Results

EA-mediated oxidative stress induces TDP-43 phosphorylation in NSC34 cells

To investigate the effect of oxidative stress on endogenous TDP-43, NSC34 cells were incubated for 12 h with EA, which is able to increase cellular oxidative stress through depletion of glutathione, (Keelan et al., 2001; Rizzardini et al., 2003). Immunoblots showed abnormal TDP-43-immunoreactive bands at 45 kDa, which suggests hyperphosphorylation of TDP-43, at EA concentration greater than 50 μM EA (Fig. 1A). The bands were immunopositive for phospho-TDP-43-specific (pTDP-43) antibodies at serine 403/404 and serine 409/410 (S403/404 and S409/410), that are seen in TDP-43 proteinopathies as pathological phosphorylation (Hasegawa et al., 2008) (Fig. 1A). In addition, phosphorylation of these TDP-43 sites was prevented by co-treatment with 2 mM NAC, a precursor of glutathione. Quantification of CM-H2DCFDA oxidation, a measure of ROS formation, showed that ROS productions was increased by EA treatment in a dose-dependent manner and was prevented by NAC (Fig. 1B). Since TDP-43 phosphorylation at S403/404 and S409/410 is exerted by CK1 and CK2 (Hasegawa et al., 2008), the effect of treatment with these inhibitors in combination with EA was examined. Both inhibitors prevented serine phosphorylation of TDP-43 in a dose-dependent manner, although CK1 inhibitor was more effective than CK2 inhibitor (Fig. 1C).

EA induces TDP-43 insolubilization and C-terminal fragmentation

To investigate the effect of oxidative stress on endogenous TDP-43 solubility, cells treated with 70 μM EA were extracted sequentially. In the immunoblots, the amount of TDP-43 in TS and TX fractions were significantly decreased, but the amount in Sar and SDS fractions were increased in a time-dependent manner (Fig. 2A). These phenomena were prevented in the presence of 2 mM NAC. Phosphorylated TDP-43 was increased in Sar fractions in a time-dependent manner and was detectable in SDS fractions 5 h after EA induction (Fig. 2A). In addition, long exposure of immunoblots with anti-TDP-43 antibody demonstrated that ~25 kDa C-terminal fragment (CTF) of TDP-43 in Sar and SDS fractions appeared evidently by EA induction, and the amount of TDP-43 CTF in SDS fraction was significantly increased at 5 h after EA induction compared with control (Fig. 2A, B).

EA induces cytoplasmic distribution of TDP-43

Immunocytochemistry showed that endogenous TDP-43 disappeared from the nucleus, translocated to the cytoplasm, and became phosphorylated at least in some population of NSC34 cells treated with 70 μM EA for 5 h, whereas this protein was localized in the nucleus and was not phosphorylated in untreated cells (Fig. 3A). Although the majority of cytoplasmic TDP-43 was diffusely distributed under EA treatment, it was also localized in stress granules (SGs), which were labeled with TIAR (Fig. 3A). The time lapse analysis of NSC43 cells expressing GFP-WT-TDP-43 demonstrated cytoplasmic distribution of TDP-43 in the majority of the cells treated with 70 μM EA, but TDP-43 consistently localized in the nucleus of cells co-treated with 2 mM NAC (Fig. 3B, C).

H_2O_2 induces C-terminal phosphorylation, C-terminal fragmentation, insolubilization, and cytoplasmic distribution of TDP-43

To confirm that the TDP-43 modifications are not induced by the specific toxicity of EA, we investigated the effects of H_2O_2, another

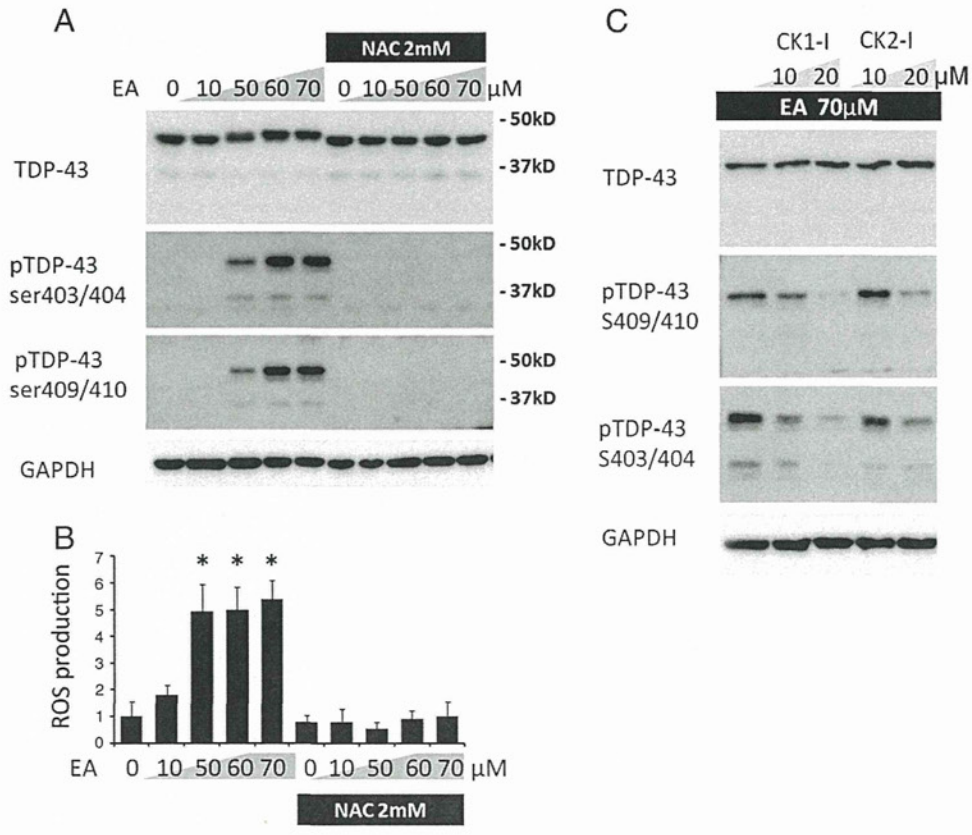

Fig. 1. TDP-43 phosphorylation induced by EA. (A) Immunoblots of NSC34 cells. EA induced TDP-43 C-terminal phosphorylation at S403/404 and S409/410 in a dose-dependent manner. The phosphorylation was prevented by 2 mM NAC. (B) Quantification of ROS by CM-H2DCFDA oxidative assay. The values relative to those of controls are shown. ROS production was increased by EA induction and suppressed by 2 mM NAC. Asterisk denotes significant difference from control ($p<0.0001$, $n=6$). Error bars indicate SD. (C) Immunoblots of NSC34 cells treated with 70 μM of EA. Casein kinase 1 and 2 inhibitors (CK1-I and CK2-I) both prevented the phosphorylation of TDP-43 in a dose-dependent manner.

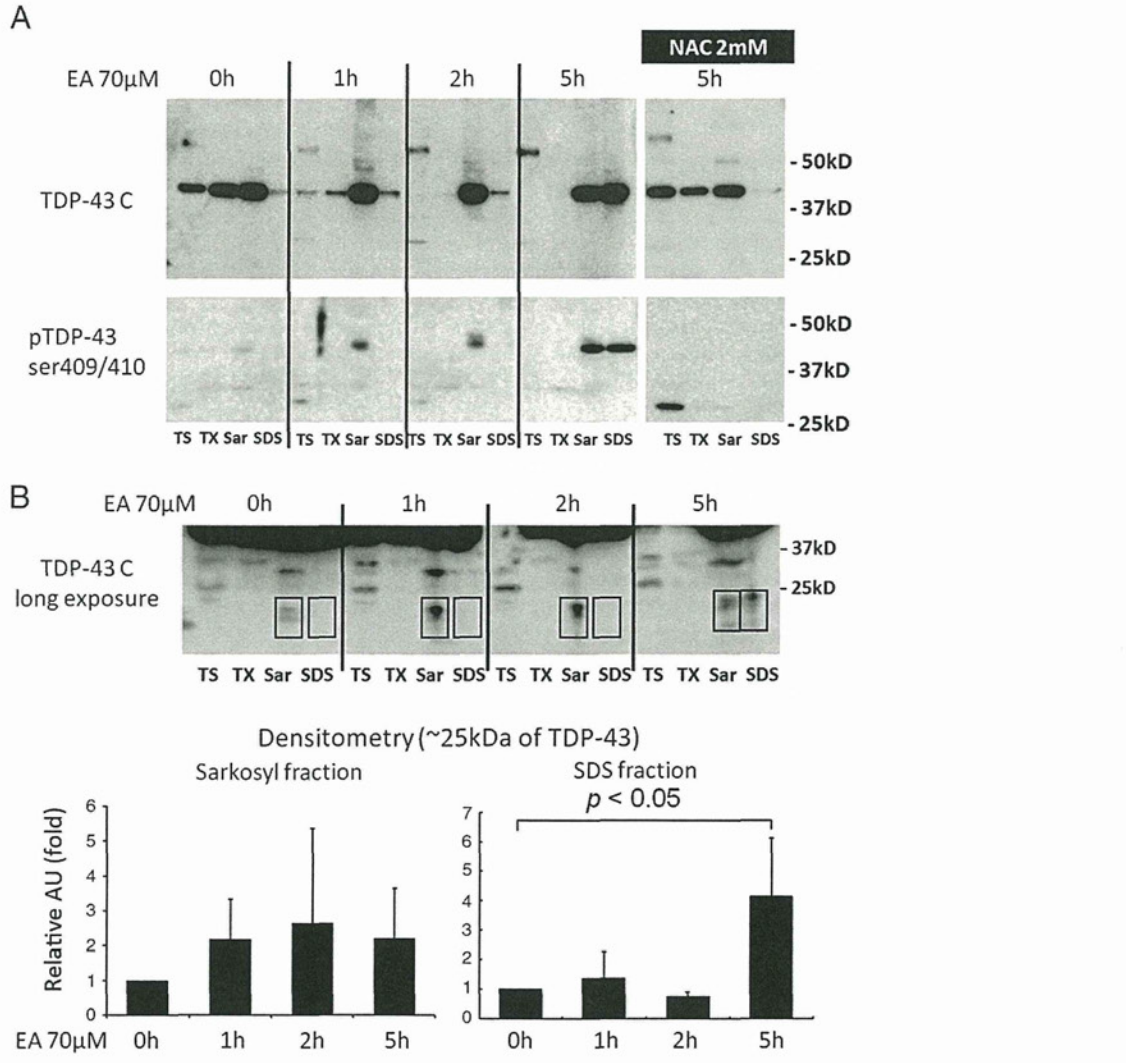

Fig. 2. Analysis of TDP-43 solubility under EA treatment. (A) Sequential extraction analysis using Tris (TS), Triton X100 (TX), Sarkosyl (Sar), and SDS buffers. The amount of TDP-43 in TS and TX fractions was decreased by 70 μM EA in a time-dependent manner, while the amount of TDP-43 in Sar and SDS fractions was increased by the treatment. These phenomena were prevented by 2 mM NAC. Phosphorylated TDP-43 (S409/410) was increased in Sar and SDS fractions in a time-dependent manner. (B) Densitometric quantitation of TDP-43C-terminal fragment (CTF). The relative intensities to controls are shown in arbitrary units (AU). Long exposure of immunoblots with anti-TDP-43 antibody (405–414) (TDP-43C) showed ~25 kDa C-terminal fragment (CTF) in Sar and SDS fractions. The amount of TDP-43 CTF was significantly increased in the SDS fraction at 5 h after EA induction (n = 3). Error bars indicate SD.

inducer of oxidative stress, on the modifications of TDP-43. Immunoblots of NSC34 cells showed that 10 mM H_2O_2 induced C-terminal phosphorylation and C-terminal fragmentation of TDP-43 (Fig. S4A). In the sequential extraction analysis of NSC34 cells, the amount of TDP-43 in TS and TX fractions was decreased by 10 mM H_2O_2, while that of TDP-43 in SDS fraction was increased by the treatment (Fig. S4B). The time lapse analysis of NSC34 cells expressing GFP-WT-TDP-43 showed that 10 mM H_2O_2 induced cytoplasmic distribution of TDP-43 (Fig. S4C).

EA induces C-terminal phosphorylation and cytoplasmic distribution of TDP-43 in primary cortical neurons

To investigate the effect of oxidative stress in neurons, 5-day in vivo (5 DIV) mouse primary cortical neurons were treated with EA for 5 h. Immunoblots showed that EA induced TDP-43 phosphorylation at S403/404 and S409/410 in a dose-dependent manner, and 2 mM NAC prevented the phosphorylation (Fig. 4A). In the time lapse analysis of neurons expressing GFP-WT-TDP-43, TDP-43 was distributed in the cytoplasm in the presence of 30 μM EA (Fig. 4B).

C-terminal phosphorylation of TDP-43 is not mandatory for its insolubilization or cytoplasmic distribution under EA

Since C-terminal phosphorylation of TDP-43 was accompanied by insolubilization and distribution to the cytoplasm in response to oxidative stress, we investigated the effect of C-terminal phosphorylation of TDP-43 using a nonphosphorylable TDP-43 (SA-TDP-43) mutant which contains serine to alanine substitutions at 403/404 and 409/410 (Fig. 5A). We used N-terminal tagged TDP-43, since C-terminal tagged TDP-43 was not detected by anti-pTDP-43 antibody in the immunoblots even under conditions of oxidative stress sufficient to phosphorylate endogenous TDP-43 (Fig. S1). As was seen with WT-TDP-43 under normal conditions, GFP-tagged and V5-tagged SA-TDP-43 were located in the nucleus (Fig. S2). In the immunoblots, endogenous and GFP-WT-TDP-43 were phosphorylated in the presence of 70 μM EA, but GFP-SA-TDP-43 was not phosphorylated even at an EA concentration of 70 μM (Fig. 5B). The time lapse analysis of NSC34 cells demonstrated that GFP-SA-TDP-43 translocated to the cytoplasm (Fig. 6A). The proportion of the cells with cytoplasmic distribution of TDP-43 under oxidative stress was not

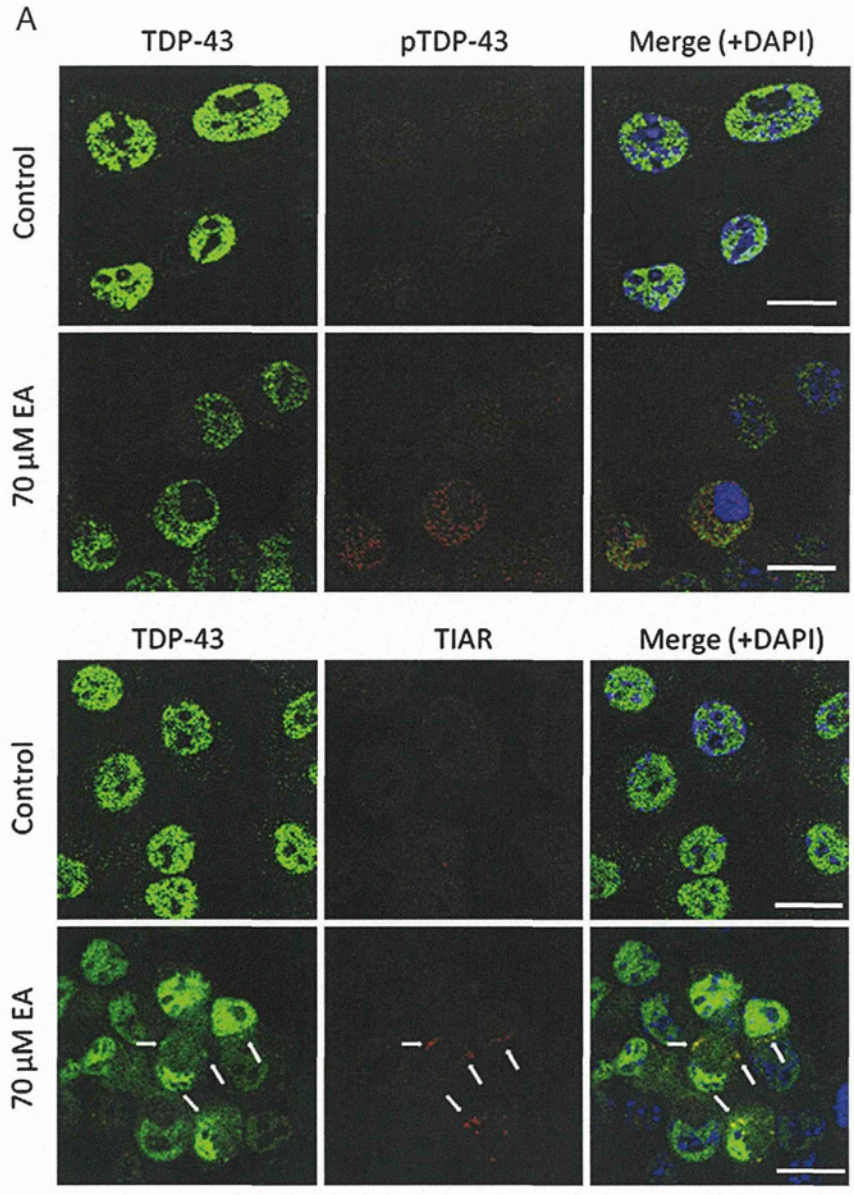

Fig. 3. Cytoplasmic distribution of TDP-43 induced by EA. (A) Immunocytochemistry of NSC34 cells. Cells were stained with anti-TDP-43 antibody (green), anti-phospho-specific TDP-43 (pTDP-43) (S409/410) or anti-TIAR antibody (red), and DAPI (blue). EA treatment (70 μM, 5 h) induced translocation of TDP-43 from the nucleus to the cytoplasm in NSC34 cells. Cytoplasmic TDP-43 was immunopositive for pTDP-43 antibody. In the control cells TDP-43 localized in the nucleus without phosphorylation. TDP-43 co-localized with stress granule marker, TIAR under EA treatment, although the majority of cytoplasmic TDP-43 was diffusely distributed. Arrows indicate stress granules. Scale bars represent 10 μm. (B) Time lapse analysis of NSC43 cells expressing GFP-WT-TDP-43. GFP and phase contrast images showed that TDP-43 was distributed to the cytoplasm when exposed to 70 μM EA, but this distribution was prevented by 2 mM of NAC. (C) The proportion of cells with cytoplasmic distribution of TDP-43 (cells with cyto-TDP) in the GFP-TDP-43 expressing cells 0 h or 5 h after EA induction without or with NAC treatment. Three areas per sample were measured. Error bars indicate SD.

different between WT- and SA-TDP-43 (Fig. 6B). Sequential extraction of NSC34 cells was performed using V5-tagged TDP-43 vectors, since the Sar-insoluble fraction of GFP-TDP-43 was abundant even in the absence of oxidative stress (data not shown). The amount of Sar-insoluble fraction of SA-TDP-43 detected was the same as was seen with WT-TDP-43. (Fig. 7A, B). These findings indicate that phosphorylation is not necessary for oxidative-stress mediated insolubilization and cytoplasmic distribution of TDP-43. Next, we performed MTS assay of NSC34 cells to investigate the effect of TDP-43 and its modifications on the cell viability. The results showed that no significant difference in the viability among the cells expressing GFP-mock, GFP-WT- and GFP-SA-TDP-43, either 0 h or 5 h after EA induction (Fig. S3).

Discussion

Post-translational modifications of TDP-43 such as C-terminal phosphorylation, insolubilization, C-terminal fragmentation, and cytoplasmic distribution are pathological hallmarks of TDP-43 proteinopathies (Arai et al., 2006; Hasegawa et al., 2008; Neumann et al., 2006). TDP-43 with defective nuclear localization signal (NLS) was shown to promote cytoplasmic aggregation, C-terminal phosphorylation, and C-terminal fragmentation of TDP-43 in cell-based studies (Nonaka et al., 2009a; Winton et al., 2008). In addition, overexpression of TDP-43 CTF lead to phosphorylation and formation of cytoplasmic aggregates (Igaz et al., 2009; Nonaka et al., 2009b). Although these observations suggest that the cytoplasmic localization

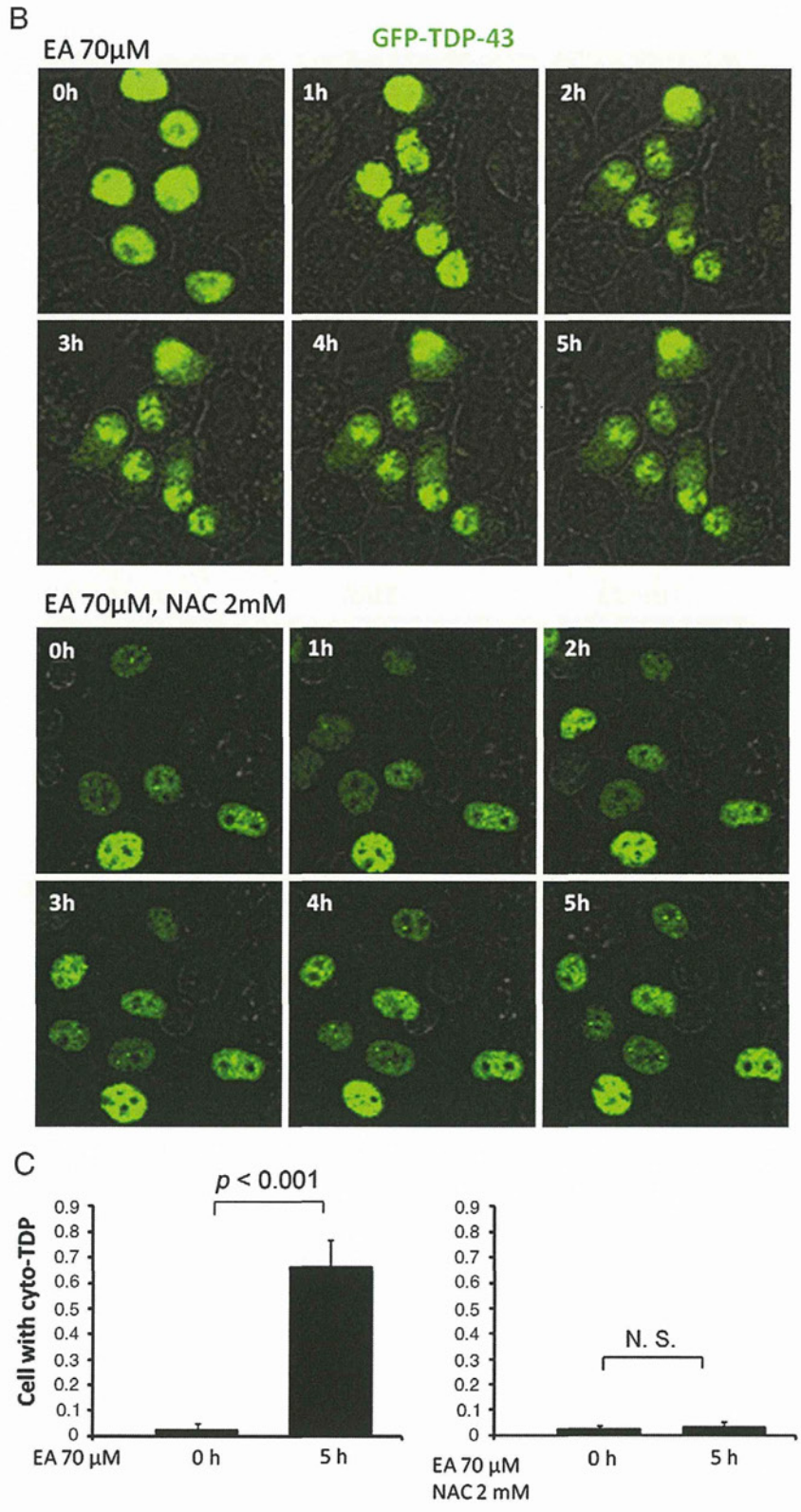

Fig. 3 (*continued*).

or fragmentation of TDP-43 facilitates its pathological modification such as aggregation and phosphorylation, the initial cause of these modifications in TDP-43 proteinopathies has not been fully elucidated. Some studies have demonstrated that artificial axonal damage induces transient cytoplasmic distribution of TDP-43 in motor neurons (Moisse et al., 2009; Sato et al., 2009), indicating that the pathological distribution of TDP-43 may result from the cellular response to neuronal injury or axonal obstruction. However, in these affected neurons, aggregation, C-terminal fragmentation and phosphorylation of TDP-43 were not observed. Furthermore, zinc-induced nuclear

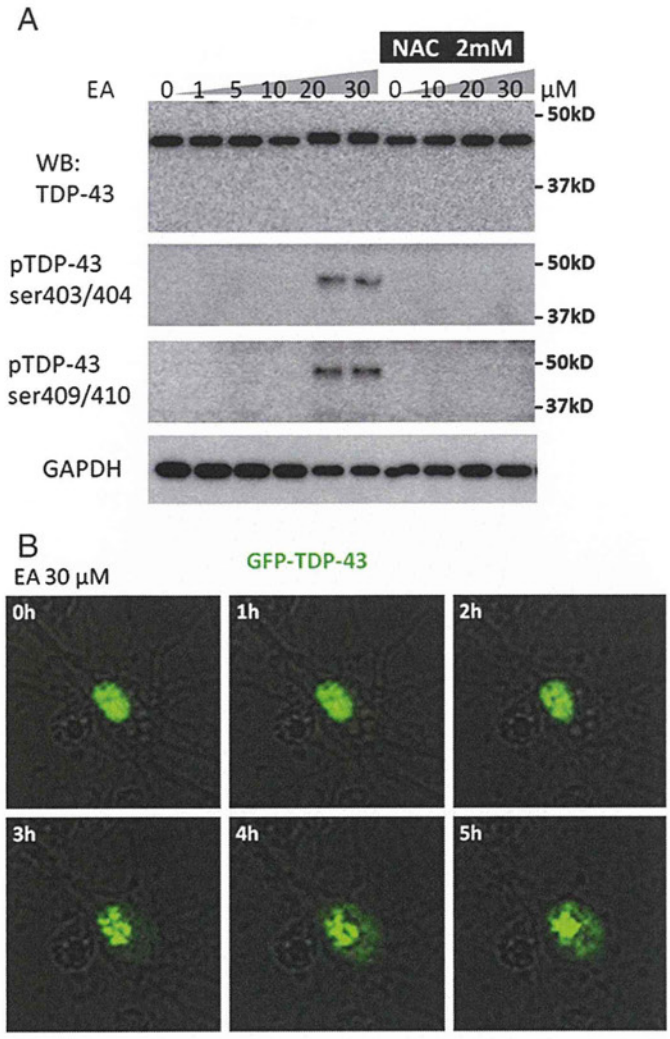

Fig. 4. TDP-43 modification induced by EA in primary cortical neuron. (A) Immunoblots of primary cortical neurons. EA induced TDP-43 phosphorylation at S403/404 and S409/410 in a dose-dependent manner, and this was prevented by 2 mM NAC. (B) Time lapse analysis of neurons expressing GFP-WT-TDP-43. TDP-43 in primary cultures was distributed to the cytoplasm in the presence of 30 μM EA.

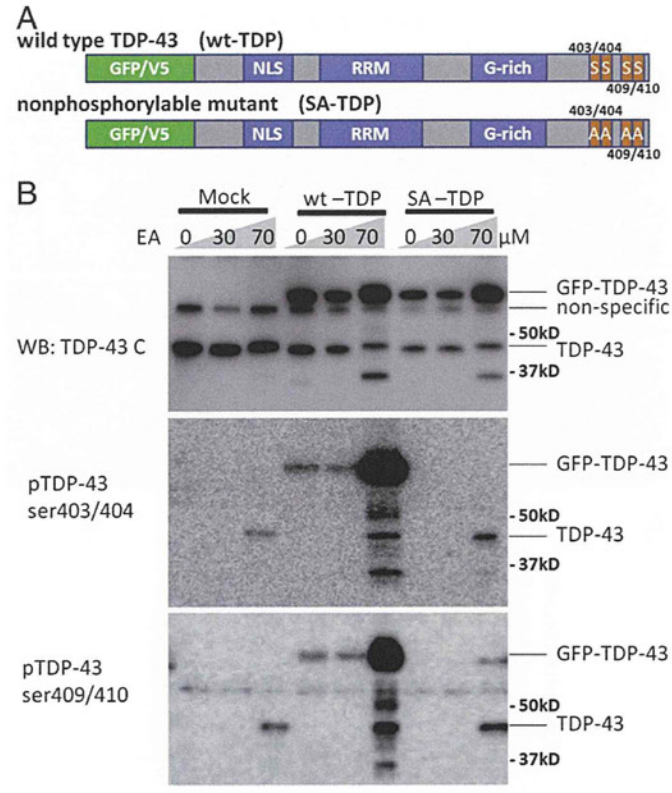

Fig. 5. Nonphosphorylable mutant of TDP-43. (A) Structures of WT- and SA-TDP-43 vectors. SA-TDP-43 contains serine to alanine substitutions at 403/404 and 409/410. (B) Immunoblots of NSC34 cells expressing GFP-WT- or GFP-SA-TDP-43. Endogenous and GFP-WT-TDP-43 were phosphorylated at both 403/404 and 409/410 by 70 μM EA, but GFP-SA-TDP-43 was not phosphorylated by the treatment.

inclusion formations have also been observed in SY5Y cells, but not C-terminal fragmentation or phosphorylation of TDP-43 (Caragounis et al., 2010).

In the present study, we demonstrated that a compound that induces cellular glutathione depletion, EA induced C-terminal phosphorylation of TPD-43 at S403/404 and S409/410 in NSC34 cells and mouse primary cortical neurons, and that NAC completely prevented this phosphorylation. In addition, inhibitors of both CK1 and CK2 also prevented the phosphorylation in a dose-dependent manner. These findings indicate that C-terminal phosphorylation of TDP-43 occurs as a consequence of oxidative stress induced by glutathione depletion and is mediated by CK1 and CK2. Furthermore, the sequential extract analysis showed that EA reduced the solubility of TDP43 and increased the amount of ~25 kDa CTF in the Sar-insoluble fraction. Additionally, EA also induced cytoplasmic distribution of TDP-43 in NSC34 cells and primary cortical neurons. The time lapse analysis showed that cytoplasmic distribution of TDP-43 was seen in the majority of NSC34 cells. Although the immunocytochemistry of TDP-43 demonstrated that cytoplasmic distribution of TDP-43 were observed only in a certain population of NSC34 cells treated with EA, this is likely due to the fact that most of damaged cells could not stay adherent to the plate during the fixation. Previous reports indicated that severe level of oxidative stress may result in apoptotic cell death, and that caspase activation induces C-terminal fragmentation of TDP-43 (Dormann et al., 2009; Zhang et al., 2007). These observations do not exclude the possibility that caspase activation contributes to TDP-43 modifications that were observed under EA treatment. The results of the present study demonstrated that H_2O_2, another inducer of oxidative stress, also causes C-terminal phosphorylation, fragmentation, insolubilization, and cytoplasmic distribution of TDP-43 as observed under EA exposure. These data suggest that oxidative stress is involved in the process of the pathological TDP-43 modifications seen in TDP-43 proteinopathies. The facts that oxidative stress is associated with aging-related disorders (Frederickson et al., 2005; Migliore, 2005) and that TDP-43 proteinopathies are aging process-related diseases may support our assumption that oxidative stress possibly mediates TDP-43 modification. A high frequency of abnormal TDP-43 pathology such as C-terminal phosphorylation has been found not only in patients with TDP-43 proteinopathies but also in patients with other neurodegenerative disease such as AD, DLB, and HD (Arai et al., 2010). Since numerous studies have demonstrated increased oxidative cellular damage in these conditions (Butterfield et al., 2007; Lovell and Markesbery, 2007; Nunomura et al., 2002), oxidative stress may be a cause of pathological TDP-43 modification in various neurodegenerative disorders.

Several studies demonstrated that TDP-43 is involved in SGs under cellular stresses including arsenite treatment and heat shock (Colombrita et al., 2009; Liu-Yesucevitz et al., 2010; McDonald et al., 2011; Nishimoto et al., 2010). Although TDP-43 was seen as a component of SGs under EA treatment, majority of cytoplasmic TDP-43 was independent of SGs and was diffusely distributed. These findings suggest that there is SG-independent mechanism for cytoplasmic distribution of TDP-43 under oxidative stress induced by glutathione depletion.

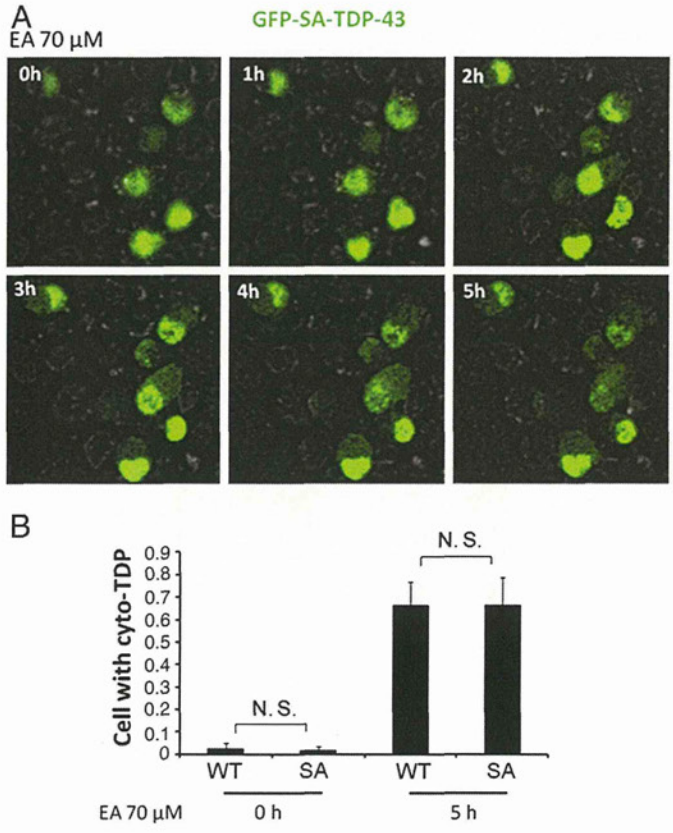

Fig. 6. The effect of C-terminal phosphorylation on TDP-43 distribution. (A) Time lapse analysis of NSC34 cells expressing GFP-SA-TDP-43. GFP-SA-TDP-43 was distributed to the cytoplasm by 70 μM of EA. (B) The proportion of cells with cytoplasmic distribution of TDP-43 (cells with cyto-TDP) in the GFP-TDP-43 expressing cells. The proportion of cells with cyto-TDP was not different between WT- and SA-TDP-43, either 0 h or 5 h after EA induction. Three areas per sample were measured. Error bars indicate SD.

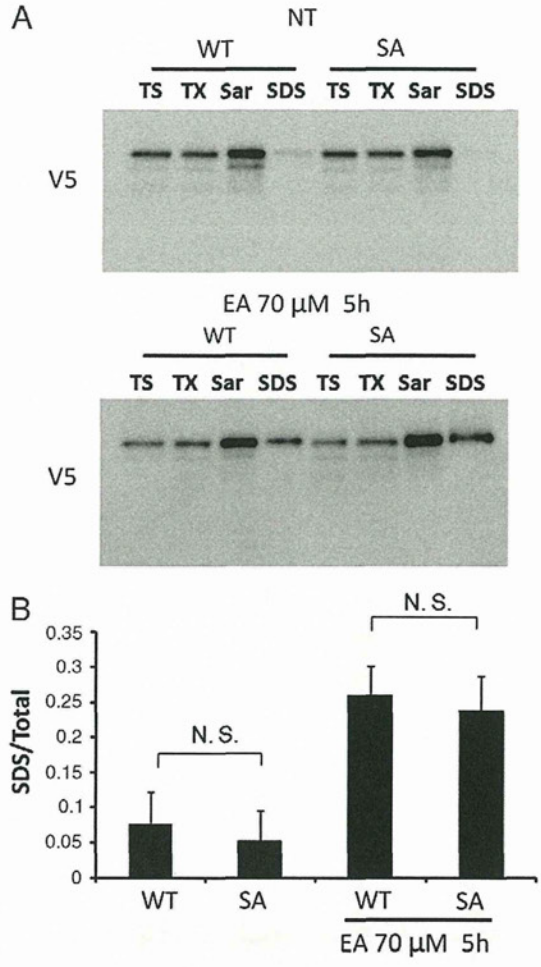

Fig. 7. The effect of C-terminal phosphorylation on TDP-43 solubility. (A) Sequential extraction of NSC34 cells expressing V5-WT- or V5-SA-TDP-43. (B) Densitometric quantitation of Sar-insoluble V5-TDP-43. Ratio of Sar-insoluble fraction from the whole fraction did not differ between WT- and SA-TDP-43 with or without 70 μM EA. Three independent experiments were performed. Error bars indicate SD.

In the present study, S403/404 and S409/410 of TDP-43 were phosphorylated together with insolubilization and cytoplasimic distribution of the protein. The hyperphosphorylation of disease marker proteins is a common feature of neurodegenerative disorders, and its relation to the pathogenesis has been intensively investigated: Tau in AD; huntingtin in HD; and alfa-synuclein in PD and DLB (Ballatore et al., 2007; Fujiwara et al., 2002; Gu et al., 2009). A number of studies have demonstrated that disease-specific phosphorylation of these marker proteins modulates aggregation and potentially influences disease pathogenesis (Azeredo da Silveira et al., 2009; Gu et al., 2009). In the present study, there was no difference between wild type and non-phosphorylable TDP-43 in the degree of insolubilization and cytoplasmic translocation under oxidative stress conditions, suggesting that C-terminal phosphorylation of TDP-43 is not mandatory for aggregation or abnormal intracellular distribution. In support with our findings, there is a study demonstrating that C-terminal phosphorylation of TDP-43 is not substantially required for the cytoplasmic aggregation (Brady et al., 2010). In addition, our results show that C-terminal tags interfere with the detection of TDP-43 phosphorylation, providing a cautionary note for cell-based and animal studies of TDP-43 with a C-terminal tag.

We further examined whether the pathological modifications of TDP-43 contribute to cell vulnerability to glutathione depletion. In the analysis of MTS assay, the viabilities of NSC34 cells were decreased by EA treatment. Although GFP-WT-TDP-43 was fully phosphorylated, insolubilized and distributed to cytoplasm in the cells treated with EA, there was no significant difference in the viability between the cells expressing GFP-mock and GFP-WT-TDP-43. In addition, the viability of NSC34 cells expressing GFP-SA-TDP-43 was not different from that of the cells expressing GFP-WT-TDP-43. These findings suggest that TDP-43 modification may not affect cell viability under oxidative stress induced by glutathione depletion.

In conclusion, we demonstrated that oxidative stress induced by glutathione depletion instigated TDP-43 modifications including C-terminal phosphorylation, insolubilization, C-terminal fragmentation and cytoplasmic distribution, and that these changes reproduce the pathological features of TDP-43 proteinopathies and other neurodegenerative diseases such as AD.

Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2011.12.002.

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Brief communication

Analysis of *C9orf72* repeat expansion in 563 Japanese patients with amyotrophic lateral sclerosis

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Abstract

Recently, a hexanucleotide repeat expansion in *C9orf72* was identified as the most common cause of both sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia in Western populations. We analyzed 563 Japanese patients with ALS (552 sporadic and 11 familial) using fluorescent fragment-length analysis of *C9orf72* and repeat-primed polymerase chain reaction analysis. Haplotype analysis was performed for 42 single nucleotide polymorphisms in patients with *C9orf72* repeat expansion. *C9orf72* repeat expansion was found in 2 patients with sporadic ALS (2/552 = 0.4%) and no patients with familial ALS (0/11 = 0%). In the probands' families, 1 primary progressive aphasia patient and 1 asymptomatic 76-year-old individual exhibited *C9orf72* repeat expansion. All of the patients with the *C9orf72* repeat expansion carried the 20-single nucleotide polymorphism consensus risk haplotype. The frequency of the *C9orf72* repeat expansion among Japanese patients is much lower than in Western populations. The existence of a 76-year-old asymptomatic carrier supported the notion of incomplete penetrance. The *C9orf72* mutation should be analyzed in sporadic ALS patients after determining their family histories not only of frontotemporal dementia but also of primary progressive aphasia.

Keywords: Amyotrophic lateral sclerosis; *C9orf72*; Incomplete penetrance; Sporadic; Aphasia; Frontotemporal dementia

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that primarily affects motor neurons in the spinal cord, brain stem, and cerebral cortex, typically leading to death within a few years. Five to ten percent of ALS cases are familial, and the remaining cases are believed to be sporadic (Valdmanis et al., 2009). A number of genes causing ALS with a dominant mode of inheritance have

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been discovered, such as *SOD1*, *TARDBP*, *FUS*, *VAPB*, *ANG*, *VCP*, *OPTN* (Ticozzi et al., 2011), and *UBQLN2* (Deng et al., 2011). Moreover, there is increasing clinical and pathological evidence for the hypothesis that ALS and frontotemporal dementia (FTD) constitute an overlapping continuum of diseases (Lomen-Hoerth et al., 2002; Neumann et al., 2006). Recently, the expansion of a noncoding GGGGCC hexanucleotide repeat in the *C9orf72* gene has been reported to be a major cause of both ALS and FTD (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011) and the most common genetic abnormality in familial and sporadic forms of both ALS and FTD, particularly in Western populations (Chiò et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). In the present study, we describe the incidence and demographic and clinical features associated with the *C9orf72* mutation in a large cohort of Japanese ALS patients. We also perform haplotype analysis to investigate whether Japanese patients have the same risk haplotype as European patients (Gijselinck et al., 2012; Laaksovirta et al., 2010; Mok et al., 2012).

2. Methods

2.1. Subjects

We obtained a total of 760 DNA samples from the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS; Appendix A). A total of 563 (11 familial and 552 sporadic) patients were diagnosed with ALS according to the El Escorial revised criteria (Brooks et al., 2000) and classified as bulbar-onset, spinal-onset, FTD-ALS, or other (see Supplementary Table 1 for details). We had determined the family histories of ALS but not FTD or primary progressive aphasia (PPA) in all of the patients when they were enrolled as patients with sporadic ALS (SALS). We recruited 197 control subjects, none of whom had a medical or family history of neurodegenerative disorders. The mean age at onset of the patients with ALS was 60.4 ± 11.7 years (range 20–86), and the mean age at sampling of the controls was 60.6 ± 10.3 years (range 26–83). All of the subjects were unrelated Japanese individuals. Written informed consent was obtained from all of the subjects. The ethical committees at the participating institutions approved this study.

2.2. Fluorescent fragment-length analysis of C9orf72 and repeat-primed PCR analysis

The normal repeat number of the GGGGCC hexanucleotide was determined in all of the patients and control subjects using genotyping primers, as previously described (DeJesus-Hernandez et al., 2011). To provide a qualitative assessment of the presence of *C9orf72* repeat expansions, we performed repeat-primed polymerase chain reaction (PCR), as previously described (DeJesus-Hernandez et al., 2011).

2.3. Haplotype analysis

We genotyped 42 single nucleotide polymorphisms (SNPs) across 232 kilobase of Chromosome 9p21, which were first described as the founder haplotype in the Finnish ALS population (Laaksovirta et al., 2010), using primers (Supplementary Table 2) to determine whether our Japanese patients carried the haplotype associated with a risk of ALS. These 42 SNPs included the 20-SNP consensus risk allele that had recently been detected in genome-wide association studies in several populations (Mok et al., 2012). We also performed haplotype analysis with 4 microsatellites (D9S1121, D9S169, D9S270, and D9S104) flanking the *C9orf72* GGGGCC repeat, as previously described (Gijselinck et al., 2012) (Fig. 1).

3. Results

3.1. Detection of C9orf72 repeat expansion

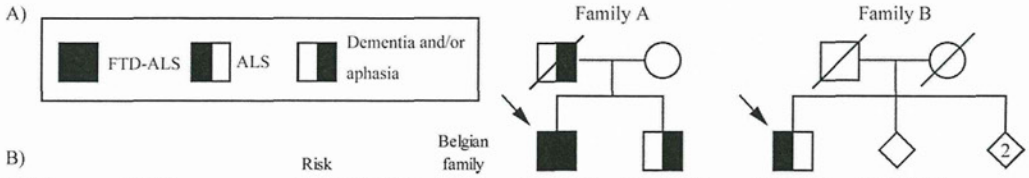
The *C9orf72* repeat expansion was found in 2 of 522 Japanese patients (2/552 = 0.4%) with SALS and none of the 11 patients (0/11 = 0%) with familial ALS (FALS) using repeat-primed PCR (Table 1). Patient A-I with a *C9orf72* mutation was classified as SALS in this study, but after detecting the mutation, we found that patient A-II (a brother of patient A-I) developed aphasia and dementia and had a *C9orf72* mutation (Fig. 1). The average repeat number based on fluorescent fragment-length analysis was 3.65 ± 2.43 (range 2–13 repeats) in 561 ALS patients without the *C9orf72* mutation. A subsequent analysis of 197 healthy controls did not detect any *C9orf72* mutation. The average repeat number was 3.69 ± 2.46 (range 2–14 repeats) in the 197 controls. The mean age at disease onset in patients with *C9orf72* mutation, including patient A-II, was 64.7 ± 6.1 years (range 57–72). The genotypes of all individuals with the *C9orf72* mutation were detected for the 20 SNPs spanning a 140-kilobase segment concordant with the recently identified risk haplotype on chromosome 9p (Mok et al., 2012) and 24 or 25 consecutive SNPs in the 42-SNP Finish risk haplotype (Laaksovirta et al., 2010) (Fig. 1, Supplementary Table 3).

3.2. Clinical presentations of individuals with C9orf72 mutation

3.2.1. Patient A-I (family A)

Patient A-I was a 65-year-old man who reported weakness in the left leg. The weakness progressed, and he developed fasciculation. At age 66, a neurological examination revealed dementia. His Mini Mental State Examination score was 23/30, and his Frontal Assessment Battery score was 13/18. He also exhibited dysarthria and weakness, atrophy, and fasciculation in the tongue and all 4 modalities. His tendon reflexes were diminished, and the plantar re-

A)



B)

Marker	Position	Risk allele	Freq JPT	Belgian family DR14	Patient A-I	Patient A-II	Patient B-I	Subject B-II
rs725804	27458939	A	A=0.081		C/C	C/C	C/C	C/C
rs10511816	27468461	T	T=0.105		**T/G**	**T/G**	G/G	G/G
rs1444533	27477874	A	A=0.553		**A/A**	**A/A**	A/G	A/G
rs1822723*	27478052	C	C=0.669		**C/C**	**C/C**	**C/C**	**C/C**
rs4879515*	27482235	T	T=0.645		**T/T**	**T/T**	**T/T**	**T/T**
rs895023	27483959	T	T=1.000		**T/T**	**T/T**	**T/T**	**T/T**
rs868856*	27489251	T	T=0.247		**T/T**	**T/T**	**T/T**	**T/T**
rs7046653*	27490967	A	A=0.250		**A/A**	**A/A**	**A/A**	**A/A**
rs2440622	27495418	A	A=0.988		**A/A**	**A/A**	**A/A**	**A/A**
rs1977661 *	27502986	C	C=0.651		**C/C**	**C/C**	**C/C**	**C/C**
rs903603*	27529316	C	C=0.341		**C/C**	**C/C**	C/T	C/T
rs10812610*	27533984	C	A=0.680		**C/C**	**C/C**	C/A	C/A
rs2814707*	27536397	A	A=0.058		**A/A**	**A/A**	A/G	A/G
rs3849942*	27543281	A	A=0.076		**A/A**	**A/A**	A/G	A/G
C9orf72	27546544..27573842			mutation	**mutation**	**mutation**	**mutation**	**mutation**
rs12349820	27553876	T	T=0.890		**T/T**	**T/T**	**T/T**	**T/T**
rs10122902*	27556780	G	G=0.738		**G/G**	**G/G**	**G/G**	**G/G**
rs10757665*	27557919	T	T=0.890		**T/T**	**T/T**	**T/T**	**T/T**
rs1565948*	27559733	G	G=0.600		**G/G**	**G/G**	**G/G**	**G/G**
rs774359*	27561049	C	C=0.070		**C/C**	**C/C**	C/T	C/T
rs2282241*	27572255	G	G=0.326		**G/G**	**G/G**	G/T	G/T
rs1948522*	27575785	C	C=0.837		**C/C**	**C/C**	C/C	C/C
rs1982915*	27579560	G	G=0.238		**G/G**	**G/G**	G/A	G/A
rs2453556*	27586162	G	G=0.448		**G/G**	**G/G**	G/A	G/A
rs702231*	27588731	A	A=0.634		**A/A**	**A/A**	A/A	A/A
rs696826*	27589657	G	G=0.983		**G/G**	**G/G**	G/G	G/G
rs2477518*	27599746	T	T=0.820		**T/T**	**T/T**	**T/T**	**T/T**
D9S270	28404862 .. 28404948			327	**327**/325	**327**/325	323/323	323/323
D9S104	28771379 .. 28771576			168	158/158	158/158	158/171	158/171

Fig. 1. (A) The pedigrees of the 2 families with *C9orf72* repeat expansion. To maintain confidentiality, several unaffected individuals who died early in families A and B are not shown. Probands are indicated by arrows. (B) The genotyping data of the single nucleotide polymorphisms (SNPs) and microsatellites. Twenty SNPs, which comprised a recently identified consensus risk haplotype (Mok et al., 2012), are shown with an asterisk. See Supplementary Table 3 for details of the analyses of 42 SNPs (Laaksovirta et al., 2010) and microsatellites (Gijselinck et al., 2012). Alleles possibly shared between our subjects and patients in Western populations are shown in bold. The genotypes of all 4 subjects with respect to the 20 SNPs were found to be concordant with the risk haplotype (Mok et al., 2012). All of the positions of SNPs and microsatellites were from NC_000009.11. Abbreviations: ALS, amyotrophic lateral sclerosis; Freq JPT, Frequency in Japanese in Tokyo from International HapMap project (International HapMap Consortium, 2003); FTD, frontotemporal dementia.

sponse was extensor on the left. He had neither dysphagia nor dyspnea. No sensory abnormalities were noted. Extensive screening for causes of motor neuropathy was negative. The diagnosis was clinically probable ALS-laboratory supported (Brooks et al., 2000) and FTD-ALS.

3.2.2. *Patient A-II (family A)*

This patient was a 57-year-old man who presented with difficulty speaking. He was believed to have suffered from a mental disease after being imprisoned because of his involvement in a fatal car accident. At age 64, he was severely dysfluent and could barely speak. Logoclonia was particularly prominent. However, he did not exhibit any violent behavior or other behavioral abnormalities. He also did not display any clinical features of motor neuron disease. Brain magnetic resonance imaging revealed severe frontotemporal lobar atrophy. PPA was considered the most likely diagnosis.

3.2.3. *Patient B-I (family B)*

Patient B-I was a 72-year-old man who presented with gait disturbance and weakness in the proximal lower extremity muscle. His family history was negative for motor neuron disease and dementia (Fig. 1). The muscle weakness and atrophy progressed and spread to the other parts of his body despite treatment with intravenous gamma globulin. At age 74, he could not roll over while sleeping. A neurological examination showed marked muscle atrophy in his arms and shoulders and prominent fasciculation in his legs. The deep tendon reflexes were decreased in his limbs, and he had no pathological reflexes. Sensations in all 4 modalities were intact. At age 75, he developed dyspnea and dysphagia and started noninvasive positive pressure ventilation and intravenous hyperalimentation. He died of respiratory insufficiency at age 76. An autopsy was not performed. The diagnosis was clinically suspected ALS (Brooks et al., 2000).