

Establishment of a standard 14-3-3 protein assay of cerebrospinal fluid as a diagnostic tool for Creutzfeldt–Jakob disease

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Periodic sharp wave complexes observed on an electroencephalographic recording and the presence of a 14-3-3 protein in the cerebrospinal fluid (CSF) are both included in the diagnostic criteria for the Creutzfeldt–Jakob disease (CJD) supplied by the World Health Organization; however, the presence or absence of the 14-3-3 protein in the CSF is sometimes difficult to discern on a western blot because of equivocal bands. The goal of this study was to establish a standard 14-3-3 protein assay and to determine the threshold level of a 14-3-3 protein that can be assayed by western blot. We searched for the most suitable isoform of the 14-3-3 protein to test for in protein assays, and the most sensitive antibody among four antibodies with an affinity for 14-3-3. We measured the levels of all 14-3-3 isoforms in 112 patients with CJD and in 100 patients with other diseases. We compared the performances of four different antibodies. We carried out a semi-quantitative analysis of γ -isoform levels using the LAS 3000 system, which was capable of producing a digital image from the luminescence on a western blot. We determined that the most suitable isoform of the 14-3-3 protein for conducting a standardized assay was the γ -isoform. Among the four commercially available antibodies for this protein, the most sensitive and specific was 18647 (IBL, Japan). We report the high repeatability of the detection of the 14-3-3 protein by this antibody to the γ -isoform, showing that western blot can be used for semi-quantitative analysis.

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As abnormal prion proteins cannot be detected at present without brain biopsy, Supplementary Methods are required for the diagnosis of the Creutzfeldt–Jakob disease (CJD). In the past, the diagnosis of CJD depended on clinical findings and on electroencephalographic (EEG) criteria. Since 1999, detection of periodic sharp wave complexes observed on EEG records, as well as the presence of a 14-3-3 protein in the cerebrospinal fluid (CSF), have been considered to be reliable diagnostic markers for CJD. Both are included in the diagnostic criteria for CJD supplied by the World Health Organization (WHO).¹ Determination of the presence or absence of the 14-3-3 protein in the CSF is sometimes confusing because of

equivocal bands, which can complicate the interpretation of assay results.

Therefore, 14-3-3 tests are time consuming and expensive, because of the necessity of obtaining judgments from multiple investigators. Thus, the development of a standard 14-3-3 assay and a valid criterion for quantitative assessment are very important. We have created a standard 14-3-3 protein assay to establish the precise diagnostic criteria for the level of the 14-3-3 protein in the CSF of patients with CJD. Our assay uses semi-quantitative analysis of digital images obtained from luminescence on western blots. Our data show that assaying for the γ -isoform of 14-3-3 in this manner produces reproducible results.

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METHODS**Patients and Disease Control Groups**

The individuals enrolled for this study were 112 patients with a confirmed diagnosis of CJD. All the 112 patients fulfilled the WHO diagnostic criteria for CJD (Table 1a). All participants were examined and CSF samples were obtained. CSF samples were collected, divided into aliquots, and stored at -80°C until use. All assays were carried out at the same time to avoid repeated freezing and thawing of the samples. The CSF samples were used within 1 month. The control group consisted of 100 patients admitted with other diseases to the Neurology departments at the Nagasaki University, the Nagasaki Kita Hospital, and at the Nagasaki Medical Center of Neurology. For the control data, we collected CSF samples

from 100 patients who suffered from one of the following disorders: dementia of Alzheimer's type (DAT) ($n=54$: male = 33, female = 21), cerebrovascular disorders ($n=7$: male = 5, female = 2), Pick's disease ($n=1$: male = 1, female = 0), Parkinson's disease ($n=5$: male = 4, female = 1), corticobasal degeneration ($n=2$: male = 0, female = 2), Huntington's disease ($n=1$: male = 1, female = 0), frontotemporal dementia ($n=1$: male = 1, female = 0), progressive supranuclear palsy ($n=3$: male = 2, female = 1), mild cognitive impairment ($n=3$: male = 1, female = 2), amyotrophic lateral sclerosis ($n=3$: male = 2, female = 1), temporal epilepsy ($n=5$: male = 3, female = 2), limbic encephalopathy ($n=3$: male = 2, female = 1), paraneoplastic cerebellar degeneration (PCD)/Lambert–Eaton myasthenic syndrome

Table 1 Profiles of (a) 112 CJD patients and (b) patients in disease control group ($N=100$ cases)

(a)		Definite cases	Probable cases	Possible cases	Total cases
Sporadic cases	Male	4	48	0	52
$N=99$	Female	3	44	0	47
Familiar cases	Male	0	2	2	4
$N=8$	Female	3	0	1	4
Iatrogenic cases	Male	0	3	0	3
$N=5$	Female	1	1	0	2

(b)	Male (cases)	Female (cases)	Total cases
ATD	33	21	54
CVD	5	2	7
Pick's disease	1	0	1
Parkinson disease	4	1	5
CBD	2	0	2
Huntington's disease	1	0	1
FTD	1	0	1
PSP	2	1	3
MCI	1	2	3
ALS	2	1	3
Temporal epilepsy	3	2	5
limbic encephalitis	2	1	3
MELAS	1	1	2
PCD/LEMS	1	1	2
undifferentiated encephalopathy	1	3	4
healthy subjects	2	2	4

ALS, amyotrophic lateral sclerosis; ATD, Alzheimer-type dementia; CBD, corticobasal degeneration; CVD, cerebral vascular disorder; FTD, frontotemporal dementia; LEMS, Lambert–Eaton myasthenic syndrome; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; MCI, mild cognitive impairment; PCD, paraneoplastic cerebellar degeneration; PSP, progressive supranuclear palsy.

We analyzed 112 CJD patients that were classified according to the criteria from the WHO. All iatrogenic cases were dura-associated with CJD.

(LEMS) ($n=2$: male = 1, female = 1), MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) ($n=2$: male = 1, female = 1), and encephalopathy owing to unknown etiology ($n=4$: male = 1, female = 3). We also obtained CSF from 4 healthy volunteers ($n=4$: male = 2, female = 2) (Table 1b). All patients or their families agreed with the aims and significance of our research, and provided appropriate informed consent.

CSF samples were collected, divided into aliquots, and stored at -80°C until they were assayed. All of the assays were carried out at the same time to avoid repeated freezing and thawing of the samples.

Overexpression and Purification of the β -Isoform and γ -Isoform of the 14-3-3 Protein in Murine 293T Cells

We obtained the full-length gene encoding the β - and γ -isoforms of the 14-3-3 protein from a cDNA library.

Full-length constructs encoding either the β - or γ -isoform of human 14-3-3 protein in addition to a His-tag were cloned into the pcDNA6/His vector (Figure 1), after which the constructs were transfected into murine 293T cell lines and overexpressed. Each protein was collected and purified thrice through an affinity chromatography column.

Semi-Quantitative Analysis of the β - and γ -Isoforms of the 14-3-3 Protein in Western Blots

We performed western blots on samples of recombinant β - or γ -isoform of the 14-3-3 protein at concentrations of 12.5, 25, 50, and 100 $\mu\text{g/ml}$, and immunoassays for the purified 14-3-3 protein, as described previously.^{2,3} We performed the 14-3-3 protein immunoassay on all samples according to previously published standard samples. A volume of 50 μl of CSF was mixed with 10 μl of sample buffer (5% glycerol, 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate, and a trace of bromophenol blue in the final solution) and boiled for 5 min. Samples were separated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (4% stacking gel with a 12% resolving gel) at 75 V for 3 h, and transferred to nitrocellulose. Immunostaining was performed by blocking the nitrocellulose membrane with Tris-buffered saline containing 0.3% Tween 20 for 30 min, followed by incubation with a rabbit polyclonal antibody against the β -isoform of 14-3-3 at a dilution of 1:500, and subsequent incubation with alkaline phosphatase-conjugated anti-rabbit IgG at a 1:1000 dilution.

Polyclonal antibodies specific for the β -isoform (sc-628 and sc-629, 1:100, Santa Cruz Biotechnology, CA, USA, or 18641, 1:4000, IBL, Gunma, Japan) and for the γ -isoform (K0203-3, 1:5000, MBL, Japan or 18647, 1:500, IBL) were obtained (see Supplementary Table 1). Protein was detected using an enhanced chemiluminescence detection kit (Amersham Buchler), and blots were photographed using a LAS-3000 (FUJIFILM) chemiluminescence detection device featuring outstanding resolution and sensitivity. By digitizing the resulting image, the LAS-3000 device facilitated quantitative image analysis and presentation. This system was

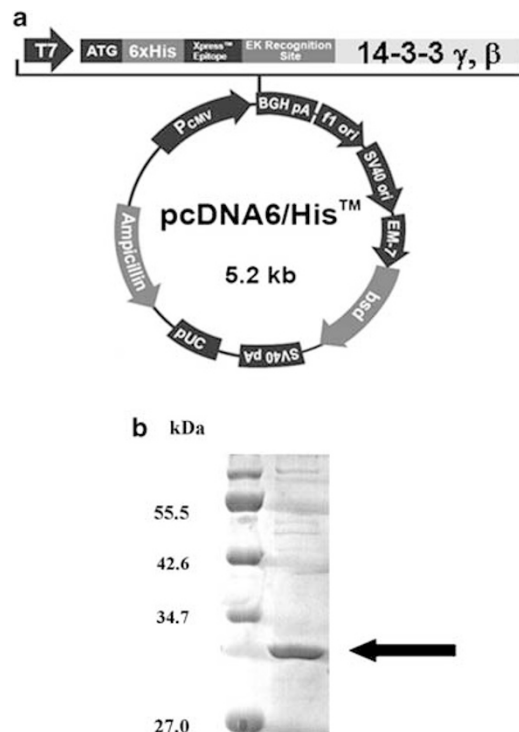


Figure 1 (a). The constructs encoding the β - or γ -isoforms of the 14-3-3 protein in pcDNA6/His vector (b). We transfected constructs encoding the β - or γ -isoform of 14-3-3 into murine 293T cell lines, and overexpressed the proteins. We collected and purified each protein. Each protein was purified thrice through an affinity chromatography column. We performed Coomassie Brilliant Blue staining to check whether the protein expressed was the β - or γ -isoform.

capable of producing a digital image from even very weak luminescence. A total of 10 replicate measurements were performed for each sample (Figure 2).

Detection of the β -isoform and γ -isoform of the 14-3-3 Protein in CSF Samples

CSF samples were collected, divided into aliquots, and stored at -80°C until use. All assays were carried out at the same time to avoid repeated freezing and thawing of the samples. Immunoassays for the 14-3-3 protein in the CSF were performed as described previously^{2,3} (Table 1a).

Blots were subjected to chemiluminescence detection and photographed using the LAS-3000 device. We created standard curves on the basis of a semi-quantitative analysis of the western blots of known amount of the β - and γ -isoforms of the 14-3-3 protein. These curves allowed standard measurements of 14-3-3 levels obtained from western blots of the patients' CSF. The following polyclonal antibodies specific for all isoforms of the 14-3-3 protein were used: 10017 for the τ -isoform of the 14-3-3 protein (IBL), 18644 for the ζ -isoform (IBL), 18643 for the ε -isoform (IBL), and 18645 for the η -isoform (IBL).

For all semi-quantitative analyses of CJD samples for the 14-3-3 protein, calibration samples of 25 and 50 $\mu\text{g/ml}$ recombinant 14-3-3 protein were included on western blots

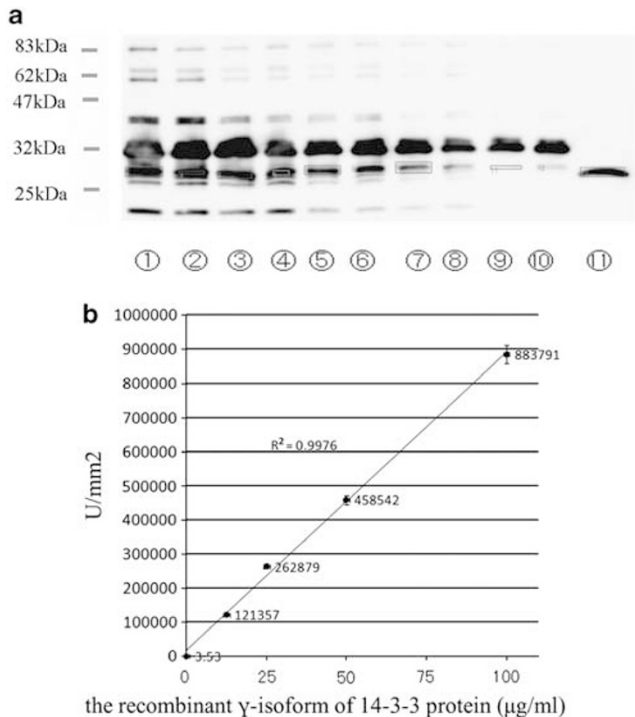


Figure 2 Semi-quantitative analysis of western blots of the β - and γ -isoforms of the 14-3-3 protein (a). We performed western blots of samples of 12.5, 25, 50, and 100 μg per lane of the recombinant γ -isoform, and immunoassays for the 14-3-3 protein 18647 (1:500) (IBL, Gunma, Japan). Protein was detected using an enhanced chemiluminescence detection kit (Amersham Buchler). We analyzed blots reacted with the chemiluminescence detection kit using the LAS-3000 device (FUJIFILM). Lanes 1 and 2: 200 μg per lane of the recombinant γ -isoform; lanes 3 and 4: 100 μg per lane; lanes 5 and 6: 50 μg per lane; lanes 7 and 8: 25 μg per lane; lanes 9–10: 12.5 μg per lane; lane 11: CSF of sporadic CJD patient (definite case). (b). The LAS-3000 device (FUJIFILM) was capable of producing a digital image from even very weak luminescence. The samples were repeated using the same methods 10 times. The detection of the γ -isoform of the 14-3-3 protein by the antibody of 18647 was excellent. We measured the detection density of western blots of samples using the LAS-3000 (FUJIFILM) twice on each assay. We computed the average and s.d. of the measured value at 20 time points. Error bars show s.d. (mean and 95% CIs). The regression line is a near perfect fit to the data ($R^2 = 0.99812$).

as a standard. If the standards deviated from the average by more than 2 s.d., the data were not used.

Resolving Power of the β -isoform and γ -Isoform of the 14-3-3 Protein by Time Course in Recombinant Protein and CSF

We measured the time course of recombinant and CSF-derived β - and γ -isoforms of the 14-3-3 protein in western blots that were stored at room temperature and at 4 °C for intervals of 0, 1/2, and 24 h. All bands detected by western blots were analyzed using the LAS-3000 (FUJIFILM) device.

All isoforms of the 14-3-3 Protein on Western blot Methods in CJD and Control Patients' CSF

A total of 20 CJD patients were 19 cases of sporadic cases (4 definite cases, 15 probable cases) and 1 case of iatrogenic

CJD. In all, 15 cases were typical classical cases of sporadic CJD. The 20 control patients included 16 patients with DAT, 1 case of limbic encephalitis, 1 case of MELAS, and 1 case of PCD/LEMS. We performed western blotting for all isoforms of the 14-3-3 protein on samples obtained from these 20 CJD patients and 20 non-CJD patients. The same polyclonal antibodies described above were used.

Genetic Analysis

Genomic DNA extracted from peripheral blood leukocytes was used to amplify the open reading frame of the PrP gene by polymerase chain reaction. The product sequences were searched for polymorphisms at codons 129 and 219 by sequencing, as described previously.

Protein analysis of PrP^{Sc} (Abnormal Prion Protein)

Brain tissues were isolated at autopsy from patients with prion disease and from unaffected participants and deep-frozen until use. For the analysis of PrP, the tissues were thawed and homogenized in buffer. Aliquots of the resuspended samples were treated with proteinase K (25 $\mu\text{g/ml}$) at 37 °C for 1 h and then analyzed by western blot (PrP^{Sc} samples) as described previously.^{4,5} Anti-PrP antibodies used for the analysis included 3F4. Signals were developed using the enhanced chemiluminescence system (Amersham Pharmacia). For the de-glycosylation of PrP, samples prepared for western blots were mixed with buffer containing N-glycosidase F (PNGase F) and incubated overnight at 37 °C. PrP^{Sc} samples were classified as type 1 (unglycosylated PrP^{Sc} of 20–21 kDa) or type 2 (unglycosylated PrP^{Sc} of 18–19 kDa) on the basis of Parchi's classification. We performed PrP^{Sc} typing in all definite cases.

Statistical Analysis

The 14-3-3 protein levels of the 112 CJD patients were compared with those of the other 100 patients using ROC analysis. Standard measures of statistical significance were used to identify true-positive, true-negative, false-positive, and false-negative results.

RESULTS

Patient Characteristics

We examined 112 patients with CJD, as determined using diffusion-weighted MR imaging (DWI), EEG, and clinical findings, and classified the cases as sporadic CJD ($n = 99$), familial CJD ($n = 8$; 5 cases resulting from a V180I mutation in the prion protein and 3 cases resulting from an M232R mutation in the prion protein), or iatrogenic CJD (dura-associated CJD; $n = 5$). (Table 1a). All CJD cases were examined by DWI, and only DWI-positive cases were used in this study. Sporadic CJD patients ($n = 99$) were divided into three groups (classical CJD, MM2-cortical form, and atypical MV1). Classical CJD patients ($n = 94$) were classified by the WHO criteria as 'definite case' ($n = 7$) or as 'probable case' ($n = 87$). Classical CJDs ($n = 94$) were divided into MM

($n = 92$) and MV ($n = 2$). Four sporadic CJD cases were of the MM2-cortical form of CJD, all definite cases, and one atypical case of sporadic CJD was MV1. The definite cases were included cases of the MM2-cortical form, two cases of the MM1 form, and one case of MV1. All cases of iatrogenic CJD and familial CJD were MM at codon 129 and EE at codon 219 of the PrP gene.

The autopsy rate was 10–20% in all CJD Japanese patients. To improve the precision of CJD patient diagnosis, the classical cases limited the typical clinical findings, the clinical time course of illness, and the laboratory findings (DWI and EEG). Shiga *et al*⁶ reported that the sensitivity and specificity of DWI was 92.3% and 93.8, respectively. DWI findings that were identified as indicative of CJD included lesions with high signal intensity areas in the basal ganglia and the cerebral cortex.

Isoforms of the 14-3-3 Protein in the CSF of CJD and Control Patients

The western blots of many CJD cases (19 of 20 cases) were positive for the β -, γ -, and η -isoforms of the 14-3-3 protein, but the β -, γ -, and ε -isoforms were detected in only a few samples (1 of 20). By contrast, the ε - or the η -isoform was identified in many other control disease patients (19 of 20), but the β -, γ -, and τ -isoforms appeared only in samples collected from patients with PCD/LEMS or limbic encephalitis caused by a wide and acute damage of the cerebral cortex and white matter. In almost all samples that were obtained from both CJD and control disease patients, the η -isoform, but not the τ -isoform, was detected.

Characterization of Four Antibodies against the 14-3-3 protein (sc-639, 18641, 18647, and K0203-3 Antibodies)

Although the commercial sample sheet stated that the sc-628 and sc-629 antibodies (Santa Cruz Biotechnology) reacted only with the β -isoform, our results contradicted this claim. In our study, this antibody recognized both the β - and γ -isoforms (Figure 3), with no difference in western blot band density between the 25 and 50 $\mu\text{g}/\text{ml}$ β -isoform samples of recombinant protein (Figure 3). By contrast, there was a difference in density between the 25 and 50 $\mu\text{g}/\text{ml}$ of γ -isoform recombinant protein samples. These results showed that the sc-628 and sc-629 antibodies reacted with the γ -isoform but not with the β -isoform. We concluded that the sc-629 antibody specifically reacted with the γ -isoform.

Similarly, although the commercial sample sheet stated that the 18641 antibody (IBL) reacted only with the β -isoform, our result showed that 18641 also reacted with a high concentration of the γ -isoform. The 18647 (IBL) and K0203-3 antibodies (MBL) reacted only with the γ -isoform.

Semi-Quantitative Analysis of the γ -Isoform of the 14-3-3 Protein on Western Blots of Recombinant Protein

We divided the γ -isoform into samples of 12.5, 25, 50, and 100 $\mu\text{g}/\text{ml}$ and performed western blots. All bands detected in

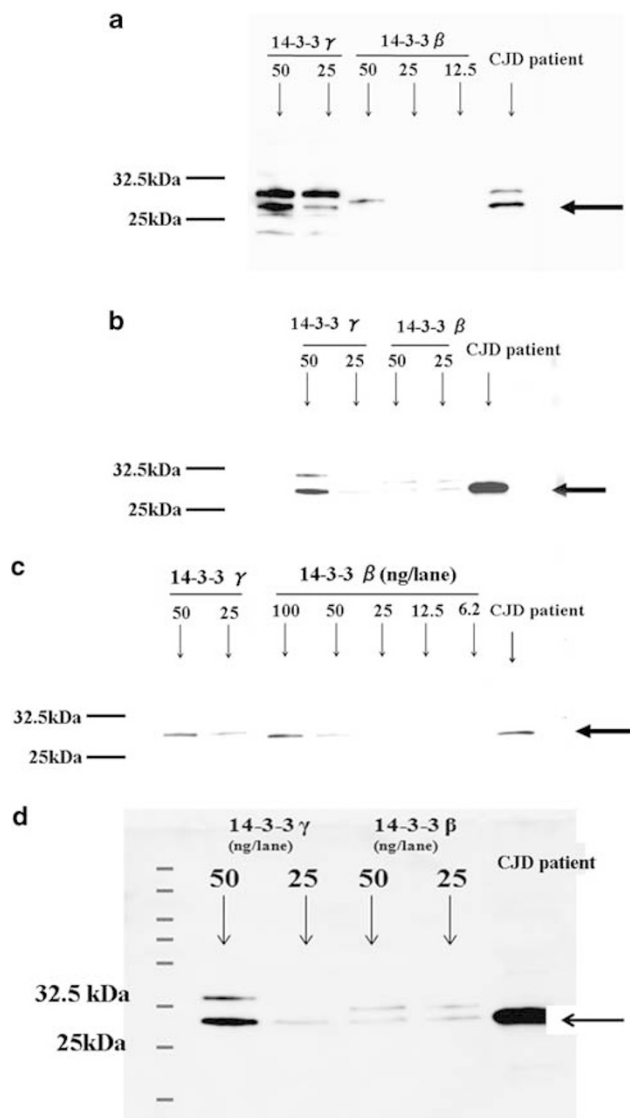


Figure 3 Staining of recombinant 14-3-3 protein (β - and γ -isoforms) by four antibodies (Sc-629; Santa Cruz Biotechnology, CA, USA), 18641 (Immuno-Biological Laboratories (IBL), Gunma, Japan), 18647 (IBL), and K0203-3 (Medical and Biological Laboratories, MBL, Japan). (a) Sc-629 (Santa Cruz Biotechnology) antibody. (b) 18641 (IBL) antibody. (c) 18647 (IBL) antibody. (d) K0203-3 (MBL).

western blots were analyzed 10 times using the LAS-3000 (FUJIFILM) device. The quantification of the β -isoform was not reproducible across replicates, but the quantification of the γ -isoform was reproducible. We tested the repeatability of the detection of the γ -isoform by the 18647 antibody (IBL) on the LAS 3000 system and found it to be excellent (Figure 2). However, we tested the repeatability of the detection of the β -isoform by the 18641 antibody (IBL) on the LAS 3000 system (data not shown) and found it to be poor. This detection did not show the repeatability of the β -isoform test, because the β -isoform was easily degraded (Figure 4). This also explained our observation that the β -isoform of the 14-3-3 protein was disassembled more easily

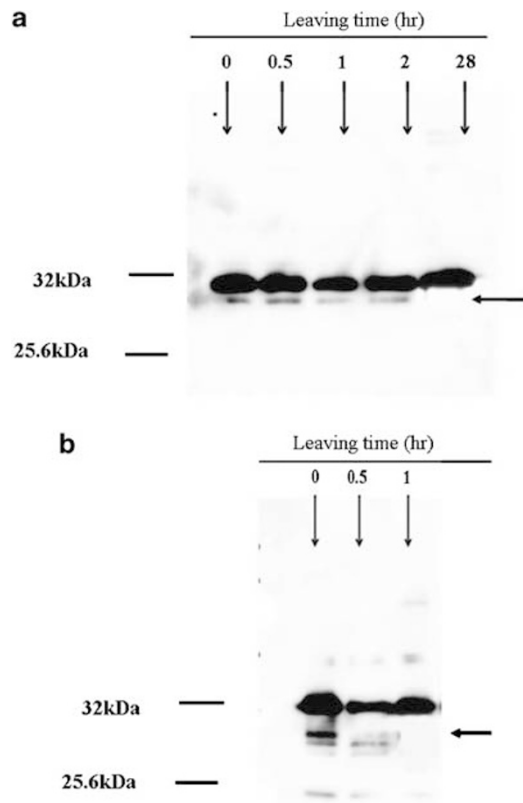


Figure 4 Resolving power of the β - and γ -isoforms of the 14-3-3 protein by time course (a). Resolving power of the recombinant γ -isoform of the 14-3-3 protein by antibody (18647 (1:500) (IBL Gunma, Japan)) with western blot method (0, 0.5, 1, 24 h). (b). Resolving power of the recombinant γ -isoform of the 14-3-3 protein by antibody (18641 (1:500) (IBL)) with western blot method (0, 0.5, 1 h).

than the γ -isoform, in both recombinant protein and CSF samples (Figure 4). These results showed that the γ -isoform was best suited for quantification of the 14-3-3 protein by western blot. In each assay of patients, and in all trials, we applied a calibration sample of recombinant protein (12.5, 2.5 $\mu\text{g}/\text{ml}$) to confirm the reproducibility of the western blot method (Figure 2b). We determined the cutoff data based on the analysis of ROC curves. A semi-quantitative analysis of the 14-3-3 protein enabled us to determine the threshold for detection of the γ -isoform (39.8 μg per lane). The sensitivity and specificity for the γ -isoform of the 14-3-3 protein were 88.4 and 81.2%, respectively (Figure 5b and Table 2). The γ -isoform levels are significantly different between CJD and control patients ($P < 0.001$; Table 3).

Stability of Recombinant and CSF-Derived β -Isoform and γ -Isoform of the 14-3-3 Protein in Western Blots

We characterized the resolving power of the β - and γ -isoforms of recombinant 14-3-3 protein (Figure 4). The β - and γ -isoforms of the recombinant 14-3-3 protein were degraded at room temperature within 24 h. The γ -isoform of the recombinant 14-3-3 protein was stable at 4 $^{\circ}\text{C}$ and at room temperature for 24 h. The β -isoform of the recombinant

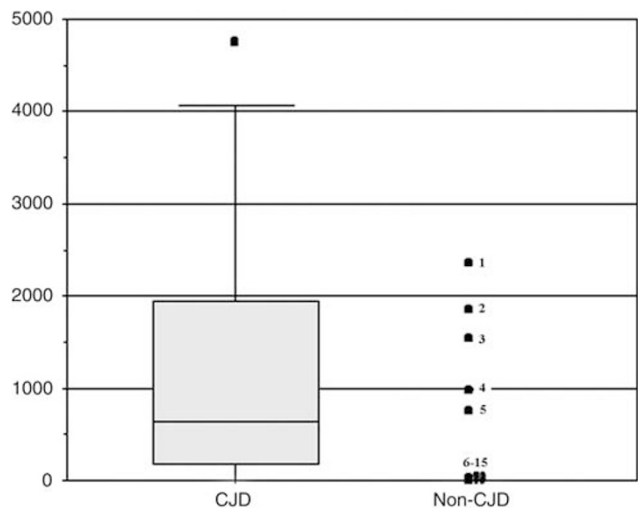


Figure 5 The results of a semi-quantitative analysis of western blots of the γ -isoform of the 14-3-3 protein in the CSF of CJD and control disease patients. The γ -isoform of the 14-3-3 protein was detected in the CSF of 15 CJD patients by western blot. (1, 3, and 4; limbic encephalopathy, 2 and 5; PCD/LEMS, 6 –15; MELAS, encephalopathy owing to unknown etiology and dementia of Alzheimer type (DAT)).

14-3-3 protein was stable at 4 $^{\circ}\text{C}$ for 24 h, but was degraded at room temperature within 24 h. Thus, the β -isoform of the recombinant 14-3-3 protein is degraded more quickly than the γ -isoform (Figure 4; Supplementary Figures 1 and 2).

We attempted to construct a standard curve using semi-quantitative analysis of western blots of recombinant β - and γ -isoforms of the 14-3-3 protein. However, a semi-quantitative analysis using the 18641 antibody was not possible, and the results were not repeatable (Figure 4; Supplementary Figures 1 and 2).

Therefore, we recommend that the γ -isoform of the 14-3-3 protein is most suitable for this assay if CSF samples are stored at 4 $^{\circ}\text{C}$ within 24 h (Figure 4; Supplementary Figures 1 and 2).

DISCUSSION

Assays of the 14-3-3 protein in CSF can be difficult to interpret because of their sensitivity to both β - and γ -isoforms of the protein. Previously, it was unclear which isoform is a more suitable marker in the CSF of CJD patients. We have found both the β - and γ -isoforms to be suitable diagnostic markers in the CSF of CJD patients, whereas antibodies for the ϵ - and η - isoforms were nonspecific (Table 2). Both the β - and γ -isoforms were detected in CJD patients, as well as in limbic encephalopathy, PCD/LEMS, and MELAS patients (Table 2 patterns 9 and 10), indicating that the β - and γ -isoforms are present in the CSF of patients with widespread and acute brain damage. We also report that the assessment of the 14-3-3 protein levels using western blots can result in false positives, making the diagnosis of CJD difficult.

Moreover, although the sc-628 and sc-629 antibodies against the β -isoform of the 14-3-3 protein, produced by

Table 2 The detection of all isoforms of the 14-3-3 protein by western blot among CJD patients' CSF and other diseases' patients' CSF

Patterns	Number	The isoform of the 14-3-3 protein					
		β	γ	η	ε	ζ	τ
<i>CJD patients (N = 20)</i>							
1	15	+	+	+	-	-	-
2	2	-	+	+	-	-	-
3	2	+	+	-	+	-	-
4	1	+	+	+	+	-	-
<i>Other disease patients (N = 20)</i>							
5	16	-	-	+	-	-	-
6	1	-	-	-	-	-	-
7	1	+	+	+	-	-	-
8	1	+	+	+	+	-	-
9	1	+	+	+	-	-	-

CJD, Creutzfeldt–Jakob disease; CSF, cerebrospinal fluid; LEMS, Lambert–Eaton myasthenic syndrome; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; PCD, paraneoplastic cerebellar degeneration.

+: positive reaction of 14-3-3 protein in western blot.

-: negative reaction of 14-3-3 protein in western blot.

1–4: classical type of CJD.

5, 6: Alzheimer-type dementia.

7: MELAS: Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, Stroke-like episodes.

8: PCD/LEMS.

9: limbic encephalitis.

Santa Cruz Biotechnology, are commonly used as the WHO standard worldwide, our results show that these antibodies react with both the γ - and β -isoforms and require a high concentration of the β -isoform to react. Accordingly, the reactions of sc-628 and sc-629 with the β -isoform in the CSF of CJD patients were weaker than those of other antibodies (Figure 1). Therefore, we conclude that sc-628 and sc-629 antibodies are not appropriate for 14-3-3 protein assays. Given that the γ -isoform of the 14-3-3 protein was best suited for detection and for semi-quantitative estimation of 14-3-3 protein levels in western blots, we recommend that the standard antibody in 14-3-3 protein assays should be either the 18647 or K0203-3 antibody to the γ -isoform.

From the point of view of sensitivity and specificity, the 18647 antibody to the γ -isoform of the 14-3-3 protein was most useful. Our data show that the antibodies to the γ -isoform of 14-3-3 (18647 and K0203-3) react only with the recombinant γ -isoform. The sensitivity and specificity of the γ -isoform were 88.4 and 81.2%, respectively. We found

Table 3 Results of the γ -isoform of the 14-3-3 protein in western blot method among 112 CJD patients

	Positive cases	Positive rate
<i>Sporadic cases (N = 99)</i>		
Classical cases (N = 93)	91/94	96.8%
129 MM	90/92	97.8%
129 MV	1/1	100.0%
MV atypical case (N = 1)	0/1	0.0%
MM2-cortical cases (N = 5)	3/5	50.0%
<i>Familial cases (N = 8)</i>		
V180I	3/6	50.0%
144 insertion	0/2	0.0%
<i>Iatrogenic cases (N = 5)</i>		
Dura associated CJD	3/5	60.0%
Total cases (N = 112)	99/112	88.4%

CJD, Creutzfeldt–Jakob disease.

that the sensitivity and specificity of the γ -isoform in CSF was clearly higher than that of the β -isoform because of its higher stability (Figure 2). In conclusion, our data confirm Shiga's data,⁶ which suggested that the γ -isoform of the 14-3-3 protein was more specific than the other isoforms. We calculated the threshold level for detection of the γ -isoform by the antibodies 18647 and K0203-3. The repeatability of the detection of the γ -isoform by the antibody of 18641 on the LAS 3000 system was excellent, establishing the utility of semi-quantitative analysis of western blots of the γ -isoform.

Until now, when using the western blot method as a diagnostic tool for CJD, it was necessary to make use of repeated measurements performed by independent scientists, because of anomalous bands that were occasionally observed. Our semi-quantitative results render this complicated procedure unnecessary. Our method has the same level of sensitivity and specificity seen in the data obtained from the study by Sanchez-Juan⁷ and Van Everbroeck *et al.*⁸

Gmitterová *et al.*⁹ reported that the ELISA assay, which measures all 14-3-3 isoforms, was very useful; however, this system is not commercially available. In addition, data from this assay is not comparable with previous data based on the β - or γ -isoform. Such comparability with previous data is another significant advantage of our method.

Finally, it is very important for clinicians to store CSF samples at 4 °C immediately after collection. Our data clearly show that, when CSF samples are left at room temperature for longer than 24 h, the results of detection by the western

blot method were different compared with storage at 4°C (Supplementary Figures 1 and 2).

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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