

Use of 14-3-3 and other brain-specific proteins in CSF in the diagnosis of variant Creutzfeldt-Jakob disease

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Abstract

Objectives—The detection of the protein 14-3-3 in the CSF has been shown to be a reliable and sensitive marker for sporadic Creutzfeldt-Jakob disease (CJD). Other brain-specific proteins such as neuron specific enolase (NSE), S-100b, and tau protein have also been reported to be increased in the CSF of patients with sporadic CJD. In 1996 a variant of CJD (vCJD) was described which is likely to be causally linked to the bovine spongiform encephalopathy agent. This study reports and compares the findings of CSF brain specific protein analysis in 45 patients with vCJD and in 34 control patients.

Methods—The CSF from 45 patients with vCJD and 34 controls were investigated for the presence of 14-3-3 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with chemiluminescent detection. Tau protein, S-100b, and NSE concentrations in CSF were measured using enzyme immunoassays.

Results—Protein 14-3-3 was detected in the CSF of 22/45 patients with vCJD and in 3/34 controls. The mean concentrations of NSE, S-100b, and tau protein in CSF were significantly raised in patients with vCJD compared with controls. The positive predictive value of CSF 14-3-3 was 86% and the negative predictive value was 63%. These values are lower than those reported for sporadic CJD. An increased CSF tau had a positive predictive value of 93% and a negative predictive value of 81%. The combination of CSF 14-3-3 and/or increased CSF tau had a positive predictive value of 91% and a negative predictive value of 84%.

Conclusions—CSF protein 14-3-3 is not as useful a marker for vCJD as it is for sporadic CJD. Increased concentration of CSF tau was found to be a sensitive marker of vCJD but as concentrations may be increased in many forms of non-CJD dementia, this may limit its usefulness as a diagnostic test.

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as transmissible spongiform encephalopathies. The most common form of CJD is sporadic in nature and is characterised by a rapidly progressive dementia with early widespread neurological signs. Definitive diagnosis depends on neuropathological examination, either by brain biopsy or at necropsy. Most cases of sporadic CJD follow a fairly uniform clinical course although there are variations in clinical phenotype, with the occurrence of relatively atypical cases. Diagnostic criteria have been developed which allow classification of patients as “probable” or “possible” without neuropathology.¹ These criteria are based on the typical clinical features and certain investigation results, originally the EEG and, more recently, CSF protein analysis. About two thirds of patients with sporadic CJD have characteristic periodic sharp wave complexes in the EEG recordings.² However, these may not appear until relatively late in the illness and, in some cases, the periodic pattern is never seen.

Increased concentrations of several proteins in the CSF have been reported in patients with sporadic CJD and one particular protein, 14-3-3, has a high degree of sensitivity and specificity for the diagnosis.³⁻⁷ After the demonstration of the diagnostic usefulness of 14-3-3, a positive CSF 14-3-3 result was incorporated into the standard diagnostic criteria for sporadic disease as a convenient and more sensitive alternative to the typical periodic EEG pattern.¹ This has contributed significantly to the clinical diagnosis of sporadic CJD by non-invasive means. Indeed, a recent report has shown that including CSF 14-3-3 in the diagnostic criteria has resulted in an increase in the number of cases of sporadic CJD identified.⁸ Other proteins, such as neuron specific enolase (NSE)^{5 9 10} S100b,^{5 11} and tau protein,¹² are also increased in the CSF of patients with sporadic CJD.

In 1996, a variant form of CJD (vCJD) was described which affects younger patients and has a more prolonged clinical course than sporadic CJD.^{13 14} These patients tend to present with early and persistent psychiatric symptoms and definitive neurological features such as ataxia and cognitive impairment develop at a relatively late stage.^{15 16} As with sporadic CJD, definitive diagnosis depends on neuropathological findings; requiring either a cerebral biopsy in life or brain examination at necropsy. Clinical diagnostic criteria have been formulated on the basis of typical clinical features and certain investigation results, most particularly a

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Creutzfeldt-Jakob disease (CJD) belongs to a family of neurodegenerative disorders known

Table 1 Positive CSF 14-3-3 results and mean concentrations of CSF NSE, S-100b, and tau protein in patients with vCJD

	Positive 14-3-3 (n)	NSE (ng/ml)	S-100b (ng/ml)	tau (pg/ml)
Definite vCJD	18 [36]	26 (18) [33]*†	0.99 (0.63) [36]*	1112 (1069) [35]*†
Probable vCJD	4 [9]	41 (34) [4]†	1.02 (0.67) [9]	806 (551) [8]†
Controls	3 [34]	14 (12) [29]†	0.44 (0.42) [34]	345 (1124) [31]†

NSE, S-100b, and tau protein results are expressed as mean (SD). Total numbers of CSF samples analysed are shown in square brackets. Statistical analysis compared the CSF protein concentrations from patients with definite vCJD with those from control patients and was performed using a Mann-Whitney *U* test.

**p*<0.001.

†Insufficient CSF to perform all analyses on all samples.

characteristic MRI finding.¹⁷ These criteria are based on relatively limited clinical experience with vCJD and need further, prospective, evaluation. The periodic EEG pattern characteristically seen in sporadic CJD has not been found in vCJD.¹⁷ There is a continuing need for non-invasive antemortem tests to help in the identification of patients with vCJD and it is important to determine whether or not CSF brain specific proteins such as 14-3-3 are as useful in the diagnosis of vCJD as they are in sporadic CJD. In this report we describe the value of the analyses of CSF 14-3-3, NSE, S100b, and tau protein in the investigation of patients with suspected vCJD.

Materials and methods

A collaborative project between the United Kingdom National CJD Surveillance Unit (NCJDSU) and the department of neuroimmunology at the National Hospital for Neurology and Neurosurgery was set up in November 1996, with the aim of determining the usefulness of brain specific protein measurements in CSF in the investigation of patients with suspected CJD. The detailed methodology of CJD surveillance in the United Kingdom is described elsewhere.¹⁸ The Department of Neuroimmunology acts as a referral centre for CSF analysis for laboratories throughout the United Kingdom and Europe, in addition to providing a service for the National Hospital for Neurology and Neurosurgery.

PATIENTS

Between November 1996 and January 2000, CSF was available from 36 patients with definite vCJD (17 female, 19 male aged 15 to 54 (mean 28 years) at notification), nine cases of probable vCJD (five female, four male aged 14 to 51 (mean 28 years) at notification), and 34 control patients (17 female, 17 male aged 14 to 79 (mean 37 years) at notification). All the patients with definite vCJD met published neuropathological criteria.¹⁹ The patients with probable vCJD were diagnosed according to clinical criteria previously described.¹⁷ The control group consisted of patients with

suspected vCJD referred to the NCJDSU during the study period, for whom CSF was available and the diagnosis was not CJD. Of these patients eight had neuropathological examination, four patients were found to have no evidence of vCJD but no alternative diagnosis was identified, two patients had Alzheimer's disease, one patient had subacute sclerosing panencephalitis, and one patient had multiple sclerosis. The diagnosis of vCJD was excluded in the remaining 26 patients for reasons that included complete or partial recovery; diagnostic tests suggestive of an alternative diagnosis; prolonged or atypical clinical course with either an alternative diagnosis probable on clinical grounds or investigations suggestive of an alternative diagnosis.

Samples were packaged on dry ice and delivered by courier to the National Hospital for Neurology, where they were stored at -20°C until analysis. All samples were identified only by the patient's initials and the CJD unit notification number. All analyses were undertaken blind to the patients' diagnostic category.

CSF PROTEIN ANALYSES

Protein 14-3-3 in CSF was detected by western blotting after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with chemiluminescent visualisation.³ Briefly, 15µl of CSF sample was mixed with an equal volume of sample buffer (0.125 M tris-Cl, containing 20% v/v glycerol, 0.2 M dithiothreitol, 4% sodium dodecyl sulphate, and a 0.02% bromophenol blue, pH 6.8) and boiled for 4 minutes. Sample proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (4% stacking gel and 10% resolving gel) for 1.5 hours at 150 V. The proteins were transferred by electroblotting to nitrocellulose for 2 hours using a current of 0.8 mA/cm². The unbound protein binding sites on the nitrocellulose were blocked using 2% non-fat milk powder in phosphate buffered saline. Immunodetection was carried out by incubation with 1:1000 dilution of rabbit anti-14-3-3 gamma (Santa Cruz Biotechnology, Germany) followed by incubation with 1:1000 horseradish peroxidase conjugated swine antirabbit

Table 2 Age at onset, disease duration, and timing of lumbar puncture in 16 patients with vCJD who were 14-3-3 positive and 15 patients with vCJD who were 14-3-3 negative

	Age at onset of disease (y)	Disease duration (months)	Time of lumbar puncture (months)	Time of lumbar puncture as % of disease duration
Positive 14-3-3 (n=16)	29.6 (9.2)	14.7 (5.3)	9.7 (2.3)	70.1 (18.1)
Negative 14-3-3 (n=15)	28.0 (11.9)	15.3 (5.1)	9.9 (4.9)	64.2 (21.8)

Results are expressed as mean (SD).

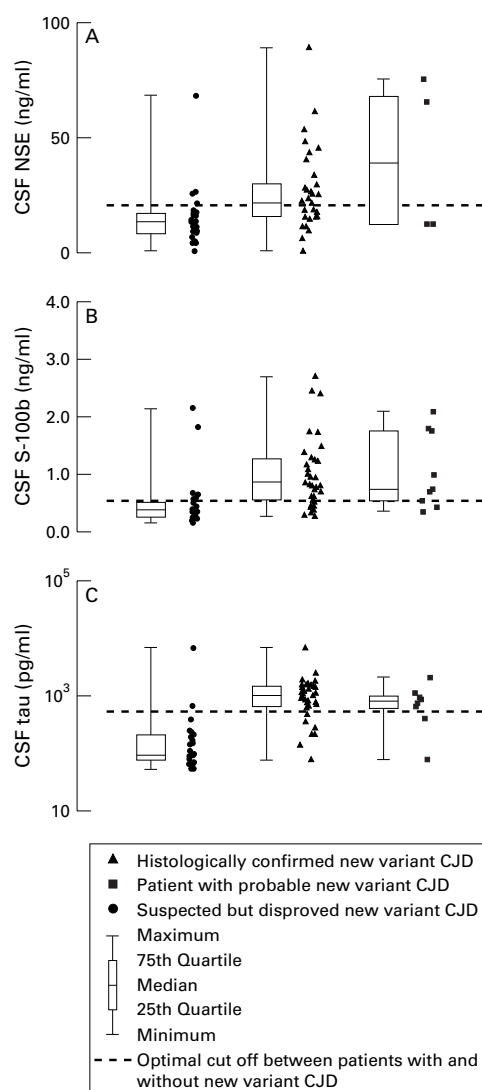


Figure 1 Combined scatter and box and whisker plots of (A) CSF NSE, (B) CSF S-100b, and (C) CSF tau protein concentrations. The scatter plots represent the individual values from which the boxes and whiskers are derived.

immunoglobulin (DAKO, Denmark) and visualisation with enhanced chemiluminescence (Super Signal ECL reagent, Pierce and Warriner, UK). A positive control (CSF from a patient with histopathologically confirmed sporadic CJD), a negative control (CSF from a patient without histological evidence of CJD), and molecular weight markers were included on each run.

Protein NSE in CSF was measured using a commercially available kit (Roche Diagnostics, Switzerland). The upper limit of normal (mean+2.5 SD) was 20 ng/ml. Protein S-100b in CSF was measured using a previously reported sandwich enzyme linked immunosorbant assay (ELISA).²⁰ The upper limit of normal (mean+2.5 SD) was 0.38 ng/ml. Tau protein in CSF was analysed using a previously reported sensitive ELISA²¹ according to the manufacturer's recommendations (Innogenetics, Belgium). This assay measures both the normal and hyperphosphorylated forms of tau protein. The upper limit of normal (mean+2.5 SD) was 315 pg/ml. The upper limits of normal for each of these proteins was obtained by the analysis of CSF samples from patients with no known organic brain disease such as headache, neuralgia, or psychological syndromes.

Results

The incidence of detectable CSF 14–3–3 and the mean concentrations of NSE, S-100b, and tau protein in the CSF of patients with and without vCJD are shown in table 1. Protein 14–3–3 in CSF was detected in 50% of patients with definite vCJD. There were no significant differences in the overall clinical features—the presence of psychiatric features, sensory symptoms, dementia, and ataxia—between the patients with vCJD who were positive for 14–3–3 and those with vCJD who were not. Information about the age at onset of illness, disease duration, and timing of lumbar puncture, both in absolute time terms and also relative to the total duration of illness, was available in 31 patients with vCJD. There were no differences between the 16 patients with vCJD who were positive for 14–3–3 and the 15 with vCJD who were not (table 2). There were no significant differences in the clinical state of the patients at the time of the lumbar puncture and all patients had signs of dementia. There were also no obvious differences in the neuropathological findings between those patients with vCJD who had detectable CSF 14–3–3 and those who did not. Of the three patients without vCJD who had a positive 14–3–3, one had multiple sclerosis, one had encephalitis, and one had no definitive neuropathological diagnosis but vCJD was excluded. The control patient with encephalitis also had markedly raised concentrations of NSE and tau protein in the CSF. Concentrations of S-100b greater than 1.0 ng/ml were found in two control

Table 3 Sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) for CSF 14-3-3, NSE, S-100b, and tau protein in the diagnosis of definite vCJD

	Positive 14-3-3	NSE >20 ng/ml	S-100b >0.5 ng/ml	tau >500 pg/ml	14-3-3 positive and/or S-100b >0.5 ng/ml	14-3-3 positive and/or tau >500 pg/ml
Definite vCJD	18/36	17/33*	28/36	28/35*	29/36	30/35*
Probable vCJD	4/9	2/4*	6/9	6/8*	6/9	8/8*
Controls	3/34	4/29*	8/34	2/31*	9/33*	3/30*
Sensitivity	50%	52%	78%	80%	81%	86%
Specificity	91%	86%	76%	94%	73%	90%
PPV	86%	81%	78%	93%	76%	91%
NPV	63%	61%	76%	81%	77%	84%
Efficiency	70%	68%	77%	86%	77%	88%

Results are expressed as number of patients with raised brain specific protein/patients investigated. Efficiency is defined as number of true positives and true negatives expressed as percentage of total number of patients investigated (except those indicated as %).

*Insufficient CSF to perform all analyses on all samples.

patients: one had cerebrovascular disease and the other had side effects from psychotropic drugs used to treat an unspecified psychiatric disorder.

The mean concentrations of CSF NSE, S-100b, and tau protein were all significantly higher in patients with definite vCJD than in control patients (table 1, fig 1 A, B, C). The mean concentrations of these proteins were also increased in patients with probable vCJD.

The optimal cut off concentrations for NSE, S-100b, and tau protein for the diagnosis of definite vCJD were found to be greater than 20 ng/ml, greater than 0.5 ng/ml, and greater than 500 pg/ml, respectively. The sensitivities, specificities, positive predictive values (PPVs), and negative predictive values (NPVs) for each of the four proteins investigated are shown in table 3. The presence of a positive CSF 14-3-3 or an increased CSF NSE concentration had a similar diagnostic potential. An increased CSF S-100b was more sensitive than 14-3-3 or NSE but was less specific, and an increased CSF tau protein had the highest sensitivity and specificity of any of the proteins investigated.

Discussion

This study shows that increased concentrations of CSF 14-3-3, NSE, S-100b, and tau protein may be found in some patients with vCJD. The sensitivities of each of these proteins for the detection of vCJD were less than those previously reported for sporadic CJD (table 4).^{3-7 9 11 12} The reason for this is unclear, but may be related to the lower mean concentrations found when compared with sporadic CJD (United Kingdom unpublished data). The mean NSE and tau protein concentrations in CSF were less than 33% and 18% of those we have found in sporadic CJD, respectively. The mean CSF S-100b concentration was 56% of that seen in sporadic CJD. It is not clear why the increases in brain specific proteins are less in vCJD, but this may reflect the differing natures of the two conditions. Proteins 14-3-3, NSE, and tau would be expected to reflect neuronal destruction whereas S100b is more related to astrocytic activity. Astrocytic gliosis is more pronounced in variant than in sporadic CJD. Other factors which may be relevant are the rate of progression of disease and the anatomical distribution of abnormality. The relatively slower progression of vCJD may give rise to lower concentrations of brain proteins, reflecting neuronal damage. The differing anatomical distributions of neuropathological change in the two forms of CJD might parallel different regional distributions of the proteins. Although the mean concentrations of brain specific proteins are lower in vCJD than sporadic CJD, our results do not suggest that measurement of these proteins can be used to clinically distinguish these two forms of the disease.

The fact that there was no difference between the mean disease duration between those patients with vCJD who were positive for 14-3-3 and those who were negative, suggests that other factors apart from the rate of neuronal loss may be important for the release of

Table 4 Reported sensitivities and specificities of CSF 14-3-3, NSE, S-100b, and tau protein in the diagnosis of histologically confirmed and clinically probable sporadic CJD

CSF Protein	Study	Sensitivity (%)	Specificity (%)
14-3-3	Hsich <i>et al</i> ³	96	96
	Zerr <i>et al</i> ⁴	94	93
	Beaudry <i>et al</i> ⁵	90	100
	Collins <i>et al</i> ⁶	91	92
	Lemstra <i>et al</i> ⁷	97	87
NSE	Zerr <i>et al</i> ⁴	80	92
	Beaudry <i>et al</i> ⁵	80	92
S-100	Otto <i>et al</i> ¹¹	84	91
	Beaudry <i>et al</i> ⁵	94	85
Tau	Otto <i>et al</i> ¹²	100	95

14-3-3. The release of CSF proteins in CJD has been shown to follow a bell shaped curve with essentially normal concentrations appearing in the early and end stage phases of the disease.²² As the timing of the lumbar puncture was similar in the two groups it is unlikely that the differences were due to missing the 14-3-3 peak. The clinical features were similar in the two groups of patients at the time of lumbar puncture, therefore it is unlikely that the differences in CSF 14-3-3 were due to performing the assay at different stages of the disease.

Tau protein in CSF has the best sensitivity and specificity for any of the proteins investigated in vCJD. Tau protein is an axonal microtubular phosphoprotein and it is unclear why it has a higher sensitivity than the other neuronal markers 14-3-3 and NSE. The combination of either a positive CSF 14-3-3 or an increased CSF tau protein has an increased sensitivity for the detection of vCJD, with only a slight reduction in specificity.

The specificities of the CSF proteins investigated are similar to those found in sporadic CJD (table 4), but none is entirely specific for CJD. Increases in each of these proteins have been reported in other diseases.^{3 4 12 23-33} Tau protein may be increased in the CSF in many forms of non-CJD dementia such as Alzheimer's disease and frontotemporal dementia, and this may limit its usefulness as a diagnostic test. Interestingly the two patients with histologically confirmed Alzheimer's disease did not have increased CSF tau protein concentrations. It is important to realise that the specificity of these proteins is highly dependent on the type of patient investigated and in this study all patients were selected by neurologists as suspect cases of vCJD using well defined criteria. If these brain specific proteins are requested as a "screening test" for vCJD in a wider group of patients their specificity will be reduced.

On the basis of the data presented here, 14-3-3 is unlikely to have the same value in identifying vCJD as it has for sporadic CJD. In particular a negative 14-3-3 does not exclude vCJD. A combination of CSF 14-3-3 and CSF tau protein may be useful but there are too few data for firm conclusions to be drawn. Clearly, it would be helpful to have data from more patients and tests continue in suspect patients

referred to the CJD unit. These tests may provide support for the diagnosis in current practice provided that their limitations are understood.

AJEG performed CSF analysis, analysed data, and codrafted the paper with RSGK. EJT discussed core ideas. RSGK was responsible for coordinating the activities of the NCJDSU and the Laboratory. MZ, GES, MAMaC, and RSGK reviewed the referred patients and were responsible for the clinical diagnoses. JMMaC organised the collection and carriage of the samples and was responsible for maintaining the database of patients and results. JWI confirmed the pathological diagnosis and reviewed the findings for the purpose of this paper. RGW is the Director of the NCJDSU. All authors contributed to the final draft of the paper. We thank Dr Drees, Hoffmann-La Roche, Switzerland for his technical and financial support. We thank M Tovell, National Hospital for Neurology and Neurosurgery for the NSE analysis. Surveillance of CJD is dependent on the cooperation of neurologists and neuropathologists throughout the United Kingdom. We are particularly grateful to the relatives of the patients for their cooperation. The CJD Surveillance Unit is funded by the Department of Health and the Scottish Home Office Department of Health.

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