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ORIGINAL ARTICLE

Elevated CSF TDP-43 levels in amyotrophic lateral sclerosis: Specificity, sensitivity, and a possible prognostic value

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Abstract

TAR DNA binding protein of 43 kDa (TDP-43) is likely to be the major pathogenetic protein in amyotrophic lateral sclerosis (ALS). A previous study has shown that levels of TDP-43 in CSF measured by an ELISA are significantly higher for ALS patients than for controls. The aim of this study was to investigate whether elevated CSF TDP-43 levels are specific to ALS, and are associated with clinical profiles in ALS patients. We measured CSF TDP-43 levels by the same ELISA in 27 ALS patients and 50 neurodegenerative or inflammatory disease controls such as Parkinson's disease, multiple sclerosis, and Guillain-Barré syndrome.

Results showed that the CSF TDP-43 levels were increased only in ALS patients. Receiver operating characteristic (ROC) analyses showed a sensitivity of 59.3% and a specificity of 96.0%. We also found that lower CSF TDP-43 levels may be associated with shorter survival time. In conclusion, the CSF TDP-43 is a potential biomarker that supports a diagnosis of ALS. Moreover, among ALS patients, lower levels of CSF TDP-43 may reflect the accumulation of TDP-43 in the cortical and spinal motor neurons and thereby shorter survival time, although this should be confirmed in larger prospective studies.

Key words: *Amyotrophic lateral sclerosis, TAR DNA binding protein of 43 kDa (TDP-43), cerebrospinal fluid (CSF), ELISA*

Introduction

TAR DNA binding protein of 43 kDa (TDP-43) accumulation is the major component of ubiquitinated protein inclusions found in patients with amyotrophic lateral sclerosis (ALS), and frontotemporal lobar degeneration (FTLD) with TDP-43 positive ubiquitinated inclusions, recently relabelled the 'TDP-43 proteinopathies' (1,2). Therefore, there is increasing evidence that the accumulation of TDP-43 in the cytoplasm of motor neurons is the pathogenetic mechanism for development of ALS.

A previous report has shown that higher levels of TDP-43 were detected in cerebrospinal fluid (CSF) samples from patients with ALS than those from normal controls by an ELISA (3), but only a small number of patients with other neurological disease has been examined, and therefore that the specificity of elevated CSF TDP-43 levels for ALS patients remains unclear. The aim of this study was to investigate whether increases in CSF TDP-43 levels are specific to ALS and whether altered CSF TDP-43 levels are associated with prognosis of ALS patients.

Methods

The study included 27 patients with amyotrophic lateral sclerosis (ALS) seen at Chiba University Hospital between 2005 and 2009. Their condition fulfilled the revised El Escorial and Awaji criteria (4,5) for at least possible ALS with rapid progression

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consistent with ALS after CSF collection. The mean time interval from ALS onset to the CSF lumbar tap is 14 months (range 3–60 months). The initial symptoms were bulbar palsy in nine patients, and limb weakness in 18. We excluded ALS patients with obvious dementia (association of FTLD).

Fifty patients with other neurodegenerative or inflammatory diseases served as neurological controls, including 15 with Parkinson's disease, multiple system atrophy or progressive supranuclear palsy, 15 with multiple sclerosis or neuromyelitis optica and 20 with Guillain-Barré syndrome or Miller-Fisher syndrome. All patients consented to use of the study for research.

Fresh CSF samples were collected from living cases and then immediately frozen without centrifugation. Samples were stored at –80°C until used for the ELISA.

TDP-43 assay by an ELISA

TDP-43 in CSF was measured using a sandwich ELISA system, as previously described with small modifications to improve the signal-to-noise ratio by decreasing the non-specific binding (3). The major difference from the previous ELISA was a decrease in concentration of the secondary antibody, from 1:10,000 to 1:30,000. The ELISA plates (Nunc MaxiSorp, flat-bottom 96-well Black Micro Well plate, Roskilde, Denmark) were coated by overnight incubation at 4°C with 0.2 µg/ml anti-TDP-43 monoclonal antibody (H00023435-M01, clone 2E2-D3, Abnova Corporation, Walnut, USA), 100 µl/well, diluted in 200 mM $NaHCO_3$ buffer, pH 9.6. The plates were washed three times with PBST (0.01 *M* phosphate buffer, 0.0027 *M* potassium chloride and 0.137 *M* sodium chloride, pH 7.4, PBS containing 0.05% Tween 20), and incubated with 200 µl/well of blocking buffer (PBST containing 2.5% gelatin) for 2 h at 37°C. The plates were again washed three times with PBST, and 100 µl of the CSF samples to be tested was added to each well.

To eliminate inter-assay variability as a confounding factor, all CSF samples were run in duplicate with the same set of standards. After washing three times with PBST, the detection antibody, anti-TDP-43 rabbit polyclonal antibody (10782-2-AP, ProteinTech Group, Chicago, USA), 100 µl/well, diluted to 0.2 µg/ml in blocking buffer, was added and the plates were incubated at 37°C for 2 h. After washing three times with PBST, the plates were incubated with 100 µl/well of goat anti-rabbit secondary antibody coupled to horseradish peroxidase (HRP) (Dako Ltd., Denmark), diluted 1:30,000 in blocking buffer, at 37°C for 1 h. After washing six times with PBST, 100 µl/well of an enhanced chemiluminescent substrate (SuperSignal ELISA Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Rockford, USA) was finally added, and then chemiluminescence in relative light units was immediately measured at 395 nm with a microplate luminometer (SpectraMax L, Molecular Device, Tokyo, Japan). The relative concentration estimates of CSF TDP-43 were calculated according to each standard curve.

Statistical analyses

All statistical analyses were performed using STATA software (Stata Corp., Texas, USA). Data were shown as mean ±SD. Univariate comparison of the CSF TDP-43 levels and clinical variables used either the unpaired *t*-test or χ^2 test. Correlations were tested with Spearman's test. Multiple comparisons were tested with ANOVA and Bonferroni procedure. Receiver operating characteristic curve (ROC) analysis was used to determine the best cut-off values for the CSF TDP-43 level. Survival curves were estimated by the Kaplan-Meier method and differences in survival were measured by the log-rank test. Multivariate analyses of the risk for death associated with selected independent variables were performed using a Cox proportional hazard model.

Results

The results of quantification of CSF TDP-43 in ALS, and neurological controls are shown in Figure 1. The CSF TDP-43 level in ALS was 29.5 ± 15.5 ng/ml, which was significantly higher

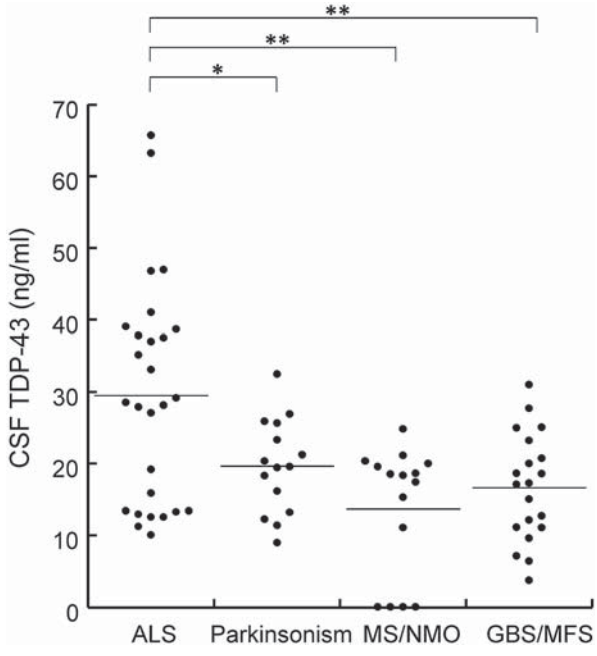

Figure 1. Dot plot of CSF TDP-43 (ng/ml) in the patient groups. Amyotrophic lateral sclerosis (ALS (n = 27)), Parkinsonism (Parkinson's disease, n = 10; multiple system atrophy, n = 3; progressive supranuclear palsy, n = 2); 15 with multiple sclerosis (MS) or neuromyelitis optica (NMO) and 20 with Guillain-Barré syndrome (GBS) or Miller-Fisher syndrome (MFS). Thick lines indicate the median value. Statistically significant difference was calculated using ANOVA, followed by Bonferroni procedure. $^{*}p < 0.05$. $^{**}p < 0.01$.

than that in Parkinsonism patients (19.7 ± 6.6 ng/ml; $p < 0.05$), multiple sclerosis/neuromyelitis optica patients (13.7 ± 9.0 ng/ml; $p < 0.01$) and Guillain-Barré/Miller-Fisher syndrome patients (16.7 ± 7.5 ng/ml; $p < 0.01$). No statistically significant differences were found in CSF TDP-43 levels among the disease control groups.

ROC analyses

The sensitivity and specificity of the CSF TDP-43 levels were determined by receiver operating characteristic curve (ROC) analysis. From the ROC curve, the optimal cut-off value was determined as 27.9 ng/ml. Using this value, analyses showed a sensitivity of 59.3% and a specificity of 96.0%.

Correlation with survival time

The median follow-up time was 10 months (range 1–46 months). During the follow-up period, 15 of the 27 patients died. We found no significant differences of survival rates in age (≥65 years ($n = 18$) versus <65 years ($n = 9$)), site of onset (bulbar onset ($n = 9$) versus upper or lower limbs onset ($n = 18$)), gender, modified Rankin Scale (mRS) at the time of collection of CSF (mRS ≥4 ($n = 6$) versus mRS ≤3 ($n = 21$)) and the duration of the disease from onset to the time of CSF collection (≥12 months ($n = 14$) versus <12 months ($n = 13$)). Survival time was significantly different by the CSF TDP-43 level. Kaplan-Meier curve showed that ALS patients with CSF TDP-43 ≥27.9 ng/ml ($n = 12$) survived longer than those with CSF TDP-43 <27.9 ng/ml ($n = 15$) from collection of CSF (log-rank, $p < 0.011$; Figure 2).

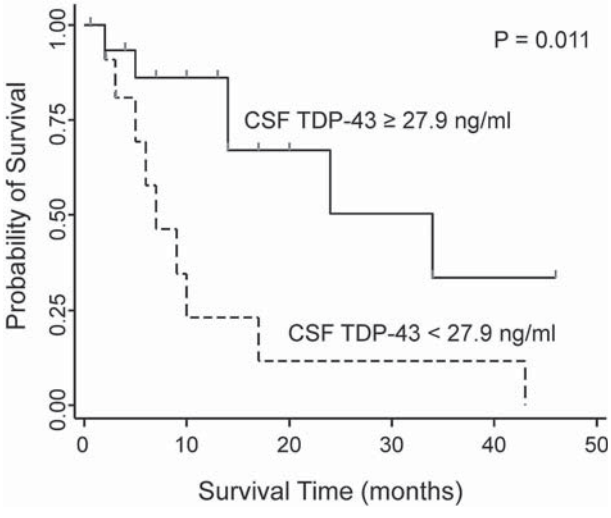

Figure 2. Kaplan-Meier survival curves in patient groups of amyotrophic lateral sclerosis according to the CSF TDP-43 levels. The cut-of value of 27.9 ng/ml was determined by receiver operating characteristic analysis. The patient group with lower CSF TDP-43 level had shorter survival time.

Multivariate analysis confirmed that the level of CSF TDP-43 <27.9 ng/ml was an independent prognostic factor in ALS patients; the hazard ratio was 3.49 (95% confidence intervals 1.1–11.1; $p = 0.034$) compared to patients with the level of CSF TDP-43 <27.9 ng/ml. Age, site of onset, gender, mRS and disease duration were not significantly related to survival time (Table I).

Discussion

This study shows that CSF TDP-43 levels are significantly increased in patients with ALS compared with the disease controls as a group, and the increase appears to be specifically seen in ALS. Using ROC analyses, the sensitivity was 59.3% and specificity 96.0%. Moreover, among ALS patients those with lower CSF TDP-43 levels might be associated with shorter survival time.

A previous study reported that ALS patients had significantly higher levels of CSF TDP-43 than the age-matched controls. The controls were 13 healthy subjects and 16 with various neurological disorders including epilepsy, cerebellar ataxia, benign positional vertigo, myelopathy, cervical spondylosis, cranial and peripheral neuropathy and myopathy. Our results showed that the CSF TDP-43 levels do not increase in other neurodegenerative and inflammatory disorders, and confirmed that the increases are specifically found for ALS patients. Our study lacked normal controls, and this is a limitation of the present study. However, we believe that the significant difference in CSF TDP-43 levels from neurological controls could suggest specific increases in CSF TDP-43 concentration in ALS.

Recently, Steinacker et al. found that using an immunoblot assay, patients with ALS and frontotemporal lobar degeneration (FTLD) had significantly higher CSF TDP-43 levels than controls, and that CSF TDP-43 level in patients with ALS plus FTLD was not statistically different from those in the ALS, FTLD and control groups (6). We did not include patients with FTLD, but our data on ALS are consistent with the study by Steinacker et al. Although the functions of TDP-43 have not yet been sufficiently understood, TDP-43 is a highly

Table I. Hazard factors for covariates affecting survival.

Variables	Hazard ratio (95% CI)	*p*-value
Age >65 years	3.165 (0.645–15.543)	0.156
Female gender	1.344 (0.338–5.355)	0.674
Bulbar onset	1.278 (0.274–5.948)	0.755
Duration of the disease <12 months	2.255 (0.487–10.448)	0.298
modified Rankin scale ≥4	0.943 (0.177–5.025)	0.946
CSF TDP-43 <27.9 ng/ml*	3.490 (1.097–11.103)	0.034

*The cut-off value was defined by the receiver operating characteristic curve.

conserved protein ubiquitously expressed in many tissues including the CNS where it is present in neuronal and glial nuclei and to lesser extent in the cytoplasm (7). The question of the increased level of CSF TDP-43 in ALS patients remains unclear. A previous study suggested that CSF TDP-43 levels might be increased in the early stages of ALS, reflecting ongoing neuronal destruction. However, our results could not confirm this finding, and showed no significant correlation between CSF TDP-43 levels and duration of the disease from onset to collection of CSF. The reasons for the discrepancy are unclear and, to solve this question, a study with larger numbers or a longitudinal study testing the same ALS patients will be required.

Our findings also showed that among ALS patients, the lower level of CSF TDP-43 may be an independent poor prognostic factor although the results of our prognostic analysis should be carefully interpreted because of its small scale. Recently, Sussmuth et al. reported that CSF concentrations of astroglial S100beta and microglial sCD14 correlated with survival time in patients with ALS (8). They proposed that the concentration of S100beta was related to astrocyte activation, which contributes directly to motor neuron destruction in ALS. However, the question regarding the reduced levels of TDP-43 in ALS patients with shorter survival remains unanswered. We speculate that reduced CSF TDP-43 levels might be related to the pathological finding that TDP-43 accumulation in the cytoplasm of motor neurons is associated with extensive TDP-43 lesions and a high level of insoluble TDP-43 in these cells. Further studies involving a larger number of patients will be required to confirm the relationship of CSF TDP-43 levels with disease progression.

Recent studies have suggested that CSF and serum antibodies against neurofilaments and CSF cystatin C could be candidate biomarkers for differential diagnostic use in ALS or the clinical course of the disease (9,10). The study also showed that the quantification of CSF TDP-43 also may be useful for the diagnosis of ALS and as a predictor of prognosis. Future studies are required to elucidate the values of these possible biomarkers.

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