Diagnostic and prognostic significance of plasma and CSF NfL, TDP-43, and tau in ALS

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Key words: 1) Amyotrophic lateral sclerosis, 2) biomarker, 3) TDP-43, 4) neurofilament light chain, 5) Simoa.

List of Disclosure

Drs. Kasai, Kojima, Ohmichi, Tatebe, Tsuji, Noto, Kitani-Morii, Shinomoto, Allsop, Mizuno, and Tokuda report no disclosures relevant to the study.

Abstract

Objective

To determine the diagnostic and prognostic significance of neurofilament light chain (NfL), TAR DNA-binding protein 43 (TDP-43), and total tau (t-tau) in cerebrospinal fluid (CSF) and plasma of patients with amyotrophic lateral sclerosis (ALS).

Methods

This was a single-center, prospective, longitudinal study. CSF and plasma samples were collected at the time of enrollment from a discovery cohort of 29 patients with ALS and 29 age-matched controls without neurodegenerative disease. In a validation cohort, there were 46 patients with ALS, and 46 control (not age-matched) patients with motor weakness resulting from neuromuscular diseases. NfL, TDP-43, and t-tau levels in CSF and plasma were measured using ultrasensitive single molecule assay (Simoa) technology.

Results

The following findings were reproducibly observed among the discovery and validation cohorts: increased levels of CSF NfL, plasma NfL, and CSF TDP-43 in ALS compared with control groups; shorter survival associated with higher levels of CSF and plasma NfL. When the CSF NfL and CSF TDP-43 levels were combined, the areas under the ROC curves (AUC) were slightly improved relative to AUCs for each biomarker alone.

Conclusion

CSF and plasma NfL may not only serve as diagnostic biomarkers but also provide a measure of disease progression. CSF TDP-43 is also useful as a diagnostic biomarker of ALS, but has no prognostic value. The combined use of CSF NfL and CSF TDP-43 may

be a useful biomarker for the diagnosis of ALS.

Key words: Amyotrophic lateral sclerosis, biomarker, TDP-43, neurofilament light chain, Simoa.

Introduction

There is an urgent need for molecular biomarkers in biofluids for the diagnosis of amyotrophic lateral sclerosis (ALS) [1]. At present, the most promising biomarker for ALS is neurofilament light chain (NfL). Elevated levels of NfL in CSF and blood plasma/serum have been reported in patients with ALS compared with controls; moreover, they were associated with poor outcomes [2-3]. TAR DNA-binding protein 43 (TDP-43) positive inclusions are found in approximately 97% of patients with ALS. This has led to the investigation of TDP-43 as a potential molecular biomarker for ALS. Overall, these studies have identified increased levels of TDP-43 in CSF from ALS patients compared with controls [4]. An elevated level of TDP-43 has also been reported in plasma from ALS patients in one case-control study [5]. However, the absolute concentrations of TDP-43 in CSF and plasma have varied across studies, suggesting that TDP-43 immunoassays are inconsistent for measuring this protein within biofluids [4]. The other candidate is Tau. Recent studies reporting elevated levels of CSF total-Tau (t-tau) in ALS patients compared with controls have generated novel interest in the diagnostic potential of t-tau for ALS [6, 7]. However, there are conflicting results [8, 9] and the prognostic significance of plasma t-tau in ALS has so far received little attention. Considering the lack of comprehensive analysis of these three biomarkers for ALS, we conducted the present study to determine the diagnostic and prognostic potential of TDP-43 and t-tau as molecular biomarkers, compared with NfL not only in CSF but also in blood plasma.

Methods

Study design, ethical approvals, and subject recruitment

All study subjects provided written informed consent before participation and the study protocols were approved by the University Ethics Committee (ERB-G-12, Kyoto Prefectural University of Medicine, Kyoto, Japan). Informed consent from patients was obtained when possible and also from the nearest relative. Study procedures were designed and performed in accordance with the Declaration of Helsinki. The discovery cohort consisted of 29 individuals with possible, probable, or definite ALS diagnosed according to the revised El Escorial criteria (the ALS group of the discovery cohort) [10] and 29 age-matched controls (the control group of the discovery cohort). All patients with possible ALS when their CSF and plasma were measured, were confirmed to show conversion to probable or definite ALS within the follow-up period. The control group participants had non-neurodegenerative diseases and presented with no neurological symptoms. They were enrolled from the registration for neurodegenerative and dementia disorders in Kyoto Prefectural University of Medicine (KPUM) from September 2009 to March 2014. All participants of the discovery cohort underwent CSF and plasma collection. The sample size of the discovery cohort was set according to the effect size of previous biomarker studies [11 12]. The validation cohort comprised 46 individuals with suspected, possible, probable, or definite ALS diagnosed with the same criteria as for the discovery cohort (the ALS group of the discovery cohort) and 46 patients with motor weakness resulting from neuromuscular diseases (the control group of the validation cohort), comprising: chronic inflammatory demyelinating polyneuropathy (CIDP: N=17), Gullain-Barre syndrome (GBS: N=18), multifocal

motor neuropathy (MMN: N=6), and inclusion body myositis (IBM: N=5). As described above, suspected and possible ALS patients were confirmed to show conversion to probable or definite ALS within the follow-up period. They were enrolled from KPUM from April 2014 to May 2018. The sample size of the discovery cohort was set based on the data from discovery cohort. Of note, not all participants in the validation cohort provided both blood and CSF samples. Because relatively young individuals were included in the control group, the ALS and control groups were not age-matched in the validation. All measurements of the biomarkers were done on a Simoa HD-1 Analyzer (Quanterix, Lexington, MA, USA) by commercially available kits. TDP43 kit used in the study was developed with antibodies against the amino acid residues between 203 – 209 and the C-terminal region and therefore mainly target the C-terminal part of the protein. For detailed information about plasma and CSF sampling, measurements of the biomarkers as well as statistical analyses see supplementary methods.

Bias

Our data are from patients who agreed to participate in this study and agreed to receive plasma collection or lumbar puncture for the diagnosis of ALS or other disorders.

Data availability statement

Any anonymized data not published in the article will be shared upon request from any qualified investigator.

Results

Patient characteristics.

The demographic characteristics of the discovery and validation cohorts are summarized in Table 1 (for clinical information and raw data on biomarker concentrations, see Supplementary Tables 1 and 2). There was no significant difference in age (P=1.000) or sex (P=0.7840) between the ALS and control groups in the discovery cohort. In the validation cohort, the median age of the control group was significantly younger than that of the ALS group (P<0.0001), while there was no significant difference in sex between the two groups (P=0.3696).

Table 1

Category	Specific diagnosis	N	Sex(M:F)	Age
The discovery cohort				
ALS		29	18:11	65.41±12.34
Control (non-neurodegenerative control)	See Supplementary Table 1B	29	19:10	66.40±9.2
	Difference between the groups:		P=1.000	P=0.7840
The validation cohort				
ALS		46	29:17	71.36±9.27
Control (patients with motor weakness from neuromuscular diseases)		46	34:12	69.83 ±20.18
	Difference between the groups:		P=0.3696	P<0.0001
	CIDP	17	14:3	60.06±14.45
	GBS	18	11:7	50.67±23.80
	MMN	6	5:1	48.50±21.03
	IBM	5	4:1	76.00±2.45

GBS: Gullain-Barre syndrome, MFS: Millar-Fisher syndrome, CIDP: chronic inflammatory demyelinating polyneuropathy, MMN: multifocal motor neuropathy

Concentrations of biomarkers in the discovery cohort.

The concentrations of TDP-43, NfL, and t-tau in the samples from the discovery cohort are summarized in Figure 1. In the case of TDP-43, both plasma (P=0.0035, Figure 1A) and CSF levels (P<0.0001, Figure 1B) of this marker were elevated in the ALS group compared with the control group. This was also the case for NfL with increased levels found in both plasma (P=0.0299, Figure 1C) and CSF (P<0.0001, Figure 1D) from the ALS group compared with the control group. Finally, t-tau levels were significantly lower in the ALS group only in plasma (P=0.0178, Figure 1E), and not in CSF (P=0.1062, Figure 1F).

ROC analysis of biomarkers in the discovery cohort. (for data, see Supplementary Figure 1)

According to ROC analysis of the discovery cohort, CSF NfL generated the highest area under the curve (AUC) value (AUC=0.8347, Supplementary Figure 1D). The second highest AUC value was observed with CSF TDP-43 (AUC=0.8205, Supplementary Figure 1B).

Correlation between levels of biomarkers in CSF and plasma in the discovery cohort. (for data, see Supplementary Figure 2)

There was a significant positive correlation between NfL levels of plasma and CSF taken from each patient with ALS in the discovery cohort (solid line, P<0.0001,). Such a significant CSF-plasma correlation was also identified in the control group (dashed line, P=0.0013) (Supplementary Figure 2B). Neither TDP-43 nor t-tau levels showed any plasma-CSF correlation in either of the groups (TDP-43 in the ALS group: P=0.2279, TDP-43 in the control group: P=0.9252, t-tau in the ALS group: P=0.1024, t-tau in the control group: P=0.3463) (Supplementary Figure 2A and C).

Biomarkers and survival times in the discovery cohort.

All members of the ALS group in the discovery cohort were included in log-rank analysis (Figure 2). Nineteen patients reached the endpoint of death, tracheostomy, or invasive ventilation during the follow-up period. Survival times ranged from 17 to 2,793 days (median: 575 days) (Supplementary Table 1B). Patients with ALS were subdivided into two groups according to the levels for each of the biomarkers: a low-level group (< median value), and a high-level group (≥ median value). When comparing the high and low level groups, significant differences were noted in plasma NfL (P=0.0248, Figure 2C), CSF NfL (P=0.0207, Figure 2D), and CSF t-tau (P=0.0124, Figure 2F), while there is no significant difference in plasma TDP-43, CSF TDP-43, or plasma t-tau (Figure 2A, B, E). The high-level groups were associated with shorter survival compared with the low-level groups, for plasma NFL, CSF NfL, and CSF t-tau. After age-adjustment in multivariate analysis, the high levels of plasma and CSF NfL still retained significant prognostic value (plasma NfL, Hazard ratio (HR) = 6.800, P=0.003; CSF NfL, HR=7.727, P=0.002), while the association between CSF t-tau and survival did not reach significance (CSF t-tau, HR=2.875, P=0.065).

Concentrations of biomarkers in the validation cohort.

The concentrations of TDP-43, NfL, and t-tau in the validation cohort are summarized in Figure 3. On comparing ALS and control groups, significant elevations of biomarker concentrations in the ALS group were reproduced for CSF TDP-43 (P=0.087, Figure 3B), plasma NfL (P=0.0031, Figure 3C), and CSF NfL (P<0.0001, Figure 3D), while neither plasma TDP-43 nor plasma t-tau levels were different between the groups, in contrast to those in the discovery cohort. CSF t-tau levels in the ALS group were significantly higher than those in the control group in the validation

cohort, although such a difference was not observed in the discovery cohort. Those significant differences were reproducibly confirmed by multiple comparison with the Kruskal-Wallis test among the ALS group and subgroups of the controls (CIDP, GBS, MMN, and IBM). Post-hoc analysis of Dunn's multiple comparison tests revealed significantly higher levels of CSF TDP-43 in the ALS group compared with those in the CIDP subgroup, CSF NfL in the ALS group compared with those in the CIDP and GBS subgroups, and CSF t-tau in the ALS group compared with those in the CIDP subgroup. Considering the age difference between the ALS and control groups, we reanalyzed those comparisons after the exclusion of individuals younger than 60 years old (Supplementary Figure 3). There was no significant difference in age between the ALS (n=42) and control (n=24) groups, consisting of individuals aged no younger than 60. In these advanced age groups, comparisons between groups regarding biomarkers showing significant differences between the groups based on raw data (CSF TDP-43, CSF NfL, plasma NfL, and CSF t-tau) were conducted. Significant elevation of CSF TDP-43 and CSF NfL and plasma NfL levels in the ALS group compared with those in controls was preserved (P=0.004 in Supplementary Figure 3A, P=0002 in Supplementary Figure 3B, and P=0.0156 in Supplementary Figure 3C, respectively), while the difference between the groups regarding CSF t-tau did not reach significance (Supplementary Fig. 3D).

Biomarkers and survival times in the validation cohort.

Not all patients with ALS in the validation cohort were included in the log-rank analysis due to missing samples. We performed survival analysis involving 20 ALS patients with plasma biomarker data and 41 ALS patients with CSF biomarker data (Figure 4). In those patients, 10 patients in plasma biomarker analysis and 18 patients in CSF biomarker analysis reached the endpoint. Survival times ranged from 28 to 1,592

days (median: 305 days) (Supplementary Table 2B). The high-level group showed significantly shorter survival compared with the low-level group for plasma NfL (P=0.0178, Figure 4C) and CSF NfL (P=0.0284, Figure 4D), corresponding with the results in the discovery cohort. However, the significant difference in CSF t-tau was not reproduced (Figure 4F). After age-adjustment, the high levels of plasma and CSF NfL still exhibited significant prognostic values (HR=12.262, p=0.041 and HR=4.83, P=0.01, respectively).

ROC analysis of composite biomarkers in discovery and validation cohorts regarding CSF TDP-43, CSF NfL and plasma NfL

Regarding the CSF TDP-43, CSF NfL, and plasma NfL that showed significant elevation in the ALS compared with control groups for both discovery and validation cohorts, we calculated composite parameters of the products of CSF NfL x CSF TDP-43, of CSF NfL x plasma NfL, and of plasma NfL x CSF TDP-43 (Figure 5). In both cohorts, the composition of CSF NfL and CSF TDP-43 provided better performance in terms of the AUC value compared to those in each biomarker alone (AUC=0.8430 and 0.9493 in the discovery and validation cohorts, respectively, whereas the discriminability in the product of CSF NfL x plasma NfL was inferior to that in the CSF NfL alone in the discovery cohort. The AUC value for composition of plasma NfL and CSF TDP-43 (0.6813) could not exceed that in CSF TDP-43 alone. The combined analyses for the CSF and plasma biomarkers in the validation were not performed because more than half of participants of the validation cohort did not underwent both plasma and CSF collection.

Combined analysis of validation and discovery cohorts regarding plasma TDP-43, CSF TDP-43, plasma t-tau, and CSF t-tau

Regarding the levels of plasma TDP-43, plasma t-tau, and CSF t-tau, for which inconsistent differences were found between ALS patients and controls when comparing the two cohorts, we conducted a combined analysis based on data from internal controls. Levels of plasma TDP-43 in the combined ALS group were higher than those in the combined control group (P=0.0137). Levels of plasma t-tau were not different between these groups (P=0.228), while CSF t-tau was significantly elevated in the combined ALS group compared with the combined control group (P=0.0006) (Figure 6). We also recalculated survival analyses in the combined ALS group for the biomarkers. Both plasma and CSF NfL levels were associated with shorter survival (P=0.0002 and P=0.0193, respectively). Those significances were still preserved after age-adjustment (HR=7.611, P<0.001 and HR=4.567, P<0.001, respectively). Meanwhile, there was no significant difference in survival between the high- and low-level groups based on TDP-43 and t-tau levels in plasma and CSF (Figure 7).

Discussion

Biomarker profiles of TDP-43, NfL, and t-tau in ALS have been comprehensively investigated [4]. However, most previous studies have focused on one or two of these biomarkers. Moreover, the diagnostic or prognostic value of plasma TDP-43 or plasma t-tau in ALS has remained uncertain because of the difficulty of stable measurement. To the best of our knowledge, this study is the first to comprehensively measure levels of all of these three candidate biomarkers, not only in CSF but also, simultaneously, in plasma. The current study showed the following three major findings that were consistent across the discovery and validation cohorts.

First, CSF NfL was significantly elevated in the ALS compared with control groups. Furthermore, the potential prognostic value of elevated levels of CSF NfL, in terms of shorter survival time, was observed after stratifying cohorts according to the median CSF NfL levels. These confirm findings gathered in retrospective case-control studies and prospective observations [2 3 13-18]. On the other hand, the AUC value used to discriminate between ALS patients and controls in our study (0.8347) was slightly lower than in a previous meta-analysis: 0.90; 95% confidence interval, 0.87–0.92 [18]. We consider that this difference may be associated with the research design, control-group choice, and ethnic differences.

Second, plasma NfL was significantly higher in the ALS group than in the controls, and higher plasma NfL was associated with a shorter survival. Those results are in agreement with observations in previous case-control studies using serum [17 19 20] and plasma [14]. Overall, these findings support the possibility that NfL not only in CSF but also plasma, can serve as a promising biomarker for the diagnosis and monitoring of disease progression of ALS. The fact that CSF and plasma NfL shared the same biomarker profile is reasonable when we consider the correlation between them in each participant of the discovery cohort. Such plasma-CSF correlation in NfL has been observed not only in patients with ALS [20] but also in patients with Alzheimer's disease, multiple sclerosis, and control individuals [21 22]. The plasma-CSF correlation in our controls was slightly irregular; actually, the association in the controls did not fit a linear correlation, in contrast to that in the ALS group. This inconsistency may be due to heterogeneity caused by the use of disease controls in this study.

Third, we noted significantly higher levels of TDP-43 in CSF of ALS patients than those in controls. This result is consistent with previous observations, including two of

our studies and one meta-analysis [11 12 23-26]. TDP-43 is considered to be a disease-specific biomarker reflecting TDP-43 pathology in the central nervous system As expected, the AUC values, representing the ability to discriminate between ALS patients and controls, were improved by combining CSF NfL with CSF TDP-43 relative to that in each biomarker alone. This observation was consistently found across the both cohort, suggesting that CSF TDP-43 could serve as a biomarker complementary to NfL in the diagnosis of ALS. CSF NfL was recently reported to have a diagnostic potential even for presymptomatic ALS [19]. However, at present, no one can predict which kind of neurodegeneration will develop in individuals with elevated CSF NfL levels due to its lack of disease specificity [27]. The combined use of CSF NfL and CSF TDP-43 may be recommended for such people suspected to have neurodegeneration with undetermined pathology. This biomarker-combination could also facilitate enrollments of clinical trials toward preemptive therapy for ALS. ~~Of note here, there is controversy regarding the validity of the hypothesis that elevation of CSF TDP-43 is specifically caused by TDP-43 proteinopathy. Immunoblotting shows that the identification of TDP-43 in biofluids by the commonly applied antibody combinations used for quantification represent a 45-kDa full-length form of TDP-43, rather than disease-specific truncated forms [23 28]. Therefore, no evidence has been reported to date that the elevation of CSF TDP-43 detected by our method results from TDP-43 pathology. Taking these facts into consideration, it is possible that increased CSF TDP-43 in ALS might simply be a consequence of neuronal cell damage, similar to NfL. To develop a more disease-specific biomarker in the future, measurements of C-terminal truncated or phosphorylated forms of TDP-43, if possible extracted from neuron-derived exosomes, would be ideal candidates.~~

Levels of plasma TDP-43, plasma t-tau, and CSF t-tau were significantly different between the ALS and control groups in both the discovery and validation cohorts, although the results were not preserved across these cohorts. In the combined analysis, the significant elevation of plasma TDP-43 and CSF t-tau in the ALS group was repeatedly observed, whereas the significant difference in plasma t-tau between the groups was not reproduced. The significant elevation of plasma TDP-43 in the ALS group agrees with one case-control study [5]. The previous measurement of plasma TDP-43 based on conventional immunoassay had the problem of low sensitivity, and actually failed to accurately quantify more than 70% of samples due to signals being lower than the detection limit [5]. In contrast, we could detect measurable signals from the whole plasma samples. This advantage may be due to the SIMOA analyzer, with 100- to 1,000-fold higher sensitivity than conventional assays [29]. This result provides evidence supporting the potential diagnostic value of plasma TDP-43 for ALS as well as usefulness of such new digital analytical platforms for the development of blood-based biomarkers of the disease.

No difference in CSF levels of t-tau were found in the discovery cohort, while levels of this biomarker were significantly elevated in the ALS group compared with the controls in the validation cohort, and on combined analysis. Previous studies have yielded similar inconsistent results regarding CSF levels of t-tau in ALS patients, which ranged between normal [16, 8, 9, 30, 31] and increased levels [6 7, 32]. This inconsistency might be linked to the inherent variability of the disease; for example, variability in release of tau from motor neurons during disease progression. Thus, differences in the disease stage and disease progression rate of enrolled patients may have contributed to the variable findings of CSF t-tau. On the other hand, levels of plasma t-tau were

significantly lower in the ALS than control group in the discovery cohort, but this result was not reproduced in the validation cohort or in the combined study. There is one published case control study on plasma t-tau in patients with FTD and controls, in which levels of plasma t-tau were not different between patients with pathogenic mutations causing TDP-43 proteinopathy (i.e., mutation of *GRN* or *C9orf72*) but they were significantly elevated in patients with *MAPT* mutations compared with controls. Our results on plasma t-tau agree with this report in that plasma t-tau levels were not different between patients with TDP-43 proteinopathy and controls.

In survival analysis all of the biomarkers except for plasma and CSF NfL failed to exhibit any prognostic value, consistently across the discovery and validation cohorts. We previously reported that lower CSF TDP-43 levels were correlated with shorter survival [12]. However, the current study did not reproduce the results in the discovery and validation cohorts, or on combined analysis. ~~This discrepancy may be due to the confounder that levels of CSF TDP-43 vary depending on the stage of ALS [11].~~ A recent study argued that higher levels of CSF t-tau are associated with shorter survival [6]. This result was consistent with that in our discovery cohort, but was not reproduced in either the validation cohort or on combined analysis. This inconsistency may have been caused by the shortness of the follow-up period in the validation cohort, which was around half of that in the previous study. Longer observation would be needed to validate the usefulness of CSF t-tau as a prognostic biomarker.

We acknowledge that the relatively small sample size was a major limitation of the study. Furthermore, as mentioned above, the short follow-up period may have weakened the statistical power to detect an association between survival and the biomarkers. In the future, case-control as well as longitudinal studies involving sufficient numbers of

Commented [K1]: I have erased the sentence because stage depending difference of CSF TDP43 was not found in my analysis, although this finding was observed in our first report).

participants with a longer follow-up period will be necessary to confirm our findings and promote the clinical application of biomarker-supported diagnosis and progression monitoring of ALS.

Conclusions

This is the first study comprehensively analyzed the three candidate biomarkers for ALS in CSF and plasma. NfL levels in CSF and plasma were significantly elevated in the ALS patients compared with controls. Moreover, higher levels of those markers were associated with shorter survival. Both may serve as not only diagnostic biomarkers but also measures of disease progression. TDP-43 levels in CSF, which were increased in the ALS patients compared with controls but were not associated with survival periods, may only be useful as a diagnostic biomarker. The discrimination ability between ALS and control was improved by the combined use of CSF TDP-43 and CSF NfL, therefore CSF TDP-43 could serve as a biomarker complementary to NfL in the diagnosis of ALS. Plasma TDP-43 and CSF t-tau may be elevated in ALS patients and, therefore, be of diagnostic value; however, the present results still need future validation in a larger cohort.

Author Contributions

T. O. and Y.K assisted with patient enrollment, data analysis, and interpretation. H.T., F.K-M., and M.S. performed laboratory work and data analysis. Y.T. and Y.N. contributed to data collection. D.A. and T.M. participated in review and revision of the manuscript. T.K. and T.T were involved with conceptualization and design of the study,

patient enrollment, data collection, interpretation of the data, and review of the manuscript. All authors reviewed the drafts and approved the final version of the manuscript.

Competing interests and funding

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Figure 1

Scatter plots of biomarkers levels in the discovery cohort.

ALS (n=29) and control (n=29). Levels of plasma and CSF TDP-43 (A, B), NfL (B, C), t-tau (D, E) are presented. Bars indicate median values. The P-value generated by Mann-Whitney's U test is shown above each graph. n.s: not significant.

Figure 2

Kaplan-Meier survival curves in ALS patients of the discovery cohort according to biomarkers levels.

(A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma t-tau, (F): CSF t-tau. The squares and circles indicate an event (death, tracheostomy, or invasive ventilation). Patients were subdivided into two groups according to the cut-off biomarker levels. The cut-off value in each graph was set as the median value of the corresponding biomarker within the ALS group. The red lines with red squares represent patients with levels of biomarkers no lower than the cut-off (the high-level group). The black lines with black circles represent those with levels lower than the cut-off (the low-level group).

Figure 3

Scatter plots of biomarkers levels in the validation cohort.

Control (n=46) and ALS (n=46). Levels of plasma and CSF TDP-43 (A, B), NfL (B, C), t-tau (D, E) are presented. Bars indicate median values. The P-value generated by Mann-Whitney's U test between the ALS and whole control group is shown above each graph. Significant differences were reproducibly confirmed by multiple comparison

421 tests with the Kruskal-Wallis test among the ALS group and subgroups of the controls
422 (CIDP, GBS, MMN, and IBM). Dashed bars and asterisks indicate significant
423 differences ($P<0.05$) between the groups by post-hoc analysis of Dunn's multiple
424 comparison procedure. n.s: not significant.
425
426 **Figure 4**
427 Kaplan-Meier survival curves in ALS patients of the validation cohort according to
428 biomarkers levels.
429 (A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma t-
430 tau, (F): CSF t-tau. Patients were subdivided into two groups according to the cut-off
431 biomarker levels. The cut-off value in each graph was set as the median value of the
432 corresponding biomarker within the ALS group. The squares and circles indicate an
433 event (death, tracheostomy, or invasive ventilation). The red lines with red squares
434 represent patients with levels of biomarkers no lower than the cut-off (the high-level
435 group). The black lines with black circles represent those with levels lower than the cut-
436 off (the low-level group).
437
438 **Figure 5**
439 ROC analyses for the composite parameters of the discovery and validation cohorts.
440 AUC values are indicated in the graphs. The title of each graph represents the biomarker
441 used as an independent variable on analysis: (A): the products of CSF NfL and CSF
442 TDP-43 in the discovery cohort; the red and blue dotted lines respectively indicate the
443 ROC curves of CSF NfL alone and CSF TDP 43 alone for reference (see Supplementary
444 Figure 1 and 3 regarding the ROC analyses of each biomarker for details). (B): the

products of plasma NfL and CSF NfL in the discovery cohort; the red and blue dotted lines respectively indicate the ROC curves of CSF NfL alone and plasma NfL alone. (C): the products of plasma NfL and CSF TDP-43 in the discovery cohorts; the red and blue dotted lines respectively indicate the ROC curves of CSF TDP-43 alone and plasma NfL alone. (D): the products of CSF NfL and CSF TDP-43 in the validation cohort; the red and blue dotted lines respectively indicate the ROC curves of CSF NfL alone and TDP-43 alone.

Figure 6

Scatter plots of biomarkers levels in combined analysis of the discovery and validation cohorts.

Analyses of plasma biomarkers involved 49 ALS patients and 47 controls; CSF biomarker analyses involved 71 ALS patients and 68 controls. Levels of plasma and CSF TDP-43 (A, B), NfL (B, C), t-tau (D, E) are presented. Because of inter-assay variation, we corrected the values of the validation cohort based on the correction formula: raw values x correction factors. The correction factors were determined as the mean value ratios between the discovery and validation assays based on four internal controls for each biomarker. Bars indicate median values. The P-value generated by Mann-Whitney's U test between the ALS and whole control groups is presented above each graph. n.s: not significant.

Figure 7

Kaplan-Meier survival curves in ALS patients on combined analysis of the discovery and validation cohorts.

Correction of interassay variation was conducted using the formula presented in Figure 5. (A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma t-tau, (F): CSF t-tau. Patients were subdivided into two groups according to the cut-off biomarker levels. The cut-off value in each graph was set as the median value of the corresponding biomarker within the ALS group. The squares and circles indicate an event (death, tracheostomy, or invasive ventilation). The red lines with red squares represent patients with levels of biomarkers no lower than the cut-off (the high-level group). The black lines with black circles represent those with levels lower than the cut-off (the low-level group).

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Supplementary Data

Supplementary Table 1

Clinical information and concentrations of biomarkers in the control and ALS groups of the discovery cohort.

Supplementary Table 2

Clinical information and concentrations of biomarkers in the control and ALS groups of the validation cohort.

Supplementary Figure 1

ROC analyses of the discovery cohort.

Supplementary Figure 2

Scatter plots of levels of TDP-43, NfL, and t-tau in plasma and CSF.

Supplementary Figure 3

Scatter plots of biomarkers levels in individuals aged no younger than 60 in the validation cohort.