white matter (a subcortical lesion), the signal on DWI decreased, while the mean diffusivity increased (14). The present patient continued to exhibit high DWI with a low ADC over one year. To the best of our knowledge, no previous reports have shown decreased ADC values in the same lesion sustained for such a long period of time. Restricted diffusion appears to be caused by high viscosity of proteinaceous fluid and a high concentration of cells. Pathologically, axonal spheroids are filled with neurofilaments and organelles, which may cause diffusion restriction in HDLS patients (17).

Several diseases of the brain have been reported in patients with decreased ADC values, including acute cerebral infarction, multiple sclerosis, Creutzfeldt-Jakob disease, abscesses, metastasis and progressive multifocal leukoencephalopathy (18, 19). In the present case, the patient's symptoms and laboratory results suggested neither ischemic stroke nor infectious disease. Moreover, the PET study showed no malignancies, including lymphoma. A normal mental status and electroencephalogram findings on the first admission did not positively indicate Creutzfeldt-Jakob disease. We did not assess the patient for the JC virus in association with progressive multifocal leukoencephalopathy because she had no immunological risk factors.

In conclusion, we herein described a case of adult-onset HDLS that initially mimicked MS both clinically and radiologically. Cases of HDLS showing a pure motor phenotype are sometimes difficult to clinically differentiate from MS.

The authors state that they have no Conflict of Interest (COI).

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Short Report

Sixty Six–Month Follow-up of Muscle Power and Respiratory Function in a Case With Adult-Type Pompe Disease Treated With Enzyme Replacement Therapy

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Abstract

We report a patient with adult-type Pompe disease treated with enzyme replacement therapy (ERT) for 5.5 years. We evaluated pulmonary function and muscle strength using 6-minute walk test, manual muscle test, and dynamometer-based measurement. The long-term ERT resulted in a substantial improvement in the pulmonary function and a possible stabilization followed by mild deterioration in muscle power measured by dynamometer and 6-minute walk test. Our data may rationalize the long-term use of ERT for adult-type Pompe disease in terms of maintaining pulmonary function.

Key Words: adult-type Pompe disease, enzyme replacement therapy, long-term efficacy, muscle power, pulmonary function

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The authors report no conflicts of interest.

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INTRODUCTION

Pompe disease (glycogen storage disease type II, acid maltase deficiency) is an autosomal recessive metabolic disorder characterized by acid alpha-glucosidase deficiency that leads to an accumulation of glycogen in lysosomes in diverse organs such as liver, heart, and skeletal muscles. It presents as a wide spectrum of phenotypes ranging from classic infant form to adult form depending largely on the age of onset. The adult-type Pompe disease manifests as insidious muscle weakness and respiratory dysfunction over years.[1] Its muscle weakness begins in shoulders, pelvic girdle, trunk, and respiratory muscles leading to loss of ambulation and necessity of artificial ventilations later in their lives.[2] In 2006, an enzyme replacement therapy (ERT) with alpha-alglucosidase (Myozyme; Genzyme) was approved for the treatment of Pompe disease. Initially, its clinical trials of ERT only focused on the infant form and showed a great benefit on both skeletal muscle function and cardiopulmonary function.[3,4] In 2010, a large randomized, double-blind clinical trial of ERT for late-onset Pompe disease including the adult type was conducted (the Late-Onset Treatment Study). This trial has revealed a dramatic improvement in 6-minute walk test (6MWT) and percentage of predicted forced vital capacity (FVC) over 20 months. Because the natural course of adult-type Pompe disease is chronically progressive[5] and untreated patients have shorter life span than general population,[6] this type may require long-term treatment. However, we still lack sufficient data to conclude the long-term efficacy of ERT and it is not clear how the balance between the efficacy of the treatment and the progression of the disease works over the long run. To elucidate the long-term effect of ERT, we report a 5.5-year evaluation in a 30-year-old patient with Pompe disease. For a quantitative evaluation of muscle power, we used a computerized dynamometer

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together with clinical evaluation using 6MWT, manual muscle testing and pulmonary function tests.

PATIENT AND METHODS

Case Presentation

The patient was a 30-year-old man who showed a gradual progression of weakness in proximal extremities for more than 10 years. He was born to consanguineous parents. One of his 2 siblings died of an unknown reason when she was an infant. Ten years before the referral to our hospital, he started to notice difficulties in climbing the stairs and rising from the bed. His muscle strength in extremities gradually declined in 3 years. At age 24, a muscle biopsy of the left biceps brachii revealed vacuolar myopathy and periodic acid-Sciff-positive deposition in myofibers. The sequence analysis of *GAA* gene showed a homozygous substitution of c. 1309 C>T, leading to an alteration of arginine at position 437 to cysteine and the diagnosis of Pompe disease was confirmed. At age 26, pulmonary dysfunction was pointed out. He was then referred to our hospital for a possible treatment with ERT.

The patient's physical examinations were normal and cranial nerves were all intact. The limb-girdle and proximal muscles showed moderate atrophy, whereas distal muscles were spared. The Medical Research Council scores (MRC scores) for muscle powers were as follows: 4 in proximal upper limbs, 4 in quadriceps femoris, 3 in hamstrings muscles, 4 in neck flexors, and 2 in paraspinal muscles. The grip strengths of his right and left hands were 30 and 37 kg, respectively. He was able to walk with waddling gait without walking device. Scoliosis was observed.

Laboratory test showed mildly elevated serum level of creatine kinase (810 IU/L) and liver enzymes (aspartate aminotransferase 68 IU/L and alanine aminotransferase 55 IU/L). The artery gas blood analysis showed reduced partial pressures of oxygen (PaO_2 71.9 mm Hg) and elevated carbon dioxide ($PaCO_2$ 50.5 mm Hg). Pulmonary function test revealed that the FVC was diminished to 2.68 L (61.2% of the expected value). Chest x-ray, electric cardiogram, and ultrasound cardiography were all normal. The magnetic resonance imaging of the thigh muscles demonstrated atrophy with fat replacement in the quadriceps and the hamstrings muscles.

Methods

ERT and Physical Therapy Regimen

The ERT, that is, intravenous infusion of recombinant human alpha-glucosidase (rhGAA 20 mg/kg body weight) was repeated every 2 weeks. Together with ERT, monthly adjunctive physical therapy (PT) was started. The patient has been fully informed and consented to participate in this study.

Evaluation of the Effects of the ERT/PT

During 5.5 years of ERT/PT, we evaluated the following measurements every 3 to 6 months on a regular basis.

Manual Muscle Testing.

Muscle strength of left knee extensors and flexors was assessed by a neurologist (N.T.) and the grades were assigned using the MRC grading scale with subdivisions.[7]

The 6-Minute Walk Test.

The 6MWT was performed in accordance with American Thoracic Society Guidelines,[8] and the distance walked in 6 minutes was recorded in meters.

Isometric Muscle Strength Measurement Using a Computerized Dynamometer.

The maximum strength of the left knee extensors and flexors was evaluated by isometric torque measurement using a computerized dynamometer (Biodex System 3; Sakai Medical, Tokyo, Japan) in the same manner as described previously.[9] The maximum values of isometric knee extension or flexion (peak torque) were measured and recorded in Newton meter (Nm) for 3 times in each direction. The maximum peak torque from the 3 trials was determined as the isometric muscle strength for each movement.

Pulmonary Function Tests.

FVC in the upright position was measured by spirometric test. Arterial blood sample was also obtained at rest with ambient air.

Immunoglobulin G Antibodies to rhGAA.

The serum level of immunoglobulin G (IgG) antibodies to rhGAA (anti-rhGAA) was assessed with an enzyme-linked immunosorbent assay at 30 and 66 months. Inhibition of the enzyme uptake was also evaluated by a flow cytometry based assay at 60 months after the start of ERT.

RESULTS

The patient received biweekly infusions with rhGAA for 66 months without any infusion-related adverse reaction. Over the observational period, he felt his symptoms remained stable and did not require any walking device or mechanical ventilation.

Manual Muscle Testing

At the baseline assessment, the modified MRC scores of his left knee extensors (quadriceps femoris muscles) was 4^+ and that of the flexors (hamstring muscles) was 3^+. The scores of the quadriceps femoris and the hamstring muscles slightly decreased to $4-$ and $3-$, respectively, in 66 months.

Six-Minute Walk Test

Figure 1 shows the time course of the walking distance in 6MWT. Before ERT treatment, he was able to walk 382 m in 6 minutes. The distance remained unchanged in the first 36 months (ranging from 381 to 398 m); however, it started to decline slightly after 42 months and ended up in 340 m.

Isometric Muscle Strength

The maximum peak torque of the left knee extensors was 33.2 Nm and that of the left knee flexors was 22.3 Nm at the baseline (Fig. 2). During the ERT, the strength of the knee extensor was consistent and remained stable, whereas that of the knee flexors slowly decreased after 42 months following a variable but yet stable period.

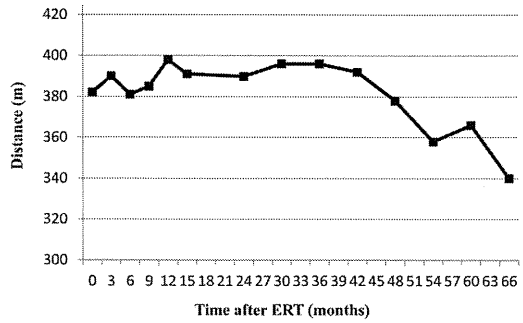

FIGURE 1. The results of the 6MWT at the baseline and after ERT. The distance (in meters) walked in 6 minutes had been stable until 42 months followed by slight reduction.

Pulmonary Function Tests

FVC at the baseline was 2.68 L and it showed significant improvement to 3.30 L at 66 months. The favorable change of the FVC was noted for the first 24 months and its effect still continued throughout the observational period (Fig. 3). Artery gas blood analysis at the baseline was PaO_2 71.9 mm Hg and $PaCO_2$ 50.5 mm Hg. Those parameters mildly improved and then showed stabilization at 66 months.

IgG Antibodies to rhGAA

The patient developed antibody against ERT. The titer value for anti-rhGAA at 30 and 66 months were 3200 and 6400, respectively. The result of the inhibition of enzyme uptake was negative at 60 months.

DISCUSSION

This case report describes a long-term effect of ERT in a patient with adult Pompe disease with regard to muscle power and pulmonary function. Our data showed that FVC improved substantially. It is also noted that the muscle function in lower extremities measured by walking distance and dynamometer seemed to be stabilized over 42 months followed by a mild deterioration. Considering the progressive nature of Pompe disease,[5] this case report may suggest a long-term benefit of ERT at least on pulmonary function.

Long-term efficacy of ERT has remained elusive for patients with adult Pompe disease.

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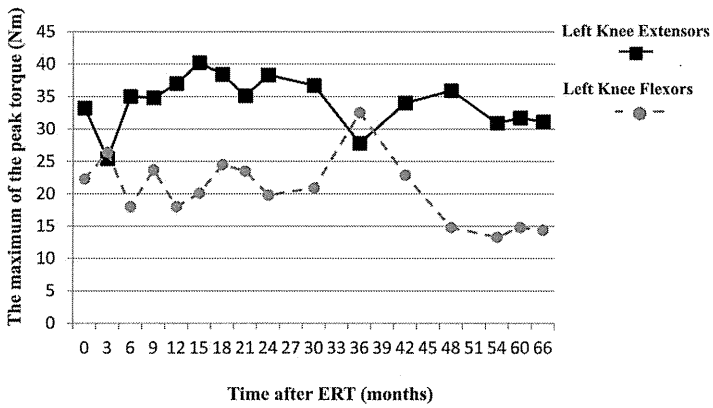

FIGURE 2. The isometric muscle strength of the left knee joint. The maximum of the peak torque of the isometric movement of the left knee extensors (shown by solid line) and the knee flexors (shown by dashed line) were measured in Newton meter separately. The knee extensors remained stable, whereas the knee flexors slowly decreased after 42 months.

Two recent studies examined long-term efficacy of ERT in patients with late-onset Pompe disease for 12–57 months[10] and 5–47 months.[11] In these studies, it was described that motor function and pulmonary function were improved or stabilized. Our data of pulmonary function are not only consistent with those studies but also provide further information about longer treatment with ERT in patients with adult-type Pompe disease. In other words, pulmonary function may have further impact beyond 5 years.

Although the pulmonary function of our patient showed the positive effect of ERT for over years, motor function of our patient took slightly different time course from the pulmonary function. During the ERT, the muscle strength and 6MWT did not show apparent reduction for the first 42 months, indicating the stabilization effect of the ERT treatment. As some studies describe that muscle strength in patients with late-onset Pompe disease significantly declines yearly without the treatment,[12,13] the muscle function of our patient would have worsened yearly over 5 years if ERT had not been started. Moreover, our observation on the motor function assessed by the 6MWT and the dynamometer revealed that the maintenance of the muscle strength may not last over the long term because the values for muscle function started to mildly decline 48 months after the initiation of ERT. Agreeing with our finding, greater improvement in the first year and less prominent improvement in the second year were documented regarding the muscle strength and pulmonary function.[14] So far, there is no clear explanation for the greater change at the beginning followed by stabilization or mild deterioration in muscle strength. It could be increased antibody against alpha-alglucosidase[15] or preceding irreversible damage in muscles.[6] In our patient, the titer for anti-rhGAA was low during the treatment, negating the possibility of the antibody hypothesis in our case. The irreversible damage to muscles of varying degree might have been the key limiting factor of ERT treatment as our patient started to present the symptom in his early adulthood and had atrophy with fat replacement in his leg muscles.

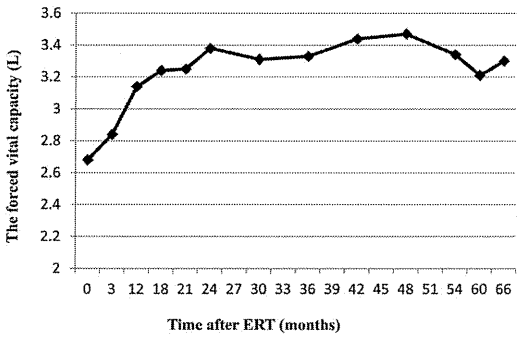

FIGURE 3. The FVC in the upright position. During the course of the treatment, the predicted FVC showed great improvement and maintained for 66 months.

Of course, this is merely a case report and does not represent the whole spectrum of adult-type Pompe disease. Nevertheless, we think it is important to report a case with longer treatment with ERT because cost for ERT is vast[16] and clinicians must know its cost-effectiveness. It is still difficult to decide at which point ERT should be terminated as ERT is the only approved disease-specified therapy for this disease.[17] In this sense, our case report may still rationalize to continue ERT with similar patients who shows decline in muscle function but has pulmonary stabilization after 4–5 years of the treatment. To confirm the long-term efficacy of ERT on pulmonary function, longer assessment of ERT in clinical setting is needed.

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Increased α-synuclein levels in the cerebrospinal fluid of patients with Creutzfeldt–Jakob disease

Takashi Kasai, Takahiko Tokuda, Ryotaro Ishii, Noriko Ishigami, Yoshio Tsuboi, Masanori Nakagawa, Toshiki Mizuno & Omar M. A. El-Agnaf

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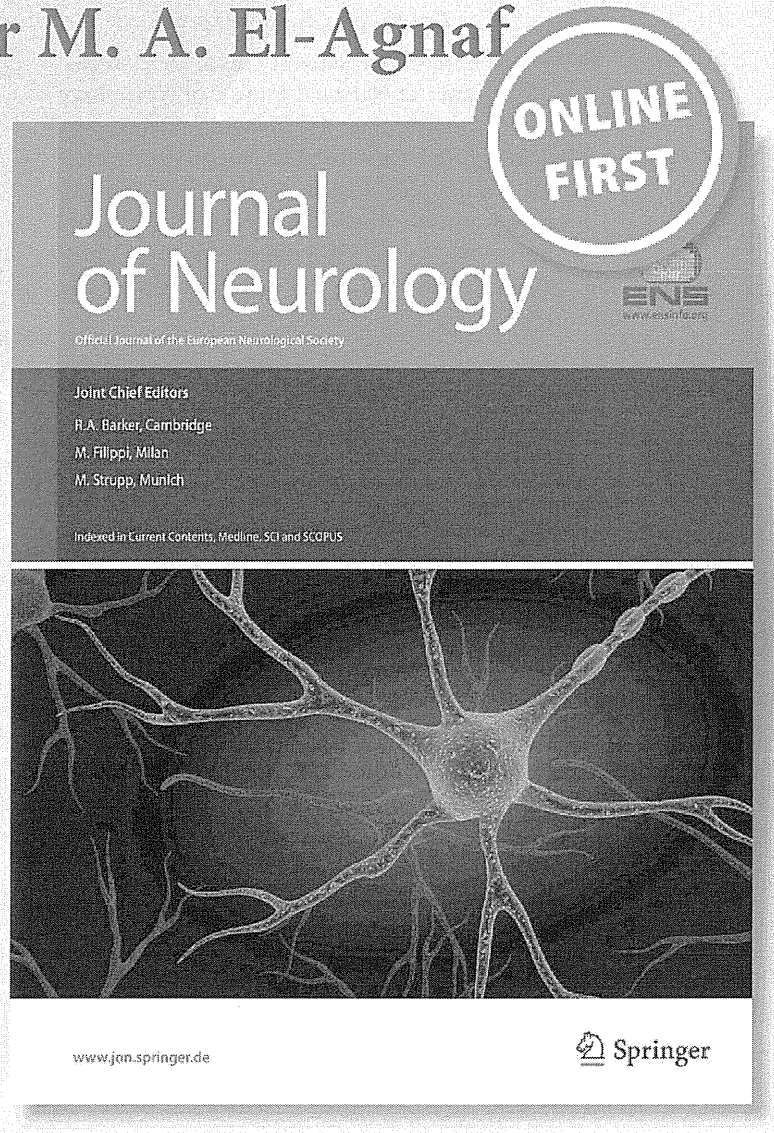

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ORIGINAL COMMUNICATION

Increased α-synuclein levels in the cerebrospinal fluid of patients with Creutzfeldt–Jakob disease

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Abstract Recent studies have shown that cerebrospinal fluid (CSF) levels of α-synuclein (α-syn) are highly elevated in patients with Creutzfeldt–Jakob disease (CJD) compared to controls. However, the diagnostic value of CSF α-syn in CJD has not been established. To confirm whether CSF α-syn is increased in CJD and is a useful marker for this disease, two independent enzyme-linked immunoabsorbent assays (ELISAs) specific for α-syn were used: ELISA 211-FL140, which is specific for full-length α-syn, and ELISA N19-FL140, which is specific for the full-length and associated C-terminal truncated forms of α-syn. CSF samples from 24 patients with CJD and 24 controls were assessed in this study. We found that samples from the CJD patients showed significantly higher levels of CSF α-syn compared to controls in both ELISA (211-FL140 or N19-FL140) tests ($P = 0.0467$ and $P = 0.0010$, respectively). However, there was a considerable overlap in the concentration ranges of the two groups of subjects. We also measured the levels of total tau (t-tau) protein in these samples and found that CSF t-tau levels were 5–10-times higher in the CJD group ($P < 0.0001$) compared with the controls. When the CSF t-tau and α-syn levels were combined, the area under the ROC curve (AUC) was slightly increased in clinically diagnosed CJD cases (AUC of 0.964) relative to an AUC of 0.943 for increased CSF t-tau alone. The combined use of CSF α-syn and t-tau levels may be a useful biomarker for the diagnosis of CJD.

Keywords Creutzfeldt–Jakob disease · Cerebrospinal fluid · α-Synuclein · Tau · Biomarker · ELISA

Introduction

Creutzfeldt–Jakob disease (CJD) is a rare neurodegenerative disease that mainly affects elderly people. The diagnosis of CJD can only be confirmed by brain biopsy; however, certain biochemical markers in cerebrospinal fluid (CSF) have been reported to be useful in the differential diagnosis of CJD from other dementia-spectrum illnesses. Several reports have demonstrated that total tau protein (t-tau) in the CSF is one of the most sensitive biomarkers in the early diagnosis of CJD [1, 2]. Tau is a microtubule-associated protein that contributes to axonal structure [3]. Therefore, axonal degeneration and the leakage of axonal proteins in the brains of CJD patients are the most likely causes of an increase in CSF levels of t-tau. However, the primary neuropathological feature in prion diseases is the loss of synapses rather than axonal degeneration [4–6], and proteins leaking from synaptic structures probably have a greater early diagnostic value than t-tau or other axonal proteins in CJD. Therefore, we investigated

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the levels of the synaptic protein α-synuclein (α-syn) in CSF from patients with CJD and healthy matched controls.

α-Syn is a protein involved in the pathogenesis of Parkinson's disease (PD) and other synucleinopathies. Recently, our group and others have developed protocols for sandwich-type enzyme-linked immunoabsorbent assays (ELISAs) specific for α-syn and have reported decreased CSF levels of α-syn in patients with PD compared to controls [7–10]. Interestingly, it has been reported that CSF levels of α-syn were highly elevated in eight CJD patients compared with controls [9]. The strong increase in the CSF levels of α-syn in CJD patients was interpreted as biochemical evidence of rapid synaptic degeneration and leakage of α-syn, which is known to be a major component of presynaptic proteins [11], in CJD. Although it has been implied that CSF α-syn could be a diagnostic marker for CJD, relatively few patients have been studied, and thus study size has been insufficient for evaluating the diagnostic value. To verify these previous findings and to evaluate the diagnostic utility of CSF α-syn for CJD, we investigated the levels of α-syn in CSF from 24 patients with CJD and 24 control subjects.

Methods and materials

Patients

CSF samples were collected from 24 patients with CJD (age 41–82, mean ± SD = 62.9 ± 10.0) and 24 gender- and age-matched control subjects (age 17–88, mean ± SD = 60.0 ± 18.3). The CJD group included two patients with familial CJD (V180I), three patients with probable iatrogenic CJD (iCJD) and 19 patients with probable sporadic CJD (sCJD). Probable sCJD and iCJD patients fulfilled the WHO diagnostic criteria for CJD [12]. The control group included neurologically normal individuals ($n = 8$) as well as subjects with peripheral neuropathy ($n = 8$), cranial neuropathy ($n = 3$), epilepsy ($n = 2$), motor neuron disease ($n = 1$), spastic paraplegia ($n = 1$), and spinal myoclonus ($n = 1$). Informed consent was obtained from the patient when possible or from the nearest relative when not possible, which was approved by the University Ethics Committee (Kyoto Prefectural University of Medicine, Kyoto, and the Fukuoka University School of Medicine, Fukuoka, Japan). The study procedures were designed and performed in accordance with the Declaration of Helsinki. Fresh CSF samples collected from living CJD patients and control cases were cleared by centrifugation at 3,000×g for 10 min at 4 °C and then stored at −80 °C until used for this study. We excluded samples with apparent blood contamination from this study following visual inspection of centrifuged CSF. Samples with pink-red discoloration indicative of hemoglobinemia were excluded.

Antibodies

The anti-human α-syn monoclonal antibody 211 (MAb211) recognizes the C-terminal portion of α-syn (residues 121–125). The anti-human α-/β-synuclein goat polyclonal antibody N19 recognizes the N-terminal portion of α-syn (residues 5–19). The anti-human α-/β-/γ-synuclein rabbit polyclonal antibody FL-140 was raised against the recombinant full-length α-syn (Fig. 1a). All antibodies were purchased from Santa Cruz Biotechnology, CA, USA.

Measurement of α-syn

CSF α-syn levels were measured using a previously reported sandwich ELISA system that was modified to achieve an improved sensitivity as low as 0.01 pg/ml [7]. Briefly, each ELISA plate (384-well ELISA plate, Nunc Maxisorb™, NUNC A/S, Roskilde, Denmark) was coated with 1 μg/ml of either MAb211 or N19 antibodies (50 μl/well) as a capture antibody in 200 mM $NaHCO_3$, pH 9.6, at 4 °C for overnight. The plate was then washed with phosphate buffered saline (PBS) containing 0.05 % Tween 20 (PBST) and subsequently incubated with 100 μl/well of a blocking buffer (PBST containing 2.5 % gelatin) for 2 h at 37 °C. After adding a cocktail of protease inhibitors (Calbiochem, CA, USA) to the CSF samples, 50 μl of these samples were added to each well and the plate was incubated at 37 °C for 3 h. After washing with PBST, 50 μl of anti-α-syn antibody FL-140 (0.2 mg/ml in the blocking buffer) was added as a reporter antibody, and the plate was incubated at 37 °C for 2 h. After washing with PBST, the wells were incubated with 50 μl/well of horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) and incubated for 1 h at 37°C. Bound HRP activity was assayed using an enhanced chemiluminescent substrate (SuperSignal Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Rockford, IL, USA, 50 μl/well), and the chemiluminescence signal was measured at 395 nm with a microplate luminometer (SpectraMax L, Molecular Devices, Tokyo, Japan). The standard curve for the ELISA was prepared using 50 μl/well of recombinant human α-syn solution at different concentrations of the protein in PBS solution. All samples and standards were run in triplicate on the same day with the same lot of standards unless otherwise noted. The relative estimated concentrations of CSF α-syn were calculated using a standard curve. In the following descriptions, the use of MAb211 as the capture antibody and FL140 as the reporter antibody is referred to as "211-FL140 ELISA", and the use of N19 as the capture antibody and

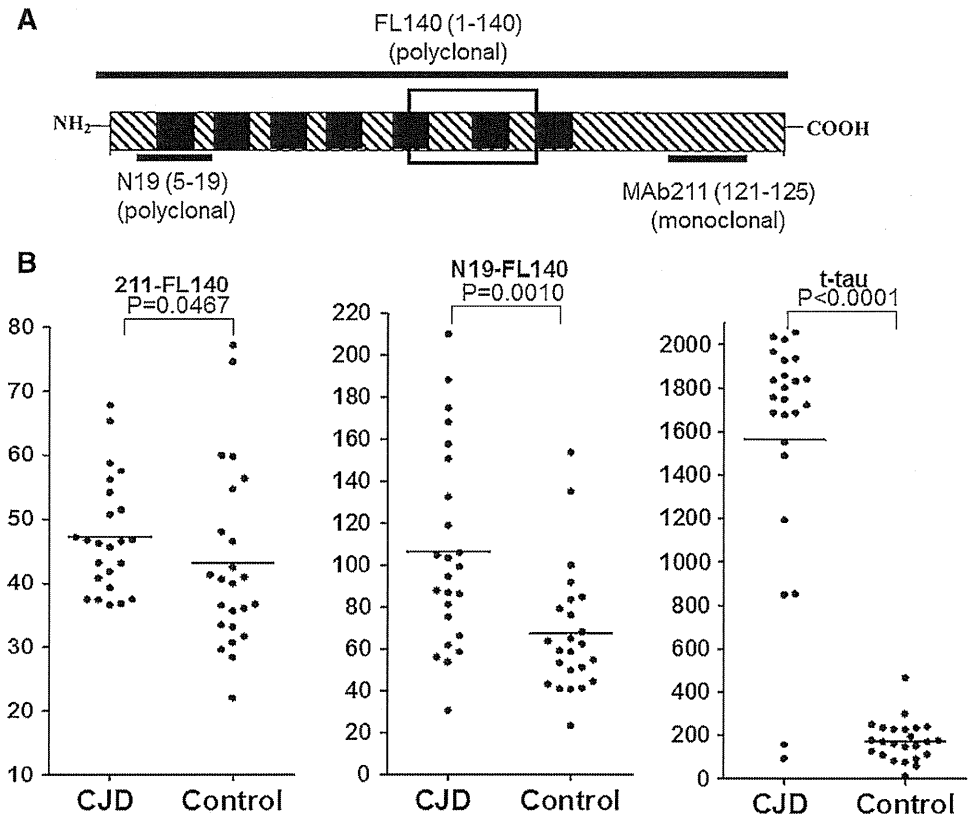

Fig. 1 **a** Recognition sequences for α-syn antibodies. Recognition sequences for α-syn antibodies used in the present study are shown as *black bars* in the schema of the primary structure of α-syn. *Black boxes* indicate imperfect lysine-rich motifs (KTKEGV), and the *white box* indicates the non-amyloid-β component (NAC) domain. **b** Scatter plots of the levels of CSF α-syn, measured using 211-FL140 ELISA and N19-FL140 ELISA, and the levels of t-tau. The CSF α-syn concentrations measured with two ELISA methods, 211-FL140 ELISA, N19-FL140 ELISA, and the t-tau levels measured using a commercial kit are shown. The concentrations of CSF α-syn in the CJD group were significantly higher than that in the age-matched control subjects for either ELISA ($P = 0.0467$ using 211-FL140 ELISA, and $P = 0.0010$ using N19-FL140 ELISA, Mann–Whitney U test). The levels of t-tau were highly elevated in the CJD group ($P < 0.0001$, Mann–Whitney U test)

FL140 as the reporter antibody is referred to as "N19-FL140 ELISA". The 211-FL140 ELISA is specific for the full-length human α-syn because of the selective affinity of MAb211 for α-syn. In contrast, N19 recognizes the N-terminal sequence of α-syn, which is shared by β-synuclein (β-syn) (Fig. 1a); therefore, N19-FL140 ELISA can detect both proteins. However, using mass spectroscopy, we have reported that neither β-syn nor γ-synuclein was detected in human CSF [9], and therefore, our N19-FL140 ELISA array only measures the α-syn present in CSF. It is noteworthy that this ELISA protocol can also detect the C-terminal truncated forms of α-syn present in CSF, which cannot be detected by the 211-FL140 ELISA protocol.

Measurement of t-tau

The levels of CSF t-tau were measured using the Fino Scholar hTau ELISA kit (Nipro, Osaka, Japan) according to the manufacturer's instructions.

Statistics

Image analysis was performed using Scion Image for Windows Version 4 (Scion Corporation Frederick, MA, USA). A comparison between the two independent groups was performed using the Mann–Whitney U test. The ROC curve and the AUC were calculated and compared between the two α-syn ELISA arrays. $P < 0.05$ was considered statically significant. Statistical analyses were performed using GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA, USA).

Results

As shown in Fig. 1b, the concentrations of CSF α-syn measured using the 211-FL140 ELISA were significantly higher in CJD patients than in controls (mean ± SD; 47.28 ± 8.98 in CJD vs. 42.33 ± 12.53 in controls,

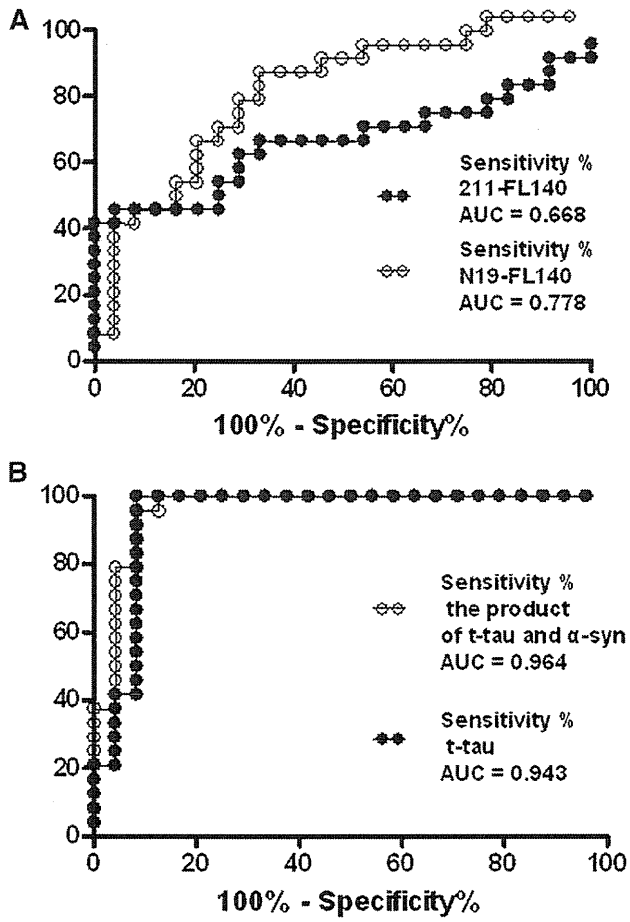

Fig. 2 **a** Receiver operating characteristic (ROC) curves for the levels of α-syn in CSF in the discrimination of patients with CJD from controls when using the 211-FL140 ELISA (*black circles*) and the N19-FL140 ELISA (*white circles*). AUC values are indicated. **b** ROC curves for the use of t-tau alone (*black circles*) and the use of combined t-tau and α-syn (*white circles*) in the discrimination of patients with CJD from controls. AUC values are indicated

$P = 0.0467$). However, there was a considerable overlap in the concentration ranges for the two groups. Receiver operating characteristic (ROC) curves used to discriminate patients with CJD from the control subjects were calculated based on ELISA data. The area under the ROC curve (AUC) for the 211-FL140 ELISA was poor (AUC = 0.668) (Fig. 2a). Using the N19-FL140 ELISA, significantly higher levels of CSF α-syn were detected in CJD patients than in controls (mean ± SD; 106.3 ± 47.23 in CJD vs. 66.38 ± 26.76 in controls, $P = 0.0010$). The AUC in the ROC curve used to discriminate CJD patients from controls improved in the N19-FL140 ELISA (AUC = 0.778) (Fig. 2a), although an overlap in the concentration ranges for the two groups was still observed, and the mean value of the signals in the CJD group was no more than twice that of the control group.

We also measured the levels of total tau (t-tau) in CJD and control samples. The mean value of t-tau in controls corresponded with previously reported levels (mean ± SD; 188 ± 103 pg/ml) [13].The CSF levels of t-Tau were highly elevated in the CJD group ($P < 0.0001$, Mann–Whitney U test) with mean values 5–10-times higher than in control patients (mean ± SD; 1,564.11 ± 548.16 pg/ml in CJD vs. 173.77 ± 93.57 pg/ml in controls). However, two CJD patients who had normal levels of CSF t-tau also had elevated levels of CSF α-syn, as measured with the N19-FL140 ELISA (87.8 and 105.8 ng/ml), that exceeded the threshold value of 77.7 ng/ml. The measured levels of CSF t-tau and α-syn in CJD patients are summarized in Table 1. When we calculated the values of t-tau and α-syn measured with the N19-FL140 ELISA, the AUC of the product (0.964) was slightly higher than that of t-tau alone (0.943) in the ROC analysis (Fig. 2b). The optimized threshold value of the product was 14,922 ng/ml, where the sensitivity and specificity of the test were 79.17 and 95.83 %, respectively.

Discussion

The CSF levels of α-syn measured with the 211-FL140 ELISA were significantly higher in CJD patients than in controls. Our data is in agreement with the results reported by Mollenhauer et al. [9]. In this earlier report, CSF α-syn levels in CJD were more than 40 times higher than in controls [9], whereas our results recorded a sizeable overlap between the concentration ranges for individual signals for CJD patients and controls. Such a discrepancy could be caused by the narrow binding specificity of MAb211, which only binds to the full-length α-syn [14]; thus, an alternative assay system (N19-FL140 ELISA) was developed in which full-length α-syn and its C-terminal truncated forms are detectable. Interestingly, the N19-FL140 assay showed better diagnostic performance than did the 211-FL140 assay. However, the large signal difference between the CJD patients and the control group reported by Mollenhauer and co-workers [9] was not reproduced in our current study. Such inconsistencies among different studies may be caused by methodological issues, including pre-analytical differences in the collection, handling, and storage of CSF samples as well as the use of antibodies that detect different species of α-syn. In fact, when we performed immunoprecipitation followed by western blotting as an alternative approach to roughly estimate the CSF α-syn levels detected with ELISA, we found that the density of the band for full-length α-syn in CJD patients was much higher than that in controls in IP-WB (Supplementary Figure). We currently have no clear

Table 1 Concentrations of CSF t-tau and α-syn measured in CJD patients

Patient No.	Sex	Age	CJD type	*PRNP* codon 129 genotyope	*PRNP* codon 219 genotyope	α-Syn (ng/ml) measured using 211-FL140 ELISA	α-/β-Syn (ng/ml) measured using N19-FL140 ELISA	t-Tau (pg/ml)
1	M	60	sCJD	ND	ND	39.25	30.48	852.03
2	M	72	sCJD	ND	ND	67.74	118.89	1,799.73
3	M	41	sCJD	ND	ND	56.19	75.33	1,719.62
4	M	54	sCJD	MM	EE	57.50	174.89	1,830.18
5	*F*	*49*	*iCJD*	*MM*	*EE*	*51.45*	*87.77*	*91.72*
6	F	59	sCJD	ND	ND	54.16	86.23	1,833.75
7	F	61	sCJD	MM	EE	45.64	56.04	1,684.48
8	F	68	sCJD	MM	EE	47.14	94.45	1,965.74
9	F	68	sCJD	ND	ND	37.48	61.78	1,934.51
10	F	82	sCJD	ND	ND	46.48	99.11	1,684.72
11	F	64	sCJD	ND	ND	41.81	157.69	847.09
12	F	49	sCJD	MM	EE	46.69	58.57	1,745.42
13	*F*	*69*	*sCJD*	*MM*	*EE*	*46.75*	*105.84*	*156.77*
14	F	60	sCJD	MM	EE	40.76	81.18	1,755.35
15	F	60	fCJD (V180I)	MM	EE	65.31	86.77	1,674.99
16	F	55	sCJD	MM	EE	50.74	132.49	2,034.30
17	F	68	sCJD	MM	EE	58.73	103.30	2,021.94
18	M	50	iCJD	ND	ND	36.79	66.11	1,191.23
19	M	62	iCJD	MM	EE	43.11	210.13	1,488.26
20	F	69	fCJD (V180I)	MM	EE	37.49	150.74	1,550.17
21	F	79	sCJD	MM	EE	43.19	188.22	1,926.43
22	F	65	sCJD	MM	EE	36.58	168.03	1,839.54
23	M	73	sCJD	ND	ND	37.43	104.62	1,855.61
24	F	73	sCJD	ND	ND	46.20	53.69	2,054.96
					Mean	47.27	106.35	1,564.11
					SD	8.91	47.23	548.16

Some cases did not agree to allow genetic testing. The *PRNP* genotypes of these patients are presented as ND (not determined). Two CJD patients had normal CSF t-tau levels (indicated by italic) and high CSF α-syn levels of as measured with the N19-FL140 ELISA

sCJD sporadic CJD, *fCJD* familial CJD, *iCJD* iatrogenic CJD

explanation for this discrepancy between our ELISA and IP-WB. However, this result suggests that detection by our ELISA of unknown α-syn species that leaked out into the CSF space in CJD patients still requires improvement. It cannot be said that the diagnostic value of the ELISA tests studied here for measuring CSF α-syn in CJD exceeds that of the conventional biomarker, t-tau. However, a few CJD patients in the present study with normal levels of t-tau showed elevated CSF α-syn levels (see Tables 1 and 2). Signal overlap is often seen in cross-sectional investigations, such as in the quantification of CSF Aβ and tau proteins as biomarkers for Alzheimer's disease [15]. In clinical practice, it has been documented that the predictive value of CSF parameters in individual subjects is often enhanced by employing more than one surrogate or biomarker. Accordingly, the combined CSF t-tau and α-syn results improved the ability to discriminate between the CJD patients and controls compared with the use of CSF t-tau alone (see Fig. 2b). The AUC value, which represents the ability to discriminate between CJD patients and controls, was 0.943 for CSF t-tau alone but was slightly increased to 0.964 by combining CSF t-tau with α-syn, implying that the CSF α-syn species measured by the N19-FL140 ELISA may be a complementary biomarker to t-tau for the diagnosis of CJD.

A potential confounder in this study is the imperfect exclusion of samples contaminated with blood. Hong et al. [8] indicated that samples contaminated by red blood cells, which are a potential source of α-syn, should be excluded using hemoglobin measures. On the other hand, not all ELISAs detect α-syn that has leaked from red blood cells. Fould et al. reported that CSF α-syn levels from four independent ELISAs, including the 211-FL140 ELISA that we used, do not correlate with hemoglobin concentrations. Therefore, she argued that these ELISAs are applicable for

Table 2 Concentrations of CSF t-tau and α-syn measured in control subjects

Patient No.	Sex	Age	Diseases	α-Syn (ng/ml) measured using 211-FL140 ELISA	α-/β-Syn (ng/ml) measured using N19-FL140 ELISA	t-Tau (pg/ml)
1	F	66	Neuropathy	33.13	51.24	80.93
2	M	64	Neuropathy	74.53	135.05	160.77
3	M	58	Neuropathy	30.69	40.52	150.67
4	M	75	Normal	39.96	54.72	225.73
5	M	72	Epilepsy	33.44	40.90	174.11
6	F	21	Epilepsy	46.52	63.73	169.48
7	F	47	MND	40.62	44.42	298.34
8	F	39	Normal	31.69	41.21	176.34
9	M	38	Spastic paraplegia	48.01	68.09	126.03
10	F	59	Neuropathy	22.00	23.22	239.02
11	F	48	Normal	59.77	83.54	248.84
12	M	17	Neuropathy	41.27	58.60	193.30
13	M	40	Normal	56.35	91.90	233.68
14	F	71	Normal	54.65	99.93	110.62
15	M	73	Normal	59.93	84.82	146.76
16	M	67	Spinal myoclonus	42.48	79.14	107.41
17	F	63	Neuropathy	77.16	153.71	234.33
18	F	76	Cranial neuropathy	28.37	53.15	228.10
19	F	60	Neuropathy	36.51	59.06	9.34
20	M	77	Normal	40.95	76.21	75.84
21	M	75	Neuropathy	35.63	49.65	169.41
22	M	88	Normal	29.62	43.10	464.68
23	F	69	Cranial neuropathy	36.05	62.25	88.90
24	M	78	Cranial neuropathy	36.70	64.85	57.79
			Mean	43.17	67.62	173.77
			SD	14.12	30.06	93.57

We found no significant difference in any parameter between the normal controls and the other neurological disease controls ($P = 0.327$ for α-syn measured using 211-FL140 ELISA, $P = 0.270$ for α-/β-syn measured using N19-FL140 ELISA, and $P = 0.327$ for t-tau)

MND motor neuron disease

CSF that may be contaminated with small amounts of blood, such as post-mortem CSF [16]. The cause of such inconsistency among reports is still unclear. Regarding the ELISA that we used in this study, we have also confirmed that no correlation exists between levels of hemoglobin and α-syn in CSF (unpublished data).

In summary, the present study found that CSF α-syn levels in patients with CJD were significantly higher than in controls when we used an ELISA protocol specific for detecting only the full-length α-syn. This result was consistently verified with an additional ELISA protocol that detects not only the full-length form but also the C-terminal truncated forms of α-syn. Although these observations were consistent with previously reported results, the large signal difference between the groups that has been previously reported was not observed in the present report. In this study, we demonstrated that the diagnostic value of CSF α-syn alone does not exceed that of CSF t-tau; however, the AUC value was slightly increased when we combined the use of both markers.

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Conflicts of interest All authors have no conflict of interest to declare.

Ethical standard All human studies must state that they have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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Case Report

Concomitant alpha-synuclein pathology in an autopsy case of amyotrophic lateral sclerosis presenting with orthostatic hypotension and cardiac arrests

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A 74-year-old man gradually developed muscular weakness in the upper extremities, followed by dyspnea and dysarthria over a 6-month period. He was admitted to our facility and diagnosed as having amyotrophic lateral sclerosis (ALS) based on clinical and neurophysiological findings. Two months later, transtracheal positive pressure ventilation (TPPV) was started. During his clinical course, orthostatic hypotension occurred a few times. He also had two episodes of transient cardiac arrest, and he died 15 months after disease onset. At autopsy, the brain, weighing 850 g, showed diffuse cortical atrophy, preferentially involving the frontal lobes. Microscopic findings included severe loss of neurons in the motor cortex, the motor nuclei of the brainstem and the anterior horns of the spinal cord, and mild loss of axons and myelin in the corticospinal tract. Trans-activation response DNA protein 43 (TDP-43) immunoreactive cytoplasmic inclusions, the pathognomonic findings for ALS, were noted in the nucleus facialis, nucleus ambiguus, and in the anterior horn of the spinal cord. In addition, Lewy bodies and Lewy neurites were found in the brainstem and in the nucleus intermediolateralis of the thoracic cord. The concomitant alpha-synuclein pathology may have been partly related to possible autonomic dysfunction underlying the two episodes of cardiac arrest.

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Key words: amyotrophic lateral sclerosis (ALS), cardiac arrest, Lewy body, orthostatic hypotension, subclinical Parkinson's disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that exclusively involves motor neurons. The autonomic nervous system (ANS) is usually spared in ALS,[1] but autonomic dysfunction has been reported in some cases.[2,3]

It was previously reported that severe sympathetic disturbances increase the risk of sudden death or cardiac arrest in patients with Parkinson's disease (PD)[4,5] or in those with multiple system atrophy (MSA).[6,7] In ALS, a similar mechanism is thought to be also responsible for sudden cardiac arrest.[8]

An autopsy case of ALS presenting with orthostatic hypotension and two episodes of cardiac arrest during the clinical course is reported. The neuropathological features of this case were examined immunohistochemically and the underlying mechanism is reviewed.

CLINICAL SUMMARY

A 74-year-old Japanese man noticed muscular weakness of the bilateral upper limbs, followed by the development of dysarthria and dyspnea over a 6-month period. On admission, he showed general gauntness. He was alert without apparent evidence of dementia. Ocular movements were normal. Severe dysarthria and dysphagia were present. Muscular atrophy and fasciculations were noticed in the tongue and limbs. The grasping power was 19 kg on the

right side and 20 kg on the left side. Tendon reflexes were brisk in both the upper and lower extremities, with no Babinski signs. Although he showed a slight forward-bent posture, he was able to walk without difficulty. It was noted that he had developed diabetes mellitus at the age 74 years. No other family member had a motor neuron disease, and neither of his parents were from the Kii Peninsula, the region where ALS-Parkinsonism-dementia complex is prevalent.

Nerve conduction studies showed no remarkable changes. Neurogenic changes were recorded in the anterior tibial muscle by needle electromyogram. According to these clinical and examination findings, he was diagnosed clinically as having ALS.

A few weeks after he was diagnosed as having ALS, his respiratory function deteriorated, and non-invasive positive pressure ventilation (NPPV) was started. Transtracheal positive pressure ventilation (TPPV) was introduced 1 week later. Orthostatic hypotension (OH) was noticed when the patient had an episode of transient loss of consciousness when his respiratory function worsened in the early stage of hospitalization. On another day, the Shellong test was performed, and a prominent decrease in his systolic blood pressure (of about 40 mmHg) was recorded. After droxydopa was administered, OH did not recur. Autonomic dysfunction was not detected in other examinations. There was no QTc prolongation on the electrocardiogram. The patient suffered two cardiac arrests. Medical personnel discovered the first episode immediately after onset, and the patient had a favorable outcome after appropriate treatment. Then he was discharged about 2 weeks after the episode. However, following the second episode of cardiac arrest, he did not recover consciousness, although his heartbeat recovered with resuscitation. It was estimated that the second cardiac arrest lasted about 10–15 min. He was readmitted and died of cardiac failure on the 18th hospital day after the second episode. The total time from clinical onset of ALS to death was approximately 15 months.

PATHOLOGICAL FINDINGS

The autopsy was performed 3 hours after the patient's death. The brain, weighing 850 g, showed diffuse atrophy, predominantly involving the frontal lobes. The proportion of cerebellum and brainstem was normal. The spinal cord was thin and atrophic throughout its entire length, with no lumbar enlargement. On the cutting surface of the cerebrum, atrophy of the cerebral cortex, dilatation of the lateral ventricles, and symmetrical ischemic changes of the lateral segments of the globus pallidus were noted. Foci of infarctions were found in the basal ganglia and midbrain. In the midbrain and pons, the substantia nigra and locus coeruleus showed slightly decreased pigmentation.

On microscopic examination, spongiosis, severe loss of neurons, and gliosis were noted both in the superficial and deep layers of the motor cortex (Fig. 1A). The loss of Betz cells was obvious in the areas of the face, tongue,

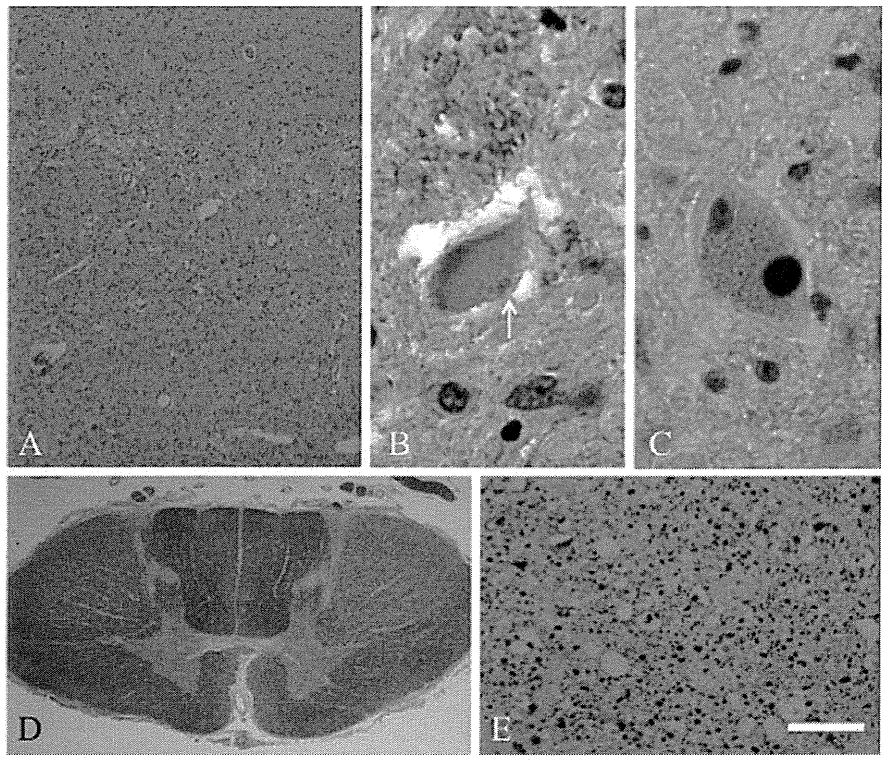

Fig. 1 Representative pathological findings of ALS. (A) Spongiosis, severe loss of neurons, gliosis, and hypoxic changes are observed in the motor cortex. (B) Bunina bodies in the nucleus ambiguus. (C) Intracytoplasmic inclusion showing immunoreactivity for TDP-43 in a neuron of the facial nucleus. (D) Myelin pallor is very mild in the lateral pyramidal tract at the level of the 4th cervical level. KB stain. (E) Large axons are decreased in the lateral pyramidal tract at the 4th cervical level. Immunohistochemistry for neurofilament. Scale bar: 150 μm (A), 15 μm (B,C), 1.4 mm (D), 30 μm (E).

upper limbs, body and in the lower limbs associated with abundant lipofuscin-laden microglial nodules. In the subcortical white matter, no myelin pallor was evident. In addition, marked hypoxic changes, such as loss of neurons, reactive astrogliosis, and capillary proliferation, were observed in the cortices. Severe neuronal loss and fibrillary gliosis were found in the facial nucleus, hypoglossal nucleus and nucleus ambiguus. The spinal cord at the cervical, thoracic and lumbar levels showed marked loss of motor neurons in the anterior horn. The lateral pyramidal tract showed faint myelin pallor (Fig. 1D), however; loss of large axons was demonstrated by immunohistochemistry for neurofilament (Fig. 1E). A few Bunina bodies were found in the nucleus ambiguus (Fig. 1B) and in the motor neurons of the lumbar (L3) spinal cord. There were round intracytoplasmic inclusion bodies immunoreactive for TAR DNA-binding protein, 43-kD (TDP-43) in the facial nucleus (Fig. 1C) and nucleus ambiguus, and round or skein inclusions were found in the motor neurons at the level of L3 and S2/S3. The distribution pattern of TDP-43 immunoreactive neuronal inclusions corresponded with the type-1 pattern reported by Nishihira *et al.*[9]

Severe loss of autonomic neurons in the nucleus intermediolateralis (IML) was found along the entire length of the thoracic cord (Fig. 2A). The number of neurons in the nucleus IML was counted from the Th1 to the Th12 levels. The average number was 3 ± 2.5/nuclear area, and an asymmetric decrease was found at the Th1 and Th2 levels. The number of large and small axons was moderately decreased in the ventral roots of the thoracic cord (Fig. 2B). A few neurons in the IML nucleus showed intracytoplasmic inclusions immunoreactive for alpha-synuclein (Fig. 2C,D).

Marked loss of pigmented neurons, intense gliosis and a few Lewy bodies were observed in the locus coeruleus. Marked hypoxic changes and loss of pigmented neurons, extracellular deposition of neuromelanin, gliosis and a few Lewy bodies were observed in the substantia nigra as well. Neurons with Lewy bodies and Lewy neurites were

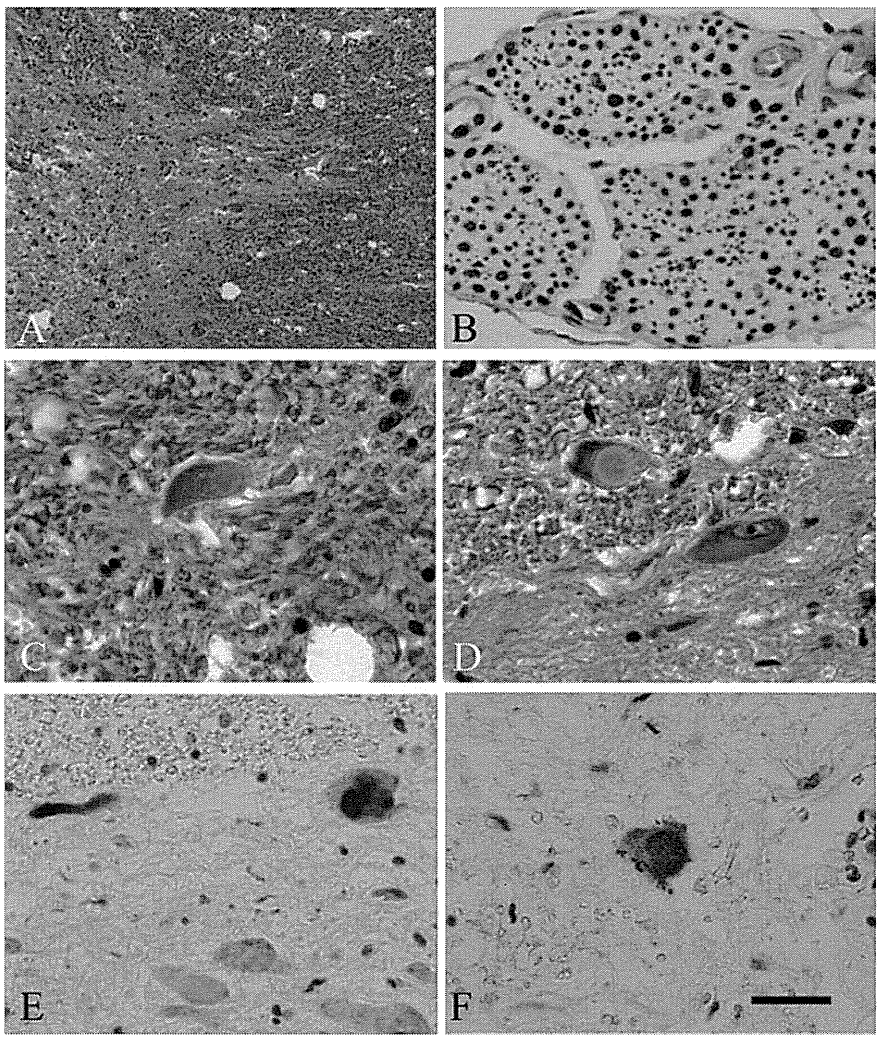

Fig. 2 Pathological findings of the intermediolateral (IML) nucleus in the thoracic cord. (A) Marked neuronal loss is found in the IML nucleus at the level of Th6. (B) Large and small axons are moderately decreased in the ventral root of Th3. (C,D) Lewy bodies are observed in the IML nucleus. (C: at the level of Th6, D: Th12). (E,F) An inclusion immunoreactive for alpha-synuclein is found in the IML nucleus. (E: at the level of Th3, F: Th6). Scale bar: 150 μm (A), 30 μm (B,C,D,E,F).

noted in the reticular formation of the brainstem and the dorsal nuclei of the vagal nerves (Braak stage 2–3). Among the visceral organs, a few alpha-synuclein-positive intracytoplasmic inclusions were found in the ganglion cells of the esophageal nerve plexus.

DISCUSSION

This patient showed slowly progressive upper and lower motor neuron signs and the main pathological findings showed loss of motor neurons corresponding to the clinical symptoms. According to both the clinical and neuropathological findings, the diagnosis of ALS was definitely established.

Sudden cardiac arrest is usually caused by cardiovascular disorders such as ischemic heart diseases, pulmonary embolism and cerebrovascular diseases. However, it also occurs in the neurodegenerative diseases, especially the ones with ANS involvement, including PD, MSA and spinocerebellar degeneration (SCD).

Shimizu *et al.* analyzed 23 respirator-dependent patients with ALS. Of these, six died from sudden cardiac arrest or anoxic encephalopathy following circulatory collapse. In eight cases, there were episodes of marked fluctuations of blood pressure before death. The authors speculated that an autonomic dysregulatory mechanism was a cause of sudden death in ALS.[2] However, OH was not described, and the value of blood catecholamines and plasma renin activity were generally high in their cases. Besides, in their autopsied cases (most of them demonstrated circulatory collapse), no neuropathological findings were found in anatomical structures related to ANS function, including the nuclei of IML. Although the detailed process remains unclear, it is indicated that some pathological mechanisms were different from those affected in the present case.

Merico and Cavinato assessed the autonomic function of 33 ALS patients and concluded that patients with prominent bulbar signs showed severe autonomic dysfunction under resting conditions.[8] Asai *et al.* studied 12 cases confirmed pathologically as sporadic ALS. All of them had not received artificial respiratory support; two of them died of sudden cardiac arrest. Both showed a clinically prolonged QTc interval on electrocardiogram and pathological loss of neurons in the IML.[10] They concluded that patients with ALS had reduced sympathetic activities in the terminal stage, resulting in an increased risk of cardiac arrest. Furthermore, in the review of autonomic impairment in ALS reported by Baltadzhieva *et al.*,[3] they suggested that autonomic disturbance may lead to circulatory collapse or sudden death.

Takahashi *et al.* analyzed 18 cases of sporadic ALS pathologically and proposed that IML neurons were primarily involved in the disease process in sporadic ALS. They also showed that there was a marked reduction of IML neurons in respirator-supported patients.[11] This characteristic pathological finding is demonstrated with progression in ALS and suggests that these neurons are vulnerable to the neurodegenerative process of this disease.[12]

Given the clinical and pathological features in these previous reports, it is suggested that a similar autonomic dysfunction affected the present patient's clinical course, although a prolonged QTc interval was not detected. Specifically, the findings that loss of IML neurons was discernible throughout the entire length of the thoracic cord may reflect autonomic dysfunction, especially sympathetic hypoactivity, which might have predisposed the patient to cardiac dysfunction. However, it is difficult to declare that the IML lesions definitely caused the cardiac arrest, because the causal relationship between them remains unclear. We consider that two distinct phenomena (i.e., the presence of IML lesions and cardiac arrest) are different manifestations of the progressive process of ALS complicated by autonomic dysfunction.

Other factors may be involved in the autonomic dysfunction of the present case. For example, diabetes mellitus might affect autonomic function to some extent, although obvious diabetic peripheral neuropathy was not detected by clinical or physiological examinations. In addition, a complication of other neurodegenerative diseases, such as PD, MSA or SCD, may have been involved in this case, since autonomic dysfunctions occur more frequently in them than in ALS.

As additional pathological findings, Lewy bodies and Lewy neurites were observed in the brainstem. Although this patient did not develop clinical symptoms of PD during his course, it was regarded as a subclinical PD, in other words, early and/or presymptomatic PD.[13]

It should be kept in mind that one of the representative symptoms of parkinsonism-like rigidity might be masked by the co-existence of spasticity due to motor neuron disease. In other words, if complicated symptoms involving different systems (i.e., motor system and extrapyramidal system) appear concurrently, it would be difficult to distinguish them from each other.[14]

From a pathological viewpoint, the present case showed ALS with subclinical PD. Similar autopsy cases have already been reported.[14–16] In these cases, the presence of PD was confirmed by the detection of Lewy bodies, even though extrapyramidal signs had not been detected during their clinical course.

Furthermore, as a quite unique finding in this case, a few Lewy bodies were also found even in the neurons of the IML nucleus in the thoracic cord. Approximately 5% to 15% of clinically normal, elderly people (older than 60 years of age) have Lewy bodies in brainstem nuclei,

and these cases have been designated incidental Lewy body disease (ILBD).[17–19] Because the affected brainstem nuclei in ILBD are the same as those in PD, ILBD is regarded as presymptomatic PD.[18] In most patients with PD, Lewy bodies are also found in the spinal cord, especially the IML nuclei.[18,19] The autonomic symptoms vary depending on the level of the spinal cord affected. When Lewy bodies appear at the lower sacral cord, this may be responsible for the mechanism of constipation in PD.[20] On the other hand, at the level of the thoracic cord, OH is frequently found.[21]

Referring to these reports, the alpha-synuclein pathology noted in this case is also consistent with early pathological alterations seen in PD. The morphological evidence of autonomic dysfunction (i.e., loss of IML neurons) or other findings (for example, ILBD) revealed by autopsy might have been related to the clinical course and outcome. If we encounter an autopsy case of ALS with sudden death, it is important to completely investigate ANS involvement from a wide clinicopathological perspective.

To summarize, it was noteworthy that ANS and extrapyramidal system involvement, in addition to motor neuron involvement, was pathologically confirmed all at once in the present case. This case may reflect a part of the widespread spectrum in ALS. Because many similar cases (ALS with PD or parkinsonism) have already been reported, it seems unreasonable to suggest that the presence of multisystem involvement in ALS is a mere coincidence. Baltadzhieva *et al.* suggested that ALS is a multisystem degenerative disease based on ANS involvement.[3] According to their suggestion, our presented case may also demonstrate an aspect of ALS as a multisystem disorder. Furthermore, from a neuropathological viewpoint by TDP-43, similar suggestions were made.[22,23] More studies are needed to elucidate the common and basic pathological mechanism to explain the process leading to such a variety of ALS. Among the literature regarding ALS (motor neuron disease, MND) with PD (parkinsonism), some reports suggested that MND might be a risk factor for parkinsonism.[24,25] Based on autopsied cases such as the present one, it is also important to consider a clinicopathological relationship between ALS and other neurodegenerative diseases represented by PD.

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