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TDP-43 levels are higher in platelets from patients with sporadic amyotrophic lateral sclerosis than in healthy controls

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ABSTRACT

TAR DNA-binding protein 43 (TDP-43) is a major pathological protein of ubiquitinated inclusions in motor neurons of sporadic amyotrophic lateral sclerosis (ALS). TDP-43 is ubiquitously expressed and the majority of TDP-43 is normally localized to the nucleus. In motor neurons of patients with ALS, TDP-43 is not localized in the nucleus, relocates to the cytoplasm, and accumulates as cytoplasmic inclusions. Based on recent reports that TDP-43 is increased in the cytoplasmic fraction of peripheral blood mononuclear cells in sporadic ALS, and several studies on platelet dysfunction in ALS patients, we investigated the TDP-43 levels in platelets from patients with sporadic ALS. We measured TDP-43 levels with a sandwich enzyme-linked immunosorbent assay in platelets separated from whole blood, and compared the TDP-43 level in platelets from sporadic ALS (n = 19) patients with platelets from non-ALS controls (n = 21). The TDP-43 concentration in platelets was significantly higher in patients with ALS compared to age-matched controls. According to sub-analysis, the TDP-43 concentration in platelets tended to increase in ALS patients with longer disease duration, as well as with lower score on the ALS Functional Rating Scale Revised (ALSFRS-R), though the differences were not statistically significant. These results suggest that ALS also affects platelets in addition to motor neurons.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive and selective loss of upper and lower motor neurons in the brain and spinal cord. Patients suffer from paralysis of voluntary muscles including respiratory muscles, leading to respiratory failure and death 3–5 years after the disease onset. The majority of ALS cases are sporadic and have unknown etiology. The study of ALS has evolved since TAR DNA-binding protein 43 (TDP-43) was identified as a constituent molecule in aggregates in the brain in frontotemporal lobar degeneration (FTLD) in 2006 (Arai et al., 2006; Neumann et al., 2006).

TDP-43 accumulates in the cytoplasm and forms aggregates in motor neurons of patients with sporadic ALS and those with familial ALS who express the mutated causative genes except for *SOD1* or *FUS*. Mutation of *TARDBP* (the gene encoding TDP-43) also causes ALS, and thus, functional abnormalities of TDP-43 cause ALS (Lattante et al., 2013). Therefore, TDP-43 plays a critical role in ALS pathogenesis.

TDP-43 is an RNA binding protein that is mainly expressed in the nucleus and is ubiquitously expressed in all tissues. In motor neurons of patients with ALS, TDP-43 is not found in the nucleus and forms aggregations in the cytoplasm. Whether the cause of ALS is due to the loss of function or the gain of a toxic function of TDP-43 has not been determined.

In ALS, pathological changes also occur in tissues other than motor neurons. De Marco et al. reported that TDP-43 is increased in the cytoplasmic fraction and decreased in the nuclear fraction of peripheral blood mononuclear cells from patients with sporadic ALS (De Marco et al., 2011). This report suggested that TDP-43 mislocalization occurs not only in motor neurons, but also in other cells.

Some studies on platelet dysfunction in ALS patients have been conducted. In particular, mitochondrial dysfunction by impairment of complex IV activities (Ehinger et al., 2015), disturbances in glutamate reuptake (Bos et al., 2006), decreases in the serotonin concentration (Dupuis et al., 2010), and microstructural abnormalities in platelets (Shrivastava et al., 2011) have been reported.

Therefore, we hypothesized that the TDP-43 abnormality is present in platelets from sporadic ALS patients and investigated the amount of TDP-43 in platelets. This analysis does not require cumbersome fractionation of organelles (i.e., cytoplasm and nucleus), which often has

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variation in accuracy. Furthermore, we also investigated whether TDP-43 in platelets of ALS patients may serve as a diagnostic biomarker.

2. Subjects and methods

2.1. Subject samples

Blood samples were obtained from 19 sporadic ALS patients and 21 non-ALS controls. Subjects were recruited at Kyoto University Hospital between January 2015 and October 2016. ALS patients were diagnosed by neurologists as possible, probable, or definite ALS according to the revised El Escorial and Awaji criteria (Costa et al., 2012). We included only sporadic ALS patients without a family history of the disease. We recruited non-ALS controls who had no neurodegenerative, infectious, or immunological diseases (Supplemental Tables 1 and, 2). Neither the ALS patients nor the non-ALS controls had dementia. We conducted genetic screening for *TARDBP* and *SOD1,* and found no mutations in any subjects. Written informed consent to participate in the study, which was approved by the ethics committee of Kyoto University Hospital (approval number: G609), was obtained from all subjects.

2.2. Platelets and preparation of lysates

A total of approximately 20 ml whole blood was drawn into EDTA tubes (VENOJectII, TERUMO, Japan) via venous puncture from each subject. We overlaid an equal volume of blood on a 1.063 barrier density reagent, which was prepared by mixing diluent (0.85% NaCl, 20 mM HEPES-NaOH, 1 mM EDTA, pH 7.4) and Optiprep (AXIS-SHIELD, UK) at a ratio of 5:22. Samples were centrifuged at 300 × *g* for 15 min at room temperature to yield a platelet-containing band. This platelet-containing fraction was carefully collected into a new centrifuge tube, mixed with saline solution, and centrifuged at 2000 × *g* for 20 min at room temperature to produce a platelet pellet. The platelet pellet was examined for purity with a microscope, and we confirmed that contamination with other blood cells (i.e., white blood cells and red blood cells) was below 0.1%. The pellet was resuspended in RIPA buffer (sc24948, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and stored at −80 °C.

2.3. Immunoblotting

Aliquots of 5 μg total protein in a lysate volume of 15 μl were loaded into wells and fractionated with 12.5% sodium dodecyl sulfate polyacrylamide denaturing gel electrophoresis (SDS-PAGE) gel. Resolved proteins were transferred to a polyvinylidene fluoride membrane. Then, the membrane was blocked with 3% bovine serum albumin in Tris-buffered saline with Tween 20 (TBS-T) and incubated overnight at 4 °C with rabbit anti-TDP-43 antibody at dilution of 1:5000 (#10782-2A, Proteintech, Rosemont, IL, USA). The membrane was washed with TBS-T buffer, incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and visualized with detection reagent (Pierce ECL western blotting substrate, Thermo Fisher Scientific, Rockford, IL, USA).

2.4. TDP-43 measurement with an enzyme-linked immunosorbent assay (ELISA)

The protein concentration of platelet lysates was quantified with the BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific). The concentration of TDP-43 in platelet lysates was measured using a sandwich ELISA kit (Human TDP-43 ELISA kit, Cat. KE00005, Proteintech). All assays were performed in duplicate according to the manufacturer's instructions. In brief, aliquots of 20 μg total protein in 100 μl were loaded into a 96-well plate. After incubation for 1 h at 37 °C, the plate was washed four times with wash buffer. Then, 100 μl detection antibody solution was loaded into each well and incubated for 1 h at 37 °C. The plate was washed four times with wash buffer, and 100 μl horseradish peroxidase-conjugated antibody was subsequently loaded into each well and incubated for 40 min at 37 °C. Then, 100 μl TMB substrate solution was loaded into each well and incubated for 10 min at room temperature in a dark room, followed by addition of 100 μl stop solution. Optical density was measured at a wavelength of 450 nm with a plate reader (Multiskan JX, Thermo Fisher Scientific).

2.5. Statistical analysis

Statistical analyses were conducted with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All data were expressed as the mean ± standard error. Statistical significance was assessed with an unpaired *t*-test.

3. Results

3.1. TDP-43 was expressed in platelets as seen with immunoblotting

Platelets are denucleated cells, and therefore, first we examined lysates of platelets from representative samples to determine whether platelets contain TDP-43. A schematic for collecting the platelets for lysates is shown in Fig. 1A. We performed immunoblotting for TDP-43 with these lysates and confirmed that TDP-43 was detected in platelets from both controls and ALS patients. Moreover, no aggregated or fragmented TDP-43 was seen, indicating that the properties of TDP-43 in platelets in ALS patients differed from those reported in motor neurons (Fig. 1B).

3.2. TDP-43 levels in platelets were increased in ALS patients

Next, we compared TDP-43 levels in platelets from ALS patients and non-ALS controls. Characteristics of the subjects are shown in Table 1 and Supplemental Tables 1 and 2 A standard curve for the ELISA is shown in Fig. 1C. The TDP-43 concentration in platelets was significantly higher in ALS patients (1.178 ± 0.184 ng/ml, n = 19) compared to non-ALS controls (0.993 ± 0.242 ng/ml, n = 21) ($p < 0.05$, Fig. 1D). The difference in the mean value between the two groups was almost 20%, although these values cannot be used as a clear disease marker due to overlap. We also confirmed that TDP-43 was increased specifically, as the levels of the internal control protein, actin, were not significantly different (data not shown).

TDP-43 levels in platelets tended to be higher in ALS patients with longer disease duration or severer symptoms.

We analyzed the data according to the duration or the severity of the disease. The median value of disease duration in all 19 ALS patients in our study was 29 months; therefore, the ALS patients were divided into two groups; within 2.5 years of disease onset and 2.5 years or more after disease onset. The TDP-43 levels in platelets tended to be higher in ALS patients with longer disease duration, though the difference was not statistically significant (< 2.5 years, 1.109 ± 0.116 ng/ml; ≥2.5 years, 1.253 ± 0.221 ng/ml; $p = 0.089$) (Fig. 1E). All patients at the time of blood sampling were alive; therefore, 'disease duration' in our study means the period of disease-onset up to the point of blood sampling.

The Amyotrophic Lateral Sclerosis Functional Rating Scale Revised (ALSFRS-R) is an instrument for evaluating the functional status of ALS patients. The total score is 48, and a lower score indicates severer symptoms. TDP-43 levels tended to be higher in ALS patients with a lower ALSFRS-R score, though the difference was not statistically significant (≤35, 1.237 ± 0.211 ng/ml; ≥36, 1.095 ± 0.084 ng/ml; $p = 0.098$) (Fig. 1F).

In addition, anti-platelet drugs, which act on platelets, did not influence the TDP-43 concentration in platelets in non-ALS controls (Fig. 1G), nor did riluzole and edaravone for treatment of ALS (data not

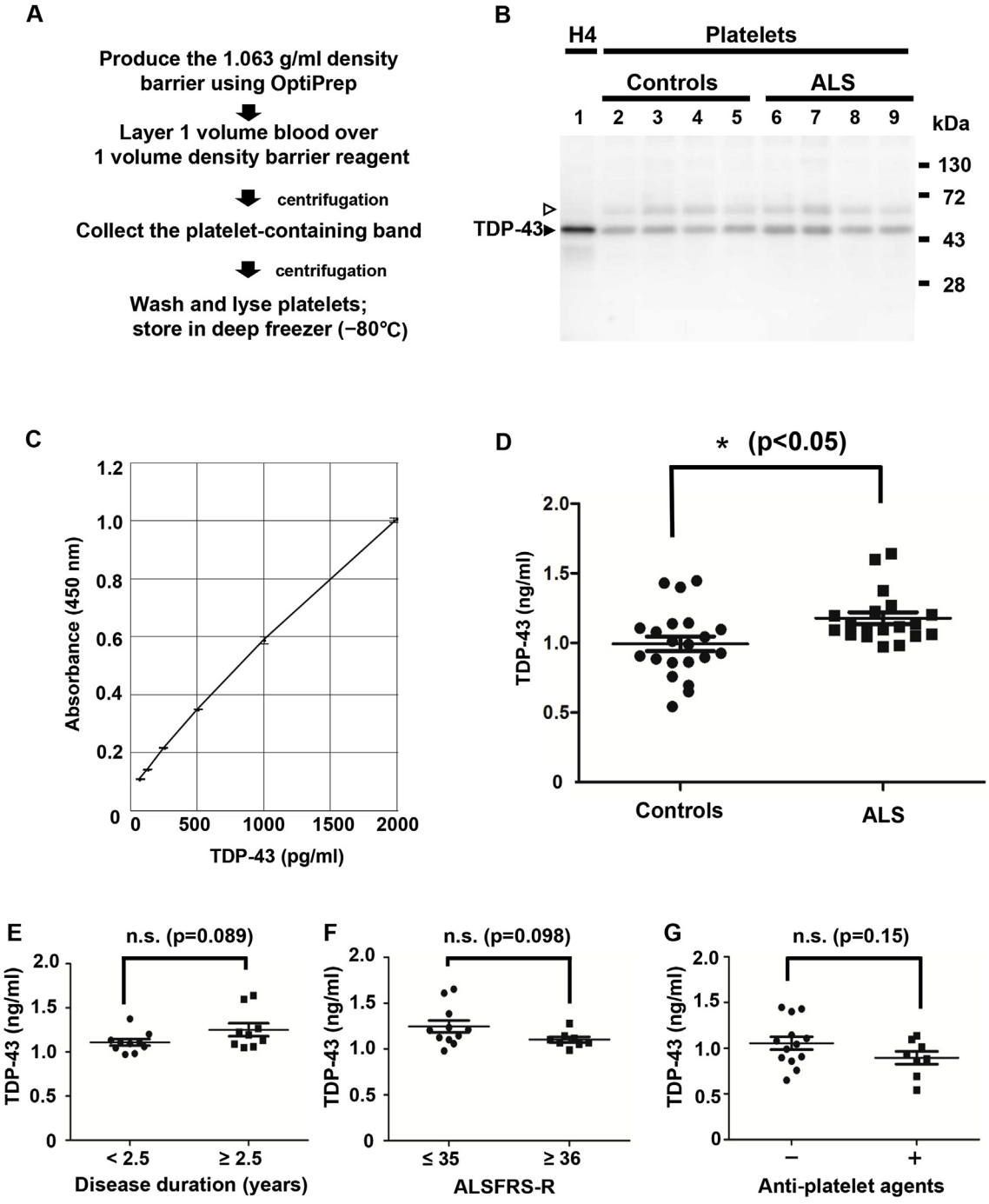

Fig. 1. Preparation of platelets, western blot for TDP-43, and TDP-43 measurement with ELISA.

A. Schematic for collecting the platelets for lysates.

B. Western blot analysis for TDP-43 in platelets.

Lane 1 is a lysate from H4 cells (human neuronal glioma cell line), lanes 2 to 5 are platelet lysates from representative non-ALS controls and lanes 6 to 9 are those from representative ALS patients. Aliquots of 5 μg total protein in a lysate were loaded in each lane. The filled arrowhead indicates TDP-43 at the predicted molecular weight. The empty arrowhead shows the non-specific band.

C. Standard curve for the TDP-43 concentration with ELISA.

D. Scatter plots of concentrations of TDP-43 in the platelets as determined with ELISA. The mean values and standard errors of the concentrations of each group are shown. The concentration of TDP-43 in the ALS group (n = 19) was significantly higher than that in the non-ALS control group (n = 21) ($P < 0.05$).

E. Scatter plots of concentrations of TDP-43 in the platelets from subjects measured using ELISA for sub-analysis in terms of disease duration. The ALS patients were divided into two groups; within 2.5 years after disease onset (n = 10), and 2.5 years or more after disease onset (n = 9). n.s., *t*-test not significant

F. Scatter plots of concentrations of TDP-43 in the platelets from subjects measured using ELISA for sub-analysis in terms of severity. The ALS patients were divided into two groups according to the ALSFRS-R score ≤35 (n = 11) and score ≥36 (n = 8). ALSFRS-R; ALS functional rating scale revised. n.s., *t*-test not significant

G. Scatter plots of concentrations of TDP-43 in the platelets from subjects measured using ELISA for sub-analysis in terms of taking anti-platelet drugs (n = 8), or not (n = 13), in non-ALS controls.n.s., *t*-test not significant.

Table 1
Demographics of the subjects.

	Controls	ALS patients	P value
Patients (N)	21	19	
Age at blood sampling (years; mean ± SD)	65.29 ± 7.37	66.42 ± 7.34	0.629
Sex (M/F)	12/9	10/9	
Anti-platelet agents (±)	11/10		–

Abbreviations: ALS, amyotrophic lateral sclerosis; SD, standard deviation; M, male; F, female.

shown).

4. Discussion

ALS is a neurodegenerative disease that selectively impairs motor neurons; however, the precise mechanism of the disease process has not been elucidated. The study of ALS advanced rapidly after TDP-43 was reported to be the major constituent protein in ubiquitin-positive, tau-negative inclusions in the cytoplasm of motor neurons of sporadic ALS and FTLD in 2006. In physiological conditions, TDP-43 exists mainly in the nuclear compartment of the cell. In disease conditions, elevated levels of TDP-43 protein have been reported in plasma from patients with FTLD (Foulds et al., 2008) and cerebrospinal fluid from patients with ALS and FTLD (Steinacker et al., 2008). Furthermore, mislocalization of TDP-43 protein in peripheral blood mononuclear cells (De Marco et al., 2011), and increased expression of TDP-43 in skin cells (Suzuki et al., 2010) have been observed in patients with ALS. Therefore, pathological changes in tissues other than motor neurons have been detected in ALS, although their significance requires careful interpretation.

A previous study demonstrated mislocalization of TDP-43 in peripheral blood mononuclear cells; however, the procedure for organelle fractionation is rather complicated. Therefore, we focused on platelets in which pathological changes in ALS have been reported. Platelets are fragments of cytoplasm that are derived from megakaryocytes in bone marrow and have no nucleus; fractionation of platelets for quantifying cytoplasmic proteins is unnecessary. Moreover, platelets have intracellular organelles such as mitochondria, and they synthesize and metabolize proteins (Weyrich et al., 2009).

First, we demonstrated with western blotting that TDP-43 is expressed in platelets. This is not counterintuitive since TDP-43 has cytosolic functions, such as mRNA stabilization, trafficking, and translation, and has been found to be present in mitochondria and endoplasmic reticulum, even though the majority of TDP-43 resides in the nucleus under physiological conditions (Gao et al., 2018). In fact, TDP-43 can be measured in platelet lysates from healthy subjects (Wilhite et al., 2017).

Second, we showed that the concentration of TDP-43 in platelets was significantly higher in ALS patients compared to non-ALS controls. We have two hypotheses to explain these results. One hypothesis is that a specific genetic background or environmental factor that causes ALS affects megakaryocytes, and TDP-43 subsequently increases in the cytoplasm of megakaryocytes before platelets are produced. Actually, levels of cytosolic TDP-43 from peripheral blood mononuclear cells of patients with ALS have been shown to be increased (De Marco et al., 2011). The other hypothesis is that after platelets are produced, they incorporate the TDP-43 circulating in the plasma or at the blood brain barrier (BBB). Platelets are reported to open the BBB via the release of platelet-activating factors (Fang et al., 2014). Intriguingly, increased TDP-43 from platelets in Alzheimer's disease has been reported as preliminary results (Wilhite et al., 2017).

The concentration of TDP-43 in platelets alone did not serve as a disease marker in ALS because we did not identify a clear cut-off value to separate ALS from control groups. A combination of TDP-43 and another protein may serve as a disease marker, similar to the amyloid β42/40 ratio in Alzheimer's disease.

Although not statistically significant, TDP-43 levels in platelets in ALS patients tended to be higher in ALS patients with longer disease duration or severer symptoms. While this might reflect the disease process in ALS, further study is needed to elucidate the mechanism.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval and informed consent

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the ethics committee of Kyoto University Hospital and the national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.12.009.

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