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14-3-3 Protein, Total Tau and Phosphorylated Tau in Cerebrospinal Fluid of Patients with Creutzfeldt-Jakob Disease and Neurodegenerative Disease in Japan

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SUMMARY

1. Sporadic Creutzfeldt-Jakob disease (CJD) is a rapidly progressive and fatal disease. Patients with CJD usually become akinetic mutism within approximately 6 months. In addition, clinical signs and symptoms at early stage of sporadic CJD may not be easy to distinguish from other neurodegenerative diseases by neurological findings. However, diagnostic biochemical parameters including 14-3-3 protein, S100, neuron-specific enorase in cerebrospinal fluid (CSF) have been used as diagnostic markers, elevated titers of these markers can also be observed in CSF in other neurodegenerative diseases. Therefore, we examined other biochemical markers to discriminate CJD from other neurodegenerative diseases in CSF.

2. We analyzed CSF samples derived from 100 patients with various neurodegenerative disorders by Western blot of 14-3-3 protein, quantification of total tau (t-tau) protein, and phosphorylated tau (p-tau) protein. All patients with CJD in this study showed positive 14-3-3 protein and elevated t-tau protein (>1000 pg/mL) in CSF. We also detected positive 14-3-3 protein bands in two patients in non-CJD group (patients with dementia of Alzheimer's type; DAT) and also detected elevated t-tau protein in three patients in non-CJD group. Elevated t-tau protein levels were observed in two patients with DAT and in one patient with cerevrovascular disease in acute phase.

3. To distinguish patients with CJD from non-CJD patients with elevated t-tau protein in CSF, we compared the ratio of p-tau and t-tau proteins. The p-/t-tau ratio was dramatically and significantly higher in DAT patients rather than in CJD patients.

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4. Therefore, we concluded that the assay of t-tau protein may be useful as 1st screening and the ratio of p-tau protein/t-tau protein would be useful as 2nd screening to discriminate CJD from other neurodegenerative diseases.

KEY WORDS: tau; phosphorylated tau; 14-3-3 protein; diagnosis; cerebrospinal fluid; dementia of Alzheimer type; Creutzfeldt-Jakob disease.

INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathy (TSE), are a group of invariably fatal neurodegenerative disorders affecting both humans and animals. These diseases include Creutzfeldt-Jakob disease (CJD), Gestmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and Kuru in humans.

Diagnosis of sporadic CJD is made based on neurological findings of progressive dementia, myoclonus, and cerebellular ataxia. The progression of clinical signs and symptoms are typically subacute. Akinetic mutism usually appears approximately within 3 months. About 70% of cases die within 6 months. Clinical findings at the early stage of sporadic CJD may resemble the symptoms of other neurodegenerative diseases including dementia of Alzheimer type (DAT). The diagnosis of CJD is made based on the clinical features, clinical course, and electroencephalogram (EEG) analysis. The biochemical detection of 14-3-3 protein in CSF samples (Zerr *et al.*, 1998) and the diffusion-weighted MRI (DW-MRI) (Demerosl *et al.*, 1999) are recently reported as useful diagnostic tools for CJD.

However, detection of 14-3-3 protein in CSF sample is useful as a diagnostic marker to discriminate CJD from other neurodegenerative diseases; 14-3-3 protein results showed false positive results in few cases among other neurological disorders. Also 14-3-3 protein could not be detected in two cases of CJD in early phase of disease progression in this study. In the late phase of disease progression, these cases showed positive result with 14-3-3 protein, which suggested that 14-3-3 protein is not a good marker for diagnosis at an early stage.

Therefore, we need to search for other biomedical markers except 14-3-3 protein in CSF to discriminate CJD from other neurodegenerative diseases.

Otto *et al.* reported the t-tau protein in CSF as a new diagnostic marker in the patients with CJD (Otto *et al.*, 2002).

We designed to compare the efficiency of 14-3-3 protein, total tau (t-tau) protein, and phosphorylated tau (p-tau) of CSF samples as diagnostic markers in CJD patients in Japan.

PATIENTS AND METHODS

We collected CSF samples from 100 patients, diagnosed CJD, DAT, cerebrovascular disorders (CVD), Pick disease, Parkinson disease (PD), corticobasal degeneration (CBD), Huntington disease, fronto-temporal dementia (FTD), progressive supranuclear palsy (PSP), and amyotrophic lateral sclerosis (ALS). We also obtained CSF from four healthy volunteers. We analyzed biochemical markers (14-3-3 protein, t-tau protein, and p-tau protein) in CSF samples.

All cases with CJD were classified as "definite," "probable," or "possible" cases by Master's criteria (Master *et al.*, 1979).

Genomic DNAs extracted from peripheral blood leukocytes were used to amplify the open reading frame (ORF) of the PrP gene by polymerase chain reactions. The products were searched for polymorphisms at codon 129 and 219 by sequencing as described.

According to the clinical criteria including EEG examination, all suspected cases of CJD were classified as "definite," "probable," or "possible" cases on Master's criteria.

ANALYSIS OF T-TAU PROTEIN AND P-TAU PROTEIN IN CSF SAMPLES

Kits form Innogentics NV (Ghent, Belgium) were used to determine t-tau protein in CSF samples derived from 100 patients. Innotest h-tau Ab and Innotest p-tau Ab were used as first monoclonal antibodies.

The ELISA is sensitive in detecting t-tau protein from 70 to 1120 pg/mL on CSF of human. ELISA test was constructed to detect both t-tau using three different phosphorylation independent antibodies (AT120:218-224, HT7, BT2:192-198) to tau protein.

Innogentics NV (Ghent, Belgium) showed that p-tau protein using one phosphorylation-dependent antibody (AT270: threonine 181, HT7) against tau protein. The ELISA is sensitive in detecting p-tau protein from 25 to 150 pg/mL on CSF of humans.

We measured the ELISA of t-tau protein and p-tau protein according to the manuals of manufacturer's instruction and using an identical standard in all experiments.

The resulting signals were measured and quantified using Labry system image station 440 nm accompanying software. These measurements were used to calculate the ratio of each signal to standard.

DETECTION METHOD OF β-ISOFORM OF 14-3-3 PROTEIN

The 14-3-3 protein immunoassay in CSF was performed on all samples according to previously published standard sample (Hsich *et al.*, 1996). Detection of the bands by polyclonal antibody against β-isoform of 14-3-3 (Santa CruzBitotech and IBL Company) was performed by using the enhanced chemiluminescence (ECL) detection kit (Amersham Buchler).

RESULTS

Selection of Patients

The profiles of 100 patients are listed in Table I. One hundred patients were divided into two groups: CJD group ($n = 13$) and non-CJD group ($n = 87$). Non-CJD group included other neurodegenerative diseases except CJD and normal subjects.

Table I. Profile of Patients ($n = 100$)

	Total	Sex	
		Male	Female
CJD patients ($n = 13$)			
Definite case	4	1	3
Probable case	9	3	6
Possible case	0	0	0
Non-CJD patients ($n = 87$)			
DAT	54	33	21
CVD	7	5	2
PD	5	4	1
PSP	3	2	1
ALS	3	2	1
CBD	2	0	2
Pick disease	1	1	0
Huntington's disease	1	1	0
FTD	1	1	0
Dementia, etiology unknown	5	4	1
Healthy cases	4	2	2

Thirteen patients were classified into four "definite" cases (one case, M232R; two cases, MM2-cortical form; one case, MM1) and nine "probable" cases (seven cases, MM1; one case, Heindenhain variant; one case, dura graft associated CJD) by Master's criteria (Master *et al.*, 1979) and Parchi's classification (Parchi *et al.*, 1999).

Detection of β-Isoform of 14-3-3 Protein

We analyzed β-isoform of 14-3-3 protein in all CSF samples. In CJD group, all 13 patients showed β-isoform of 14-3-3 protein on CSF samples at least at certain clinical phase. In two cases of early stage CJD, 14-3-3 protein was detectable. In these cases, second assay performed with CSF obtained 1 month later showed positive bands. On the other hand, two patients with DAT in non-CJD group were positive for 14-3-3 protein.

ELISA Assay of t-Tau Protein and p-Tau Protein in CSF Samples

t-Tau protein levels in CSF were also determined (Fig. 1). Titers of t-tau in CJD group (1248 pg/mL) ranged higher than non-CJD group (16,087 pg/mL) (mean ± SD: 7.174 ± 6552 pg/mL) (Fig. 1 and Table II). In DAT group, levels of t-tau protein ranged between 117 and 1389 pg/mL (mean ± SD: 387.37 ± 280.2 pg/mL). The levels of CVD patients ranged between 172 and 1300 pg/mL (mean ± SD:

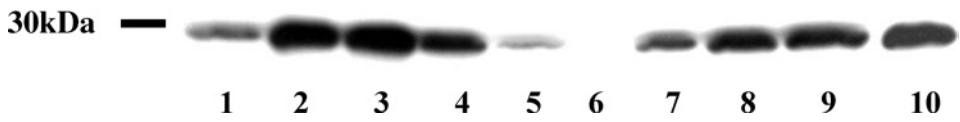

Fig. 1. The detection of β-isoform of 14-3-3 protein immunoblotting assay from 10 patients with CSF. Lanes 1, 2, 3, and 4: possible cases of sporadic CJD; lane 5: healthy subject; lanes 7and 8: DAT; lanes 9 and 10: definite cases of sporadic CJD.

Table II. CSF Concentration of t-Tau Protein and p-Tau Protein and Results of 14-3-3 Protein in 100 Patients

Diagnosis	Positive 14-3-3 protein	t-Tau protein[a]	p-Tau protein[a]	p-Tau/t-tau protein ratio (10^{-2}) ± SD
CJD	13/13	7174 ± 6588	36.38 ± 5.42	1.147 ± 1.079
DAT	2/54	387.37 ± 275.5	55.17 ± 32.1	18.36 ± 18..38
CVD	0/7	657.86 ± 612.4	63.11 ± 31.4	9.593 ± 7.588
PD	0/5	198.73 ± 44.4	33.98 ± 19.50	16.90 ± 2.781
PSP	0/3	319.7 ± 81.4	45.0 ± 12.3	14.47 ± 3.996
ALS	0/3	86.03 ± 54.45	18.9 ± 10.3	25.17 ± 8.597
CBD	0/2	266.4 ± 0.7	34.96 ± 31.97	27.39 ± 20.80
Pick disease	0/2	267 ± 100.4	35.3 ± 3.15	13.22 ± 4.01
Huntington's disease	0/1	157	21.47	13.68
FTD	0/1	370	69.44	18.77
Dementia, etiology unknown	0/4	290.5 ± 181.9	55.18 ± 29.1	20.04 ± 7.821
Normal control	0/4	95.30 ± 51.11	24.53 ± 4.162	30.03 ± 12.17

[a]Median (pg/mL) ± SD.

623.4 ± 612 pg/mL). t-Tau levels of CJD patients were significantly higher than those of the non-CJD group ($p < 0.001$). t-Tau levels of CJD were higher than those of CVD and DAT ($p < 0.05$).

Levels of p-tau protein ranged between 27 and 44.48 pg/mL (mean ± SD: 36.38 ± 4.86 pg/mL) in CJD. In DAT, titers ranged between 22.0 and 178.9 pg/mL (mean ± SD: 55.2 ± 31.56 pg/mL) (Fig. 2 and Table II). In CVD, titers ranged between 20 and 95.23 pg/mL (mean 55.7 ± 31.6 pg/mL).

The Ratio of p-Tau Protein/t-Tau Protein in CSF Sample

The ratio of p-tau protein/t-tau protein (p/t ratio) was calculated with all CSF samples. The p/t ratio of patients with CJD ranged between 0.181×10^{-2} and 3.21×10^{-2} (mean ± SD: $1.147 \times 10^{-2} \pm 1.079 \times 10^{-2}$). In contrast, it ranged between 4.40×10^{-2} and 145×10^{-2} (mean ± SD: $18.36 \times 10^{-2} \pm 18.38 \times 10^{-2}$) in DAT (Fig. 4 and Table II), and in CVD it ranged between 2.64×10^{-2} and 23.8×10^{-2} (mean ± SD: $9.593 \times 10^{-2} \pm 7.588 \times 10^{-2}$). Particularly among patients with higher levels of t-tau protein (>1300 pg/mL), p/t ratio of CJD patients stayed lower than the other patients with DAT or CVD ($p < 0.001$). The lowest was the patients with CJD among other groups. Also difference of p/t ratio between CJD group and non-CJD group was significant ($p < 0.001$). The ratio showed no overlap with any other single case in non-CJD group.

DISCUSSION

We checked the level of t-tau protein and p-tau protein in CSF samples derived from 100 patients with CJD, non-CJD with various neurodegenerative diseases, and normal subjects. Our data indicated the significance of t-tau protein and p-tau protein as diagnostic markers of CJD.

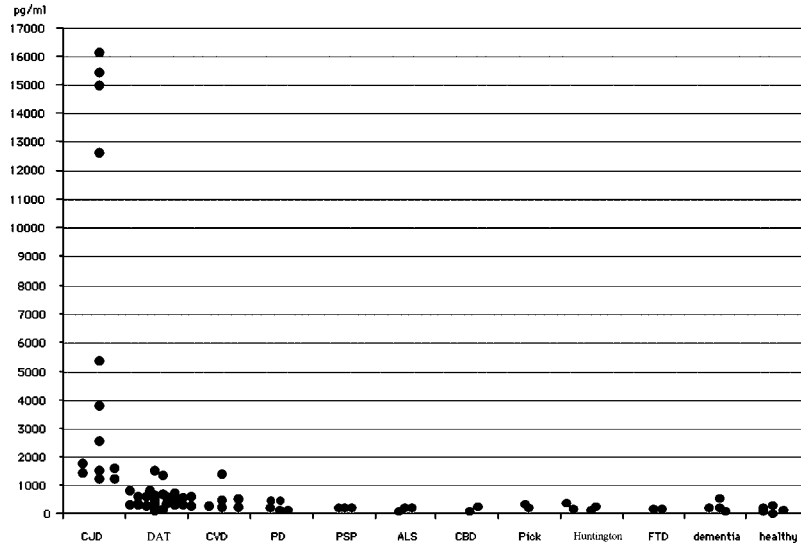

Fig. 2. The concentration of t-tau protein in CSF of patients with CJD, other neurodegenerative diseases, and normal subjects. Black hallmark corresponds to the one case. The abbreviations are indicated as following: CJD, Creutzfeldt-Jakob disease; DAT, dementia of Alzheimer type; CVD, cerebrovascular disorders; PD, Parkinson disease; PSP, progressive supranuclear palsy; ALS, amyotrophic lateral sclerosis; CBD, corticobasal degeneration; Pick, pick disease; Huntington, Huntington disease; FTD, fronto-temporal dementia; healthy, normal subjects.

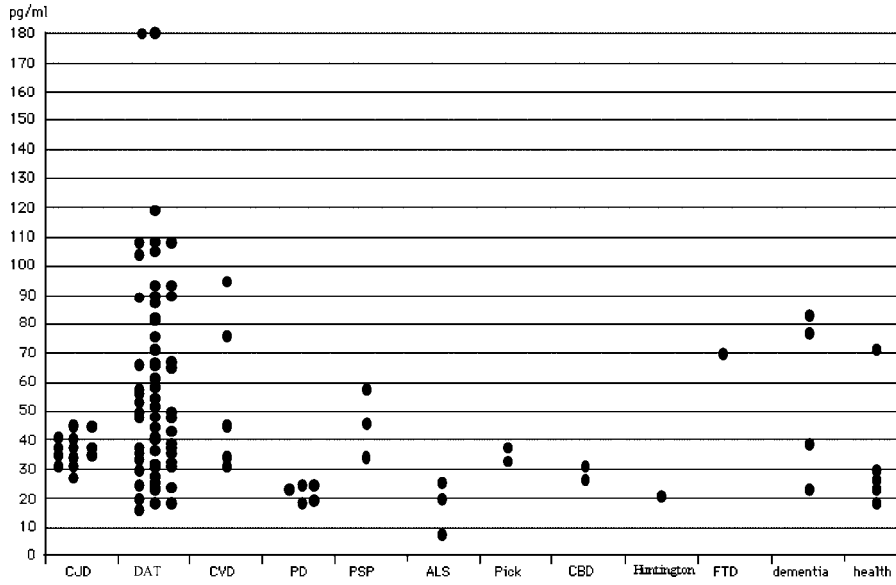

Fig. 3. The concentration of p-tau protein in CSF of patients with CJD and other neurodegenerative diseases. Black hallmark corresponds to the one case. Cases analyzed in this study were identical to the patients in Fig. 1. The abbreviations are mentioned in the legend of Fig. 1.

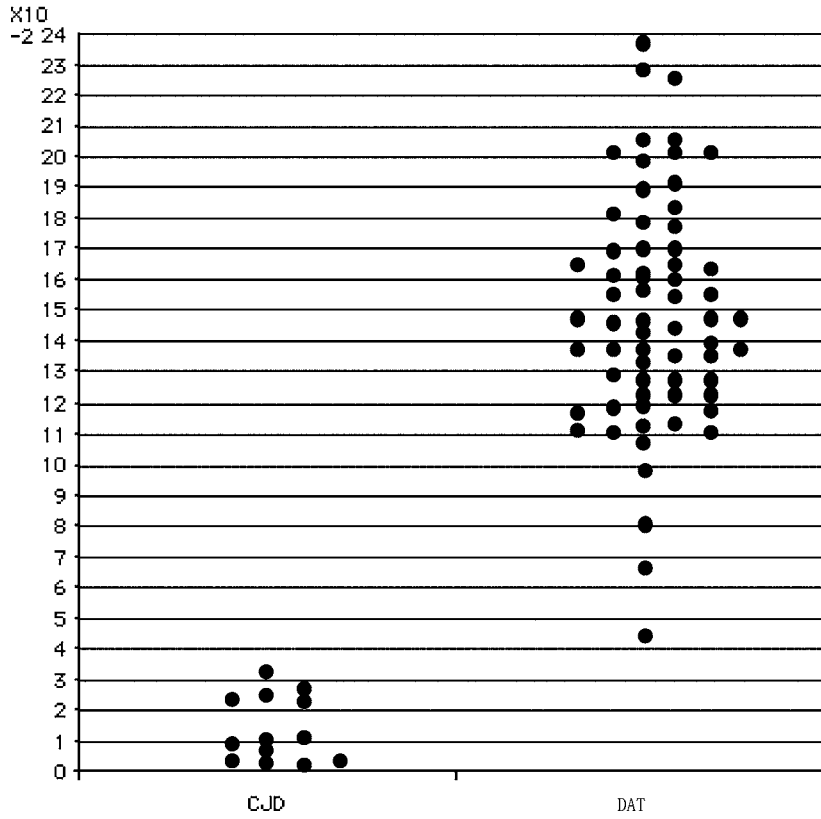

Fig. 4. The comparison of ratio of p-tau protein/t-total protein in CSF of patients with CJD and DAT. Black hallmark corresponds to the one case. The number of patients were 67 with CJD or DAT.

Sixteen out of 100 patients showed >1000 pg/mL of t-tau protein in CSF. These 16 patients comprised all 13 patients with CJD and three patients of non-CJD groups (two patients with DAT and one patient with CVD).

Judging from our data, the best results for the sensitivity and the specificity were obtained at a cut-off of 1260 pg/mL. According to receiver operating characteristic (ROC) curve analysis by SPSS software, the sensitivity was 92.3%, and the specificity was 97%. On the other hand, we could not distinguish two patients with DAT from within CJD patients by using both t-tau protein and 14-3-3 protein.

Neuropathological significance of DAT is formation of neurofibrilary tangles (NFT), which is originated by phosphorylation of tau protein. In contrast, spongiform changes, astrocytic gliosis, and the accumulation of PrP^{Sc} but no NFT accumulation can be observed in CJD brain. The phosphorylation of tau protein is not involved in the pathogenetic process of CJD brain damage.

Thus, we focused on the phosphorylated tau in CSF of patients with DAT and other neurodegenerative diseases.

Therefore, we designed to measure the p/t ratio to discriminate CJD from DAT. According to our results of p/t ratio, all patients with DAT were identified at the ratio of >0.04, but all patients with CJD were identified at <0.04 (Fig. 3 and Fig. 4). We could clearly distinguish CJD from DAT by detecting the p/t ratio.

As a conclusion, we recommend to use t-tau protein (>1000 pg/mL) and as a first screening test, and the p/t ratio as a second screening test in CSF.

t-Tau levels of CJD in our study showed slightly lower than previously reported (Otto *et al.*, 2002 and Van Everbroeck *et al.*, 2003). CSF materials used in this study were collected at early stages. Nine out of 13 cases of CJD were examined within 3 month after onset. On the other hand, CSF samples were mainly obtained from 4 to 12 month after onset in previous report, which is middle stage of clinical course of CJD (Van Everbroeck *et al.*, 2003). In general, t-tau levels in middle stage were showed higher than in early stage (data not shown).

We serially examined t-tau protein of CSF with CJD in clinical course. However, the abnormal findings of the diffusion-weighed MRI and 14-3-3 protein were not detected in two cases of CJD in early stage, high concentration of t-tau protein (2841 and 1460 pg/mL) could be detected. One month after the first assay, we found typical changes to CJD by diffusion-weighed MRI, positive 14-3-3 protein bands, and an elevated t-tau protein (10,634 and 3495 pg/mL). This result indicates that t-tau protein may be a possible diagnostic marker of CJD at early stage.

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