscript{4}. Other notable biomarkers are S100 and tau proteins, which are non-specific for prion disease, however are dysregulated compared to healthy controls\textsuperscript{5}. However, testing methods are lacking in disease specificity and general detection sensitivity.

In preliminary experiments for this study, prion disease was passaged into female Sprague Dawley rats. CSF was collected from these animals during the clinical stage of disease and compared to uninfected age-matched control samples via mass spectrometry by Dr. Lingjun Li at the University of Wisconsin at Madison. This analysis identified 10 proteins that were upregulated when compared to uninfected control samples. Western blot analysis was then chosen as a method of verifying results obtained from the mass spectrometry data. This study examines 3 of the 10 that proteins that were found to be upregulated in the CSF of clinically infected rats when compared to uninfected controls. Initial steps are to identify antibodies that react with these proteins and optimize detection using western blot analysis of uninfected control CSF and brain. It is necessary to confirm these observations in human subjects, and therefore protocol establishment is necessary. Identification of specific biomarkers could ultimately aid in the preclinical, antemortem detection and prevention of spread of the disease and offer further understanding of the disease for possible treatments and cures.

\textit{Proteins of Interest}

The 14-3-3 group is a specific group of proteins that have many regulatory roles in the cell, such as cell cycling, transcription and apoptosis\textsuperscript{6}. They are highly expressed in
the brain, skeletal muscle and heart, and not normally at high levels in CSF. There are 7 isoforms and the mass spectrometry analysis revealed the isoforms gamma (γ), epsilon and zeta/delta to be upregulated in prion disease; this study focuses on γ. 14-3-3 is a good biomarker of CJD as it has been shown to be more upregulated when compared to other neurodegenerative diseases; however it is still upregulated to an extent in other neurodegenerative diseases.

Enolase 2, or NSE was found to be elevated at early clinical stages of CJD, and has been shown to have neuroprotective and neurotropic properties. NSE is an enzyme in the glycolytic cycle that converts D-glyceraldehyde 3-phosphate to pyruvate using Mg2+ as a cofactor.

Another upregulated protein demonstrated by the mass spectrometry analysis was RNaseT2. It is a ubiquitous enzyme that in humans is encoded by the RNASET2 gene. It is well conserved through eukaryotic and bacterial cells as well as viruses and can have general roles in cellular housekeeping through RNA recycling or aiding in preventing pathogens. It is involved in cell proliferation, nitrogen storage and phosphate scavenging.

NCAM is a transmembrane protein involved in neuron-to-neuron adhesion. Mass spectrometry analysis revealed that NCAM protein expression levels remain the constant between clinically infected rats and their respective age matched controls, and is therefore considered for an adequate control.

Methods

CSF and brain samples were collected from four uninfected, female Sprague Dawley rats that were approximately two months of age. The rats were anesthetised using
isoflurane and lumbar puncture completed in the cisterna magna. The spines were then
severed and brains harvested. Two left hemispheres and 2 right hemispheres were
homogenized to 10% weight/volume (wt/v) using RIPA cell lysis buffer and aliquoted
into 30 µL pooled and individual stocks and stored at -80°C. 1% brain homogenate was
then prepared using RIPA buffer. Using a 96 round bottom well plate and bovine
albumin serum (BSA) as a standard, samples were loaded using 1/6 dilutions Pierce’s
BCA assay reagents were prepared according to manufacturers’ instructions deposited,
then the plate incubated at 37°C for 2 hours, this was repeated for CSF. After
incubation, using the spectrophotometer (SpectraMax M5), the absorbance was read at
562nm and samples adjusted accordingly for total protein.

**Western blot**

A serial dilution of brain homogenate was made (Table 1), boiled with sample
buffer in a 1:1 ratio and stored at -20°C. CSF samples from individuals and pooled were
mixed with sample buffer in a 1:5 ratio of sample buffer to CSF, and stored at -20°C.

The general protocol was to install the 15 well polyacrylamide gels in the cassette
with 1X MOPS solution plus antioxidant to maintain the proteomic reduced state, load
samples along with the All Blue Ladder and Laemmli buffer for the blank. All samples
were mixed with Laemmli buffer and boiled for 10 minutes prior to loading.
Electrophoresis was run at 60V for 1 hour, then 120 V until samples migrated to the
bottom of the gel. The transfer to PVDF membranes was performed at 40 V for 1 hour in
transfer buffer. The membranes were blocked with skim milk in TBS-T for 30 minutes.

The membranes were incubated with the primary antibodies for 14-3-3γ 1:1000
(Figure 1) and 1:500 (Figure 7), NSE 1:200 (Figure 2), NCAM 1:1000 (Figure 4) and
1:500 (Figure 5A), and RNase T2 1:1000 (Figure 3). The membranes were washed using TBS-T six times for four minutes each and placed in secondary goat-anti-rabbit-HRP (GaR), 1:10 000, for all but NCAM which required rabbit-anti-goat-HRP (RaG), 1:30 000 and 1:60 000. Secondary antibody without primary served as a control, GaR 1:5000 and RaG 1:30 000 (Figure 6). Enhanced chemiluminescence (ECL) was used to visualize the proteins on X-ray films.

**Results**

The calibration of detection of positive biomarkers using uninfected rat CSF and brain homogenate are as follows:

**14-3-3γ**

Protein concentration for 14-3-3γ in the pooled rat CSF was determined to be of similar concentration to 0.01 µg/µL (Figure 1) when compared to the same molecular weight bands detected in the serial dilutions of brain homogenate at the expected 33kDa. This antibody also detects a band just under the 75kDa marker in brain homogenate that looks similar in size to albumin, and 2 smaller ones below it at 55 and 60kDa. None of the other antibodies detected any albumin in the brain homogenate.

**NSE**

NSE was similar to 0.004 µg/µL (Figure 2) as determined by the same size band in the brain homogenate ladder at the expected 47kDa. It is important to note that there was observed non-specific binding with the molecular weight ladder and the NSE antibody.

**RNase T2**
Only a weak signal was detected for RNaseT2 (Figure 3) in the CSF at 30kDa, and in a different molecular weight in brain homogenate, 48kDa. 

**NCAM**

The approximate size of the different isoforms of NCAM are as follows: transmembrane 140/180 kDa, GPI-linked 120/125 kDa, soluble fragment: 110 kDa. NCAM was not detectable in the brain homogenate dilutions, however did react with some bands in CSF (Figure 4). This was later determined to be the same bands in CSF as RoG alone; nonspecific binding possibly covering whatever band was detected in the CSF at 75, 49, and 35kDa, as demonstrated by higher primary antibody concentration, 1:500, and lower secondary antibody, 1:60 000 (Figure 5B). These concentrations also detected NCAM in the 1 µg/µL. There is a possible band for the transmembrane fragment in the CSF of individual 4 (Figure 4), however the other isoforms are not visibly detected in CSF or brain homogenate

It is important to note that all of the primary antibodies as well as RoG cross reacts with albumin (MW 66kDa) in the CSF. Individual 4 had a higher protein concentration than the other individuals for 14-3-3γ, NSE, and albumin (providing that is the protein to which nonspecific binding of the antibodies occurs).

**Secondary antibodies**

GaR secondary antibody does not bind non-specifically to any proteins on the western as evidenced by a blank X-ray film (Figure 6). RoG has high background noise over the entire membrane and it appears that some of the same bands are visible in the CSF with only secondary antibody at 75, 49, and 35kDa, compared to with both NCAM primary and RoG (Figure 5B, 4).
**Time and temperature variations**

For the varying times of heating in Laemmli buffer, the sample that was not heated at all and the sample that was heated at 50°C for ten minutes had much more higher molecular weight proteins than the other treatments (Figure 7).

**Discussion**

The goal of optimizing detection methods using western blot for prion diseases was tested using CSF and brain homogenate of uninfected rats with aim in identifying antibodies adequately sensitive for preclinical diagnostics. Most of the antibodies tested proved adequate as they were able to detect the proteins in the CSF of uninfected which, when compared to infected, were shown to be a much lower concentrations.

Anti 14-3-3γ binds to other proteins, possibly albumin and IgG in the brain and CSF. However, bands do differ between the samples. This is expected, as the albumin should be higher in CSF than brain. On the other hand, it has been found that 14-3-3γ is more relevant to Parkinson’s disease, or cancer rather than CJD specifically, and that the zeta/delta isoform is a superior candidate for sporadic and variant CJD as it is found in the amyloid plaques while the other isoforms are not\textsuperscript{10,11}.

Anti-NSE binds to the molecular weight ladder, as well as albumin. More research needs to be done in regards to what exactly the anti-NSE is binding to in the ladder, as it is not just one but all of the markers. This is problematic as it speaks to a decreased specificity, another antibody should be tested instead. Specificity and sensitivity are both important in identifying preclinical markers, as they are much less abundant than in clinical stages. Albumin is more abundant than any of the proteins
probed for in CSF and makes it difficult to visualize on a western blot needing maximum exposure as well as antibody ratio.

The fact that the secondary antibody produces so much background noise without any primary antibody is a concern. One possible solution to eliminate background and would be to block with BSA instead of skim milk\textsuperscript{12}. BSA contains only one protein while milk contains many, including the phosphoprotein casein. Others have shown cross reactivity between casein and antibodies for phosphorylated proteins like NCAM\textsuperscript{13,14}.

The time and temperature variations of heating in Laemmli buffer also need to be investigated further as the lower temperature treatments have more higher molecular weight proteins in the CSF samples. It is possible that the high heat treatment of the CSF damages what minimal proteins that are there.

The 50kDa band in the CSF blots could be IgG, however, further investigation is needed. Neither IgG nor albumin from the blood showed up in the highly diluted brain homogenate. At higher concentrations they might be visible, such as 0.003 \(\mu\)g/\(\mu\)L and above. Higher concentrations of brain homogenate might have also helped detect NCAM, as it seemed to be visible in the 1 \(\mu\)g/\(\mu\)L.

\textit{Limitations}

The antibodies are temperature sensitive. RaG, GaR, NCAM and 14-3-3\(\gamma\) are to be kept at 4\(^\circ\)C. During the experiment, the refrigerator malfunctioned and the antibodies warmed to room temperature for approximately 8 hours; this could have affected their efficacy. Working with very low concentrations of BH combined with low volumes of
sample buffer leaves room for human error also. Any discrepancy will have a great effect at such low concentrations. More repeats of the experiment might aid in overcoming this. The number of rats used in the pooled samples might have had an effect on the results as well. A sample number of 4 is not adequate to compare to an entire population, using a larger sample size for further investigations would be a good recommendation. The antibodies at concentrations used likely would detect stronger signals in infected samples due to them being upregulated when compared to healthy controls, however access to those was unavailable. Determining the relative thickness of the bands detected in with all of the antibodies in CSF compared to the brain homogenate ladder needs to be confirmed with Photoshop.

A lumbar puncture is somewhat invasive and painful, and is not normally prescribed unless a problem surfaces requiring a CSF sample. Furthermore, it is important to consider that by the time the procedure is referred, CJD will have already progressed to clinical representation at the late stages of disease. Perhaps this test would be better recommended for patients such as those 1/2000 UK vCJD carriers, as a routine screening before a surgery so as to prevent the unknown spread of the disease, or a requirement once a certain age is attained. Finding an acceptable and reliable marker in the CSF of CJD patients could lead to more accessible and less invasive means such as blood or urine samples providing the established biomarkers are present. This is important as once a person is suspected of having the disease, their quality and longevity of life will have greatly decreased. Different testing/diagnostic methods have their own benefits and/or shortfalls (Table 2).
Conclusion

Neurodegenerative diseases have many biomarkers in common. The levels and quantities, however, vary between the different neurodegenerative disorders. Current practices only diagnose clinical and post-mortem subjects. Detecting specific biomarkers, or group of biomarkers in preclinical prion disease in rats will develop the understanding of the biology and aid in the development of therapeutic interventions, preventions, or, at the minimum, a delay of clinical onset. The next step would be to test the results in humans, and if comparable to begin administration of said treatments to cater to preclinical CJD positive individuals needs.
References:


Figures

Figure 1. **Anti-14-3-3γ 1:1000 to GaR-HRP 1:10 000.** 14-3-3γ is detectable at approximately 33kDa. The antibodies also detect a band in the brain homogenate just under the 75kDa marker, which is similar in size to albumin which is what is hypothesized to be the similar bands in the CSF.

Figure 2. **Anti-NSE 1:2000 to GaR-HRP 1:10 000.** NSE is detected at approximately 47kDa in both brain and CSF. Albumin cross reacts with the NSE antibody in the CSF. NSE antibody also reacts with the molecular weight ladders.
Figure 3A. **Anti-RNaseT2 1:1000 to GaR-HRP 1:10 000.** The protein was only slightly detected at 30kDa. In brain homogenate the protein is much larger at 48kDa. B. Coomassie reveals total protein levels.

Figure 4A. **Anti-NCAM 1:1000 to RaG-HRP 1:30 000.** There is a possible band for the transmembrane fragment in the CSF of individual 4, however the other isoforms are not detected in CSF or brain homogenate. Albumin is detected as well as an unknown band slightly below that. B. Coomassie stain reveals total protein levels.
Figure 5 A. **Anti-NCAM 1:500 to RaG-HRP:60 000.** The combination of antibodies detects 3 bands in the 1 µg/µL brain homogenate at 75, 49, and 35kDa and in CSF 1 band at 55kDa. There is a lot of background noise over the membrane.

B. **RaG-HRP 1:30 000.** This antibody binds without any primary to the entire membrane and has its own bands on what appears to be albumin only in the CSF, there are three at 75, 65 and 55 kDa. This is the same molecular weight as with anti-NCAM and R α G-HRP together.

C. Coomassie stain of NCAM 1:500 to R α G-HRP membrane.

D. Coomassie stain of R α G-HRP membrane.
Figure 6. **GaR-HRP 1:5000.** This antibody does not bind non specifically to the membrane.

B. Commasie reveals total protein.

Figure 7. **14-3-3γ 1:500 to GaR-HRP 1:10 000.** 14-3-3γ was detected in brain homogenate as well as CSF, more so in individual 4, no boiling in sample buffer, as well 50 degrees celsius. According to the other proteins, it seems the higher and longer heat treatments greatly increased the breakdown of the larger proteins.
Table 1. **Serial dilutions of brain homogenate.**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Brain homogenate added (µL)</th>
<th>Final concentration (µg/µL)</th>
<th>RIPA added (µL)</th>
<th>Total volume (µL)</th>
<th>Sample Buffer added (µL)</th>
<th>Total volume (µL)</th>
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<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>0.05</td>
<td>26.5</td>
<td>30</td>
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<td>60</td>
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<tr>
<td>2</td>
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<td>0.03</td>
<td>28</td>
<td>30</td>
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<td>60</td>
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<td>0.022</td>
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<td>5</td>
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<td>6</td>
<td>0.4</td>
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<td>0.26</td>
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<td>8</td>
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</table>
**Table 2. Diagnostic tools; tests and substances.** Different testing methods, proteins and bodily fluids have positive and negative facets when diagnosing CJD.

<table>
<thead>
<tr>
<th></th>
<th>Positives</th>
<th>Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MRI</strong></td>
<td>Non invasive</td>
<td>Not specific to prion disease, and within prion disease there is variation</td>
</tr>
<tr>
<td><strong>EEG</strong></td>
<td>Non invasive</td>
<td>Different forms of prion disease have different findings</td>
</tr>
<tr>
<td><strong>14-3-3</strong></td>
<td>Easily detected, more abundant than PrP&lt;sub&gt;Sc&lt;/sub&gt;</td>
<td>Non specific for prion disease</td>
</tr>
<tr>
<td><strong>NSE</strong></td>
<td>Easily detected, more abundant than PrP&lt;sub&gt;Sc&lt;/sub&gt;</td>
<td>Non specific for prion disease</td>
</tr>
<tr>
<td><strong>Tau</strong></td>
<td>Easily detected, more abundant than PrP&lt;sub&gt;Sc&lt;/sub&gt;</td>
<td>Non specific for prion disease</td>
</tr>
<tr>
<td><strong>S-100</strong></td>
<td>Easily detected, more abundant than PrP&lt;sub&gt;Sc&lt;/sub&gt;</td>
<td>65% sensitivity, non specific for prion disease</td>
</tr>
<tr>
<td><strong>Brain biopsy</strong></td>
<td>Accurate, PrP&lt;sub&gt;Sc&lt;/sub&gt; accumulates in the neurons</td>
<td>Invasive</td>
</tr>
<tr>
<td><strong>Tonsil biopsy</strong></td>
<td>IHC 100% detected for vCJD&lt;sup&gt;17&lt;/sup&gt;</td>
<td>Only detects vCJD&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RT-QuIC</strong></td>
<td>80% sensitivity and 100% specificity&lt;sup&gt;19&lt;/sup&gt;</td>
<td>More clinical, not preclinical&lt;sup&gt;19&lt;/sup&gt;, we would like to know before PrP&lt;sub&gt;Sc&lt;/sub&gt; accumulates, perhaps there is a biomarker that reflects initial infection, or the genetic variants which would lead to prevention and/or early treatment of the disease.</td>
</tr>
<tr>
<td><strong>Nasal swabs</strong></td>
<td>sCJD&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Not in mucous, only epithelium</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td>Sensitivity of 92.9% and a specificity of 100.0%&lt;sup&gt;16&lt;/sup&gt;</td>
<td>PrP&lt;sub&gt;Sc&lt;/sub&gt; only in urine of vCJD not sCJD, and only 40 to 100 oligomeric particles of PrP&lt;sub&gt;Sc&lt;/sub&gt; per milliliter of urine</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>Low invasive</td>
<td>Background PrP&lt;sup&gt;Sc&lt;/sup&gt; is higher than other tissues, also other proteins&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CSF</strong></td>
<td>Low invasive</td>
<td>Closer to brain than other fluids, less protein than other fluids/tissues like blood including PrP&lt;sup&gt;19&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>