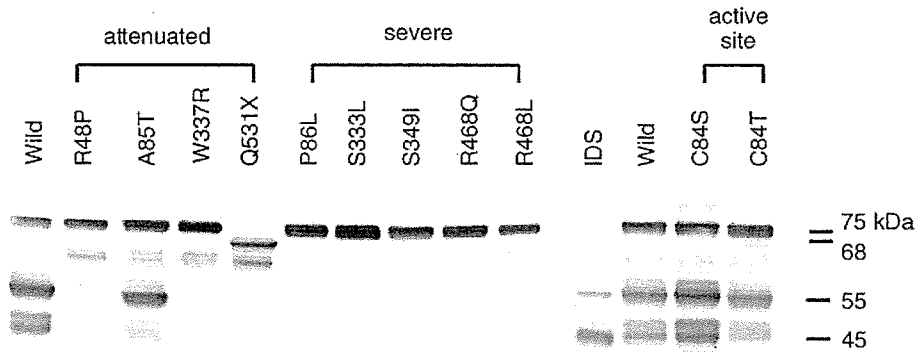

Fig. 1 Western blot analysis of IDS proteins in stably transfected CHO cells. Aliquots of cell extract (20 μg of protein) were analysed by SDS-PAGE (10%), followed by immunoblotting using an anti-human IDS monoclonal antibody

IDS structure

The x-ray crystal structures of the human arylsulfatase B (*N*-acetylgalactosamine-4S sulfatase; 4S) (Bond et al 1997) and human arylsulfatase A (ASA) (Lukatela et al 1998) have been reported. These sulfatases share 24% and 29% identity with *N*-acetylgalactosamine-6S sulfatase (GALNS). Using ASA and 4S structures as the templates, we could successfully construct a fine tertiary structural model of the human GALNS that gave us a good explanation of the 32 different mutations (Sukegawa et al 2000). IDS also shares about 20% identity with these sulfatases. In the present study, we constructed a tertiary structural model of human IDS, using our original programs. The structural model of IDS had a monomeric form with two domains. The main structural feature of the larger domain was a beta-sheet with 10 strands sandwiched between alpha-helices. The smaller domain consisted of a four-stranded anti-parallel beta-sheet with an orthogonal alpha-helix (Fig. 2). The tertiary structure of IDS protein showed that putative catalytic residues should include Asp45, Asp46, Cys84, Lys135 and Asp334, which corresponded to highly conserved residues in the sulfatase family (Bond et al 1997; Lukatela et al 1998; Sukegawa et al 2000). The active centre C84 should be posttranslationally modified to a formylglycine during processing of the protein (Schmidt et al 1995).

Mutations in the active centre, C84

A nonsense mutation C84X on this active centre, Cys84, of IDS has been reported in a Hunter patient (Vafiadaki et al 1998), but no missense mutation has been reported. There has been a report of two artificially engineered mutations on Cys 84, namely C84A and C84T (Millat et al 1997). The results showed that C84A had a significant reduction of the normally processed protein when expressed, indicating a structural alteration of the protein, while C84T showed a lesser reduction. Our aim here was to have a reference for molecular phenotype analysis, including protein structure, so we used two kinds of substitutions on this C84 by serine or threonine. Especially, the serine substitution should be the most structurally conserved alteration from cysteine, almost preserving the atomic radius or polarity of the side-chain of the amino acid. The substitution from cysteine to serine or threonine should modify the active site with very slight structural changes, but should produce a drastic alteration of the protein activity. As expected, the protein with C84S or C84T mutation was well expressed and normally processed, producing mature forms without any enzymatic activity (Fig. 1, Table 1). This result was used as a reference for discussion of the molecular phenotypes of the IDS protein.

Mutations found in the severe phenotype

P86L: P86 is found adjacent to the active site residue, C84, and is a part of the core of the major domain of IDS. The alteration by leucine, a more bulky hydrophobic residue, can affect the stability of the major domain structure. Previously, we identified a truncated form of mRNA of IDS from the patient with this mutation, suggesting an activation of a cryptic splice acceptor site (Isogai et al 1998). However, the results here indicate that the effects of the mutation should also be considered from structural aspects, in addition to the abnormality of the transcription steps.

S333L: The S333 residue is adjacent to the active site residues and the part of the beta-sheets making up a hydrophobic core of the major domain. Substitution from serine to leucine at this position should loosen the core structure.

S349I: S349 is buried in the edge of the core domain and it is on the packing interface between the major domain and the subdomain of IDS. The alteration of this residue to a large hydrophobic residue, isoleucine, may structurally alter the core domain, and also the higher-order conformation.

R468Q, R468L: R468 geometry was not modelled on the present structural model so as to exclude the possibility of being misled by to the ambiguity of the alignment in this part. In our previous paper, we speculated that substitutions of R468 by large hydrophobic residues—tryptophan or

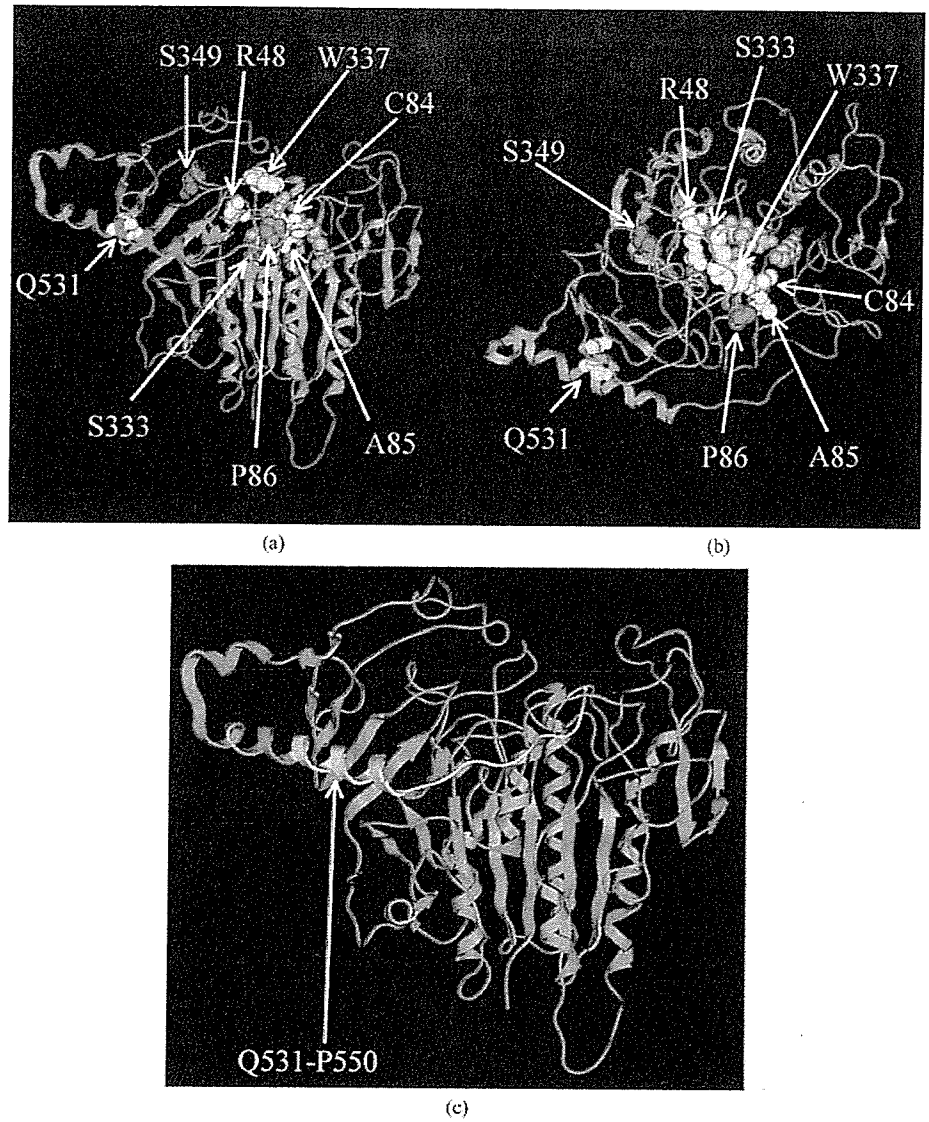

Fig. 2 Location of mutated residues in a tertiary structural model of IDS. (a, b) The active site centre, C84 residue, is shown as yellow spheres and the other active site residues are shown as orange spheres. The residues related to the severe clinical phenotype are shown in red and those related to the attenuated clinical phenotype are shown in cyan. (c) The deleted C-terminal fragment, Q531-P550, in Q531X mutant protein is indicated as yellow

leucine—should affect the electrostatic field for substrate entrance into the active site cavity, resulting in an inactive enzyme, simply by referring to the model constructed by other automatic modelling software (Kato et al 2005). However, the results of western blot analysis showed only primary precursors, indicating certain structural changes in these proteins (Fig. 1). This residue should be within the subdomain structure of IDS (Kato et al 2005; Kim et al 2003), but further studies are needed to clarify the molecular effects of these mutations. In addition, R468Q mutation has been found in several patients with severe phenotype, but was also detected in a patient with an intermediate (attenuated) phenotype (Goldenfun et al 1996). Further studies should be done to elucidate phenotype–genotype correlation.

Mutations found in the attenuated phenotype

R48P: The side-chain of R48 stretches in the opposite direction from the active site and arginine has a hydrophobic root in its side-chain. Thus, the substitution of R48 by a smaller hydrophobic residue, proline, could be tolerated.

A85T: The position of this A85 is closely adjacent to the residue C84, suggesting a significant effect on the active site geometry, but the change to the relatively small threonine residue could be partially tolerated in combination with the excellent structural stability, as shown in Fig.1. A85T is found widely around the world, but there has been no clear phenotype–genotype relationship (Kato et al 2005). Further studies, including evaluation methods of clinical phenotypes,

need to be performed for better understanding of the correlation (Kato et al 2005).

W337R: This change is of a large hydrophobic residue to a large hydrophilic residue, i.e., it is not conservative. However, the side-chain of W337 stretches to the solvent and this feature could assist tolerance to the arginine, which has an ionized residue on the top of the side-chain in this position. Moreover, the body of the arginine side-chain has a long stretch of hydrophobic parts, suggesting an additional feature for tolerance.

Q531X: The deletion mutant Q531X would produce polypeptides with a loss of 20 amino acids in the C-terminus, but the deleted part contains only two short helices and short loops in the subdomain of IDS. The mutant protein should have most of its catalytic domain and subdomain, preserving residual enzymatic activity.

Structural stabilizers as possible therapeutic agents

Findings on the relationship between genotypes and clinical phenotypes have considerable relevance and significance in the selection of patients for therapy and in predicting the response to therapy. Recently, Tanaka and colleagues (2004) have developed an attractive therapeutic approach for genetic diseases that result from misfolded/aggregated proteins. They showed that trehalose reduced polyglutamine aggregates, stabilized the partially unfolded polyglutamine-containing protein, and increased survival in a cellular model and in a transgenic mouse model of Huntington disease. Fan and colleagues (1999) have shown that it is possible to use the small molecule 1-deoxygalactonojirimycin, a potent competitive inhibitor of alpha-galactosidase A, to rescue the misfolding and mistrafficking associated with certain Fabry disease mutations. We preliminarily investigated whether trehalose has an influence on the stability and/or activity of the mutant IDS proteins. The enzyme activity increased severalfold in the trehalose-treated cells with a mild mutant cDNA (data not shown).

Small compounds, such as trehalose or glycerol, may work as stabilizers of IDS protein and may provide an effective treatment for some patients with Hunter disease. Knowledge of the distribution of the mutations and their functional effects on catalytic or stability properties of IDS could have significant implications for the design and/or development of new therapeutic agents for enzyme, chaperone, or genetic therapeutic approaches.

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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:

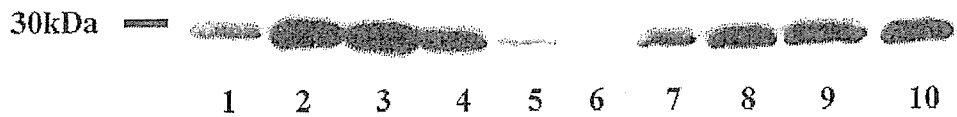

Fig. 1. The detection of β-isoform of 14-3-3 protein immunoblotting assay from 10 patients with CSF. Lanes 1, 2, 3, and 4: possible cases of sporadic CJD; lane 5: healthy subject; lanes 7and 8: DAT; lanes 9 and 10: definite cases of sporadic CJD.

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.

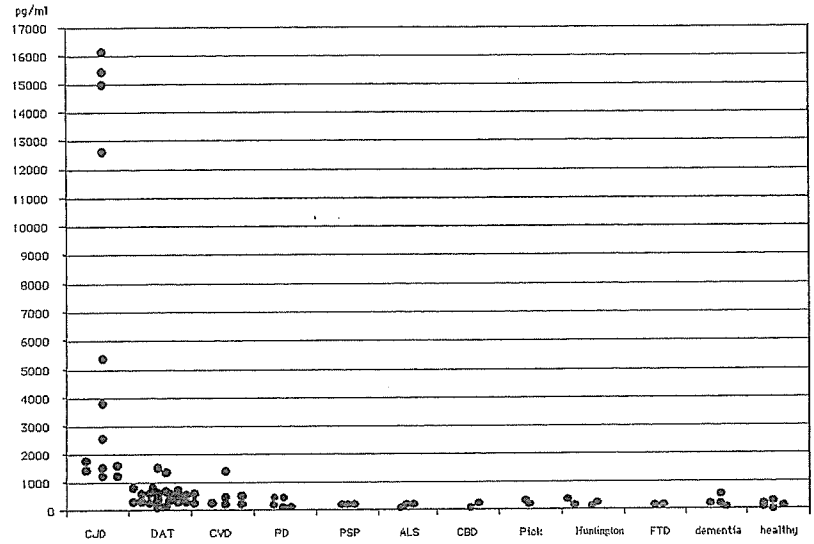

Fig. 2. The concentration of t-tau protein in CSF of patients with CJD, other neurodegenerative diseases, and normal subjects. Black hallmark corresponds to the one case. The abbreviations are indicated as following: CJD, Creutzfeldt-Jakob disease; DAT, dementia of Alzheimer type; CVD, cerebrovascular 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.

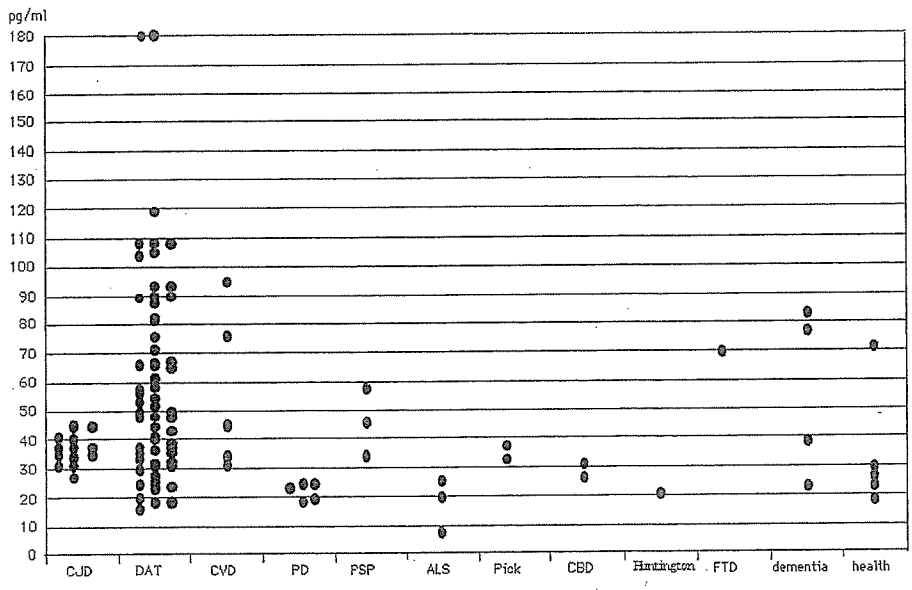

Fig. 3. The concentration of p-tau protein in CSF of patients with CJD and other neurodegenerative diseases. Black hallmark corresponds to the one case. Cases analyzed in this study were identical to the patients in Fig. 1. The abbreviations are mentioned in the legend of Fig. 1.

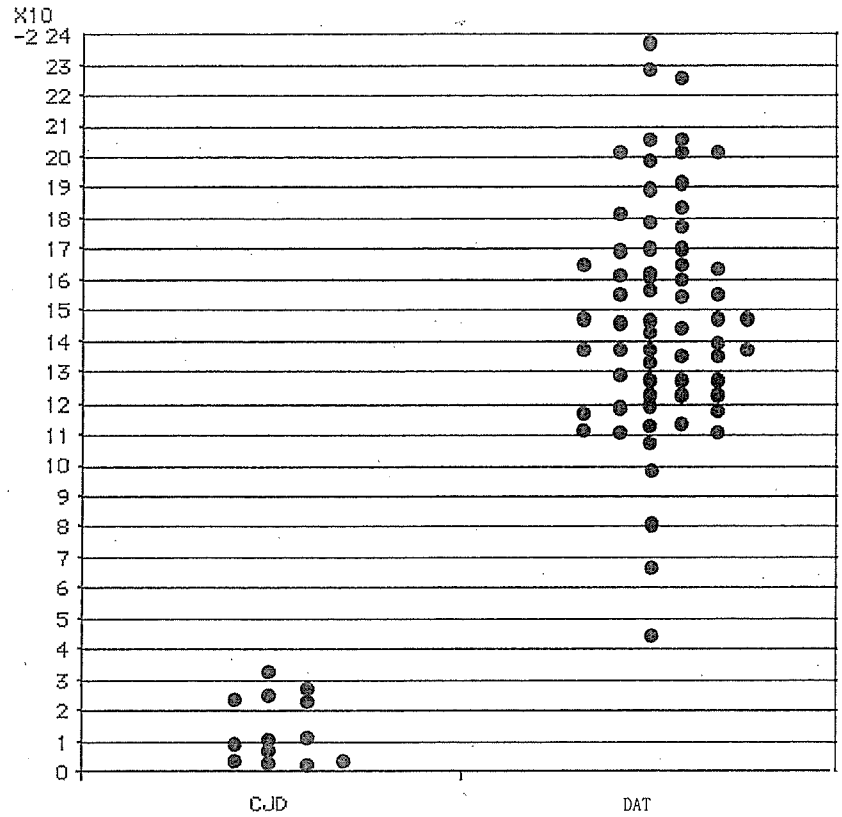

Fig. 4. The comparison of ratio of p-tau protein/t-total protein in CSF of patients with CJD and DAT. Black hallmark corresponds to the one case. The number of patients were 67 with CJD or DAT.

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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Total Tau Protein of Cerebrospinal Fluid As an Early Diagnostic Marker for Creutzfeldt-Jakob Disease

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Abstract

The most sensitive and reliable marker at early stage of Creutzfeldt-Jakob disease (CJD) was clarified among several diagnostic markers. To ascertain what was the most sensitive marker for CJD patients within 6 weeks from onset, we researched and compared among diffusion-weighted MRI (DWI), total tau (t-tau) protein and 14-3-3 protein of cerebrospinal fluid (CSF) ; concluding that t-tau protein of CSF was the most sensitive.

Introduction

The diagnostic criteria of Creutzfeldt-Jakob disease (CJD) have depended on clinical findings and electroencephalographic criteria. The detection of abnormal prion protein (PrP^{Sc}) without brain biopsy has yet to be established ; therefore, we must depend on supplementary methods. A clinical diagnosis of CJD can be supported by a biochemical marker of cerebrospinal fluid (CSF). We have often used 14-3-3 protein of CSF as a reliable diagnostic marker, and 14-3-3 protein is included in the WHO diagnostic criteria[2]. Some also reported that 14-3-3 protein could not be detected in some cases at an early phase of the disease, or at the late phase of disease progression.

Otto et al.[2] reported total tau (t-tau) protein, except for 14-3-3, as a new diagnostic marker in the patients with CJD. We also identified that t-tau protein was more prominent than the other diagnostic markers, including the 14-3-3 protein for CJD patients in Japan[3].

Shiga et al.[4] reported that diffusion-weighted MRI (DWI), a sensitive noninvasive test, is a diagnostic procedure for CJD. However, not all hospitals have MRI scanners, or even if a hospital has an MRI scanner, they are always not provided when there is DWI.

Recently, because clinicians can administer drugs from a very early stage, it has become important to identify at an early stage of CJD what is the most sensitive and reliable marker from among the various ones proposed.

To ascertain what was the most sensitive marker for CJD patients within 6 weeks from onset, we researched and compared among DWI, t-tau protein and 14-3-3 protein of CSF.

Methods

1. Detection of β-isoform of 14-3-3 protein of CSF

14-3-3 protein immunoassay in CSF was performed on all samples according to a previously reported paper[3]. Detection of the bands by polyclonal antibody against β-isoform of 14-3-3 antibody (SC639, Santa Cruz Biotechnology, Santa Cruz, CA, or IBL Company) was performed using an enhanced chemiluminescence (ECL) detection kit (Amersham Buchler).

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Table 1 Summary of t-tau and 14-3-3 protein (including NSE and S-100b) of CSF and diffusion weighted MRI in 20 CJD patients in early stage

No.	Age	Sex	Type	d.l.	d.w. (wk)	CSF				MRI	
						t-tau protein (pg/mL)	14-3-3 protein	NSE (ng/mL)	S-100b protein (ng/mL)	DWI	Flair image
1	64	f	sp	Probable	4	3,414	+	120	4.73	+	−
2	73	m	sp	Probable	4	2,068	+	30	1.64	+	−
3	67	m	sp	Probable	4	9,055	+	56	0.86	+	−
4	76	m	sp	Probable	4	4,645	+	59	0.60	+	−
5	80	f	sp	Probable	4	8,766	+	71.68	0.84	+	−
6	77	m	sp	Probable	2	10,671	+	120	0.60	+	−
7	63	f	sp	Probable	4	1,814	−	10	1.28	+	−
8	69	f	sp	Definite	6	4,917	+	32	0.91	+	−
9	54	f	sp	Definite	6	1,317	−	18	1.76	+	+
10	67	m	sp	Probable	4	3,055	+	44	1.96	+	−
11	70	f	sp	Probable	4	2,841	−	36	1.91	+	−
12	70	f	ia	Probable	6	9,787	+	61	0.85	−	−
13	67	f	sp	Probable	5	2,657	−	16	1.33	−	−
14	70	f	fa	Probable	6	3,358	+	22	0.60	+	+
15	63	m	sp	Probable	4	3,125	+	18	0.84	+	−
16	63	m	sp	Probable	4	3,530	+	26	0.90	+	−
17	64	f	sp	Probable	6	2,630	+	48	0.90	+	−
18	51	f	ia	Probable	6	3,930	+	53	0.62	+	−
19	74	m	sp	Probable	5	4,574	+	35	0.98	+	−
20	74	f	sp	Probable	6	3,666	+	42	1.86	+	−

No : patients number, f : female, m : male, sp : sporadic CJD, ia : iatrogenic CJD, fa : familiar CJD, + : positive, − : negative, d.l. : diagnostic level based on WHO and the Master's criteria, d.w. : duration from onset to diagnostic examination, t-tau protein : total tau protein, NSE : neuron-specific enolase, DWI : diffusion-weighted image.

Codon 129 was Met/Met homozygous in all 20 cases examined while codon 219 was Glu/Glu homozygous in all 11 cases examined.

2. DWI technique

Scans were performed on a number of units. A 1.0 T or 1.5 T MR unit was used, including T1-weighted and fast spin-echo T2 weighted images. The DWI technique we used was in accord with that of other authors[4].

3. Measurements of biochemical markers in CSF (t-tau protein, S-100b protein and NSE)

The analysis of t-tau protein in CSF was done as before[3]. The method of analysis of S-100b protein in CSF samples utilized chemiluminescent enzyme immunoassay (CL-EIA) of S-100b protein. Antibody of S-100b protein (DAKO Japan) was fixed on 96 well microplates, and the 2nd antibody was anti-rabbit IgG peroxidase-linked species F (ab') fragment (DAKO Japan). The range of measurement in S-100b was 0.001-2.0 ng/mL. Final measurements were with an NSE protein analysis kit, sandwich ELISA with ^{123}I immunoradioassay (EIKEN Chemistry Company) with a measurement range in NSE of 2-200 ng/mL.

Results

The period of "early stage" was not defined, and so we assumed a definition for the duration on "early stage" as being within 6 weeks from onset. From among 44 CJD patients, we selected 20 that met the condition of having high-intensity brain lesions detected by DWI during time course (Table 1). We had determined 1,260 pg/mL as the cut-off point for data of t-tau protein as described[3]. All 20 patients had abnormal data (Table 1). The 14-3-3 β protein produced by Santa Cruz Biotechnology detected 14/20 cases, whereas the IBL antibody detected 17/20 patients on Western blots.

Analyses of all 20 patients with CJD identified sensitivities as ; NSE (12/20 : 60%), S-100b protein (9/20 : 45%), t-tau protein (20/20 : 100%), 14-3-3 protein (16/20 : 80%) and DWI (18/20 : 90%). The sensi-

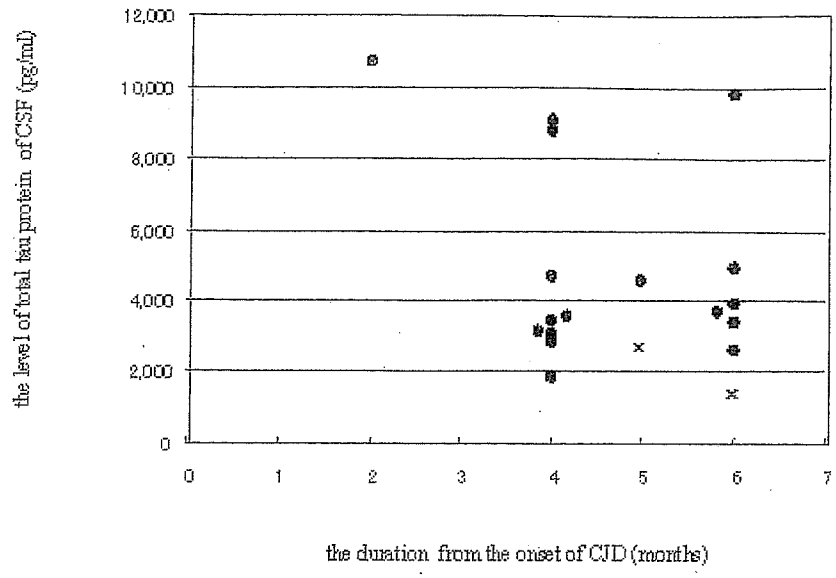

Fig. 1 The relationships between the duration from the onset of CJD and total tau protein of CSF
● : CJD cases detected by DWI, × : CJD cases undetected by DWI.

tivity of flair MRI was 10% (2/20). For the prion protein gene in all 20 cases examined, codon 129 was Met/Met homozygous, while codon 219 was Glu/Glu homozygous. And the level of t-tau protein didn't always depend on the duration (Fig. 1).

Discussion

As we couldn't discuss relative comparisons with these markers without the "early stage" period being defined, we assumed a definition : that "early stage" was within 6 weeks from onset. One reason for this was that many CJD patients visited the hospital for various reasons within 6 weeks of onset ; another reason was that 50% of CJD patients displayed akinetic mutism and appeared as power spectral density (PSD) on EEG within 2 months.

Several researchers have reported that the measurement of t-tau protein, 14-3-3 protein and NSE had high diagnostic impacts for differential diagnosis of CJD. But we were confused about the judgment of results by Western blots in some cases since 14-3-3 protein was the qualitative assay for Western blots.

Van Everbroeck et al.[5] reported that the course of the disease was divided into three stages. They showed the average of t-tau protein at an early stage ; results that support our data. However, Van Everbroeck et al.[5] and Otto et al.[2] didn't research for correlations of sensitivity between DWI and t-tau protein.

In Japan the usefulness of DWI as a diagnostic procedure has spread rapidly because MRI has been considered a sensitive noninvasive diagnostic test of CJD. However, not all hospitals have MRI scanners, or even if a hospital has a scanner, such scans are always not provided when there is DWI. Our study reported that the sensitivity of DWI was 90% at an early stage, but 100% at final stage. Shiga's[4] study reported that the sensitivity of DWI was 92.3% at final stage. Mendez et al.[6] reported studies of CJD in which DWI and 14-3-3 protein of CSF were both performed. However, neither Shiga nor Mendez discussed the difference of sensitivity between t-tau protein and DWI.

Our data clearly showed that the sensitivity of t-tau protein was greater than that of DWI at an early stage. We didn't emphasize research into t-tau protein in CSF ; measuring t-tau protein was taken as sufficient, but we focused on data that showed sensitivity was 100% in double positive cases of t-tau protein and DWI. The two methods strongly complement each other.

Recently we have doubted the significance of the diagnostic criteria of PSD on EEG, as we believe the sensitivity of CJD patients is lower than that with DWI and t-tau protein of CSF. We think that both of these tests need to be included as diagnostic criteria.

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