

中野今治	本邦初のパーキンソン病遺伝子治療	日本老年医学会雑誌	45	9-13	2008
嶋崎晴雄 中野今治	放射線照射による脊髄障害と神経障害	Brain and Nerve	60(2)	115-121	2008
中野今治	孤発性 ALS 古典型「病理」	Clinical Neuroscience	26	277-279	2008

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nomoto, T., Okada, T., Shimazaki, K., Yoshioka, T., Nonaka-Sarukawa, M., Ito, T., Takeuchi, K., Katsura, K., Mizukami, H., Kume, A., Ookawara, S., Ikeda, U., Katayama, Y., Ozawa, K.	Systemic delivery of IL-10 by an AAV vector prevents vascular remodeling and end-organ damage in stroke-prone spontaneously hypertensive rat.	Gene Ther			<i>in press</i>
Nonaka-Sarukawa, M., Okada, T., Ito, T., Yamamoto, K., Yoshioka, T., Nomoto, T., Hojo, Y., Shimpo, M., Urabe, M., Mizukami, H., Kume, A., Ikeda, U., Shimada, K., Ozawa, K.	Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats.	J Gene Med	10	368-74	2008
Suzumura, K., Hirano, T., Son, G., Iimuro, Y., Mizukami, H., Ozawa, K., Fujimoto, J.	Adeno-associated virus vector-mediated production of hepatocyte growth factor attenuates liver fibrosis in mice.	Hepatol Int	2	80-88	2008
Liu, Y., Okada, T., Shimazaki, K., Sheykholeslami, K., Nomoto, T., Muramatsu, S., Mizukami, H., Kume, A., Xiao, S., Ichimura, K., Ozawa, K.	Protection against aminoglycoside-induced ototoxicity by regulated AAV vector-mediated GDNF gene transfer into the cochlea	Mol Ther	16	474-80	2008

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Wakamatsu M., Ishii A., Iwata S., Sakagami J., Ukai Y., Ono M., Kanbe D., <u>Muramatu S.</u> , Kobayashi K., Iwatsubo T., Yoshimoto M.	Selective loss of nigral dopamine neurons induced by overexpression of truncated human $\alpha$ -synuclein in mice.	Neurobiol Aging	29(4)	574-585	2008
Fukushima F., Nakao K., Shinoe T., Fukaya M., <u>Muramatu S.</u> , Sakimura K., Kataoka H., Mori H., Watanabe M., Manabe T., Mishina M.	Ablation of NMDA receptors enhances the excitability of hippocampal CA3 neurons.	PLoS One	4(1):	e3993	2009

## 研究成果の刊行物・別刷



## Novel *SACS* mutation in a Belgian family with saccin-related ataxia

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Received 21 May 2007; received in revised form 15 July 2007; accepted 20 July 2007

Available online 22 August 2007

### Abstract

The authors describe the four patients in the first known Belgian family with autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS). A novel homozygous missense mutation, NM\_014363.3: c.3491T>A in exon 9, of the *SACS* gene was identified in the present family, which results in an original amino acid of methionine to lysine substitution at amino acid residue 1164 (p.M1164K). Although the cardinal clinical features, i.e., spastic ataxia with peripheral neuropathy, in our patients were similar to those in Quebec patients, our patients exhibited some atypical clinical features, e.g., teenage-onset and absence of retinal hypermyelination. The present family is from Wallonia, and there could be shared ethnicity with the families of Charlevoix–Saguenay.

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**Keywords:** ARSACS; The *SACS* gene; Belgian family; Teenage-onset

### 1. Introduction

Autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS; OMIM 270550) is an inherited neurodegenerative disorder characterized by early-onset spastic ataxia, dysarthria, nystagmus, distal muscle wasting, finger and/or foot deformities, and retinal hypermyelination. It was originally described, with a high prevalence among inhabitants of the Charlevoix–Saguenay region of north-eastern Quebec in Canada, in the late 1970s [1].

In 2000, the gene responsible for ARSACS (*SACS*) was mapped to 13q11. The *SACS* gene was initially reported to consist of a single gigantic exon spanning 12.8kb with an 11.5-kb open reading frame (ORF), which encodes the protein saccin [2]. However, eight exons upstream from the gigantic one were very recently found, and thus the new ORF is 13,299 bp long. To date, approximately 30 mutations have

been reported in Quebec and non-Quebec patients [3–5], and ARSACS is now global [6]. We report here a novel *SACS* mutation in a Belgian family with ARSACS.

### 2. Methods

We encountered four patients in a Belgian family with teenage-onset ataxia. This family is from Wallonia, and more exactly from 'les Ardennes'. There was distant consanguinity between the two pairs of parents (II-1 and II-2, and II-3 and II-4), but the detailed relationship was unknown (Fig. 1). Detailed neurological examination was performed on the whole family, i.e., the patients and unaffected individuals.

Blood samples were obtained with informed consent from 12 individuals in this family. Genomic DNA was extracted from peripheral blood leukocytes. Using 38 appropriate primer pairs (all primer sequences are available on request), the coding exons of the *SACS* gene were amplified by PCR from 200 ng of genomic DNA, and then sequenced directly with an ABI PRISM 3100 genetic analyzer; the analysis was

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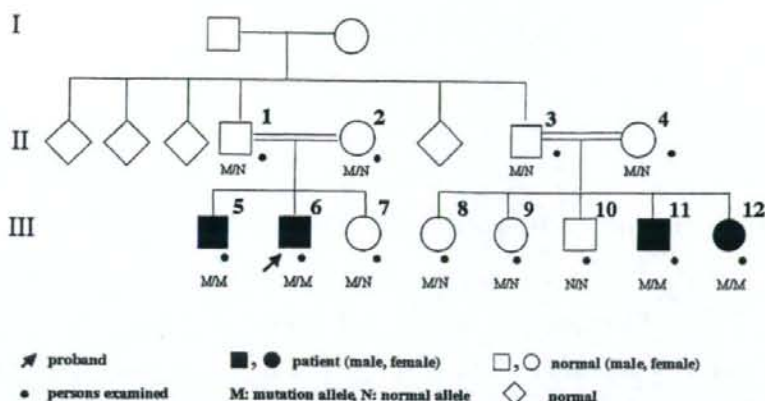


Fig. 1. Pedigree of the Belgian family with ARSACS. There was distant consanguinity between the two pairs of parents (II-1 and II-2, and II-3 and II-4), but the detailed relationship was unknown. The gender is concealed for some individuals, denoted by diamonds, to maintain the anonymity of the family.

performed with Sequencing Analysis software, ver. 5.2 (Applied Biosystems), as described previously [7–9]. When sequence analysis revealed a novel missense mutation in the Belgian family, we examined 114 Belgian control chromosomes in order to exclude polymorphisms. The PCR products containing the novel mutation with specific primers (1410F, 5'-TTGGCTCCTCACTTCCTCTTGTG-3'; 1962R, 5'-ACTGGTGTGTTGGGGCTTGCT-3') were digested with *Nco I* (Takara Bio Inc.) at 37 °C overnight, subjected to electrophoresis on 3.5% NuSieve 3:1 agarose gels, and then stained with ethidium bromide. The wild-type PCR products harbor three *Nco I* sites, one of which is destroyed by the novel missense mutation.

This study was approved by the Medical Ethical Committee of Jichi Medical University.

### 3. Results

#### 3.1. Clinical study

We found four patients (III-5, III-6, III-11, and III-12) with progressive gait unsteadiness in a Belgian family. The age at onset was 13, 12, 12, and 13 years old, respectively, and the age at examination ranged from 21 to 43 years old. There were no obvious abnormalities in their first decade. All of the patients, however, complained that their gait and running became slow and unsteady at the start of their second decade. It became difficult for them to do sports or gymnastic exercise in their school days.

Neurological examination revealed that cerebellar ataxia with leg spasticity was a cardinal clinical feature in all four patients (Table 1). Furthermore, all patients showed absent ankle jerks and pes cavus. The Babinski sign was present in three patients, but equivocal in one patient with hyperreflexia. Distal amyotrophy was apparent in three patients. Myelinated retinal nerve fibers were not apparent in one patient examined ophthalmologically. Mini Mental State Examination was 30/30 in all patients. Brain MRI was performed in all patients,

which revealed cerebellar atrophy, particularly in the vermis (data not shown). Motor nerve conduction velocity examination in one patient (III-6) revealed mild reduction in the median and ulnar nerves, and moderate reduction in the common peroneal nerves. A compound muscle action potential was not evoked in the posterior tibial nerve. A sensory nerve action potential was not evoked in the radial and sural nerves (data not shown). The other three patients showed similar data in the peripheral nerve conduction study.

#### 3.2. Molecular study

A homozygous missense mutation, NM\_014363.3:c.3491T>A in exon 9 of the *SACS* gene, was identified in our patients (Fig. 2), which results in an original amino acid of methionine to lysine substitution at amino acid residue 1164 (p.M1164K). This substitution was found to be heterozygous in the four unaffected parents (II-1, II-2, II-3,

Table 1

Summary of the neurological findings in the patients in the Belgian family

Patient	III-5	III-6	III-11	III-12
Age (years)	43	41	34	33
Sex	M	M	M	F
Age at onset (years)	13	12	12	13
Age at examination (years)	43	39	35	21
Cerebellar ataxia	+	+	+	+
Dysarthria	+	+	+	-
Nystagmus	+	+	+	+
Distal amyotrophy	+	+	+	-
Absent ankle jerks	+	+	+	+
Babinski sign	+	+	+	*
Hyperreflexia	+	-	+	+
Leg spasticity	+	+	+	+
Finger deformity	+	?	?	-
Pes cavus	+	+	+	+
Retinal myelinated fibers	NE	-	NE	NE
MMSE	30/30	30/30	30/30	30/30

M: Male, F: female, MMSE: Mini Mental State Examination, NE: not examined, \*: equivocal.



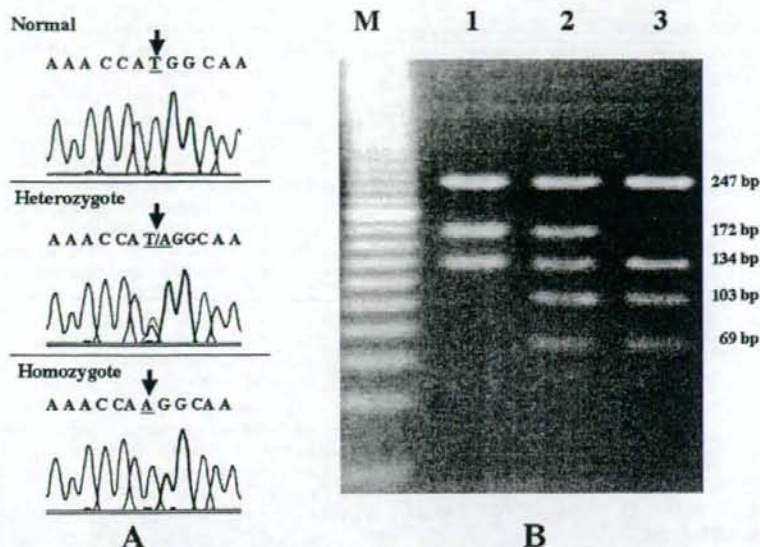


Fig. 2. A. Identification of a novel missense mutation of the *SACS* gene in the Belgian family. The sequences in a normal control, a carrier, and a patient are shown in the upper, middle, and lower panels, respectively. The arrow indicates the NM\_014363.3: c.3491T>A substitution with a heterozygous or a homozygous state. B. The PCR products after *Nco* I digestion. Lane M, 20 bp size markers. Lane 1, the *Nco* I-digested PCR products with a homozygous NM\_014363.3: c.3491T>A substitution (patient) gave three bands (247, 172, and 134 bp). Lane 2, the *Nco* I-digested PCR products with a heterozygous NM\_014363.3: c.3491T>A substitution (carrier) gave five bands (247, 172, 134, 103, and 69 bp). Lane 3, the wild-type *Nco* I-digested PCR products gave four bands (247, 134, 103, and 69 bp).

and II-4) and three siblings (III-7, III-8, and III-9), but not in the other unaffected one, III-10 (Fig. 1). Thus, this novel missense mutation in exon 9 of the *SACS* gene was found to be cosegregated with the disease in the Belgian family with autosomal recessive transmission. Furthermore, this mutation was not found in 114 Belgian control chromosomes.

#### 4. Discussion

Although mutations of the *SACS* gene were originally identified in Quebec patients [2], *SACS* mutations have been reported in non-Quebec patients including ones in Japan, Italy, Tunisia, Turkey, and Spain so far [3–5,7–13]. In this study, we examined the first Belgian family with a *SACS* mutation. The present family is from Wallonia, and there could be shared ethnicity with families of Charlevoix–Saguenay.

Genetically, the present missense mutation (NM\_014363.3: c.3491T>A) is considered to be responsible for our patients' disease, because this novel mutation was cosegregated with the disease in the Belgian family with autosomal recessive transmission. Furthermore, the mutation was not found in 114 Belgian control chromosomes. In addition, p.M1164K leads to a change in the secondary structure predicted by the PROF program [14]. To date, although the genotype is expanding upstream from the original gigantic exon 9 [5,7], more than 90% of the causative *SACS* mutations, including ours, have been detected in exon 9 [3,4]. Furthermore, two important

domains, i.e., the DnaJ molecular chaperone homology domain and the HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domain, correlate with chaperone-mediated protein folding in the *SACS* protein, and are encoded by exon 9. Thus, exon 9 may play an important role in the function of saccin, and it should be preferentially analyzed when performing a molecular test on ARSACS.

The cardinal clinical features and MRI findings in our patients are similar to those in Quebec ones with ARSACS reported previously, such as a progressive ataxic gait, dysarthria, nystagmus, leg spasticity, the Babinski sign, hyperreflexia, foot deformity, pes cavus, normal intelligence, peripheral neuropathy, and atrophy of the cerebellar vermis [1–4]. In addition, the neurophysiological data closely resemble those previously reported [3,4,8,9], suggesting that severe axonal degeneration and diffuse peripheral demyelination are general in ARSACS.

One patient, however, showed the absence of retinal hypermyelination. Although this feature is atypical in Quebec patients [1], it has sometimes been reported in non-Quebec ones [4,5]. Thus, the present study confirmed that retinal hypermyelination is variable in non-Quebec patients.

Furthermore, all our patients showed teenage-onset ataxia (12–13 years old). Since the age of onset in ARSACS usually ranges from infancy to preschool age (from 2 to 5.5 years old) [4], that in our patients was much later than that in Quebec and most non-Quebec patients, except for in two non-Quebec families (age of onset, 10 and 12 years old)

[5,11]. The identification of teenage-onset cases of different races suggests that the onset age in ARSACS might vary.

Meanwhile, since the clinical features of teenage-onset ARSACS are similar to those of autosomal recessive spastic ataxia such as SPG30 (mean onset age, 17.5 years) [15] and SAX2 (onset age, before 15 years) [16], it is necessary to discriminate these diseases in patients with teenage-onset spastic ataxia.

In conclusion, this is the first documentation of a Belgian ARSACS family. Since the present family is from Wallonia, identification of a novel mutation of the *SACS* gene would be of interest. As more *SACS* mutations are identified, the clinical spectrum of saccinopathies might expand.

#### Acknowledgements

The authors thank the family for participating in this study. This work was supported by a grant from the Research Committee for Ataxic Diseases (Y.T.) of the Ministry of Health, Labor and Welfare, Japan.

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# Phosphorylated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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**Objective:** TAR DNA-binding protein of 43kDa (TDP-43) is deposited as cytoplasmic and intranuclear inclusions in brains of patients with frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). Previous studies reported that abnormal phosphorylation takes place in deposited TDP-43. The aim of this study was to identify the phosphorylation sites and responsible kinases, and to clarify the pathological significance of phosphorylation of TDP-43.

**Methods:** We generated multiple antibodies specific to phosphorylated TDP-43 by immunizing phosphopeptides of TDP-43, and analyzed FTLD-U and ALS brains by immunohistochemistry, immunoelectron microscopy, and immunoblots. In addition, we performed investigations aimed at identifying the responsible kinases, and we assessed the effects of phosphorylation on TDP-43 oligomerization and fibrillization.

**Results:** We identified multiple phosphorylation sites in carboxyl-terminal regions of deposited TDP-43. Phosphorylation-specific antibodies stained more inclusions than antibodies to ubiquitin and, unlike existing commercially available anti-TDP-43 antibodies, did not stain normal nuclei. Ultrastructurally, these antibodies labeled abnormal fibers of 15nm diameter and on immunoblots recognized hyperphosphorylated TDP-43 at 45kDa, with additional 18 to 26kDa fragments in sarkosyl-insoluble fractions from FTLD-U and ALS brains. The phosphorylated epitopes were generated by casein kinase-1 and -2, and phosphorylation led to increased oligomerization and fibrillization of TDP-43.

**Interpretation:** These results suggest that phosphorylated TDP-43 is a major component of the inclusions, and that abnormal phosphorylation of TDP-43 is a critical step in the pathogenesis of FTLD-U and ALS. Phosphorylation-specific antibodies will be powerful tools for the investigation of these disorders.

Ann Neurol 2008;64:60–70

Tau-negative and ubiquitin-positive inclusions (UPIs) that include neuronal cytoplasmic inclusions (NCIs), neuronal intranuclear inclusions (NIIs), and dystrophic neurites (DNs) are the pathological hallmarks of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) with or without clinical features of motor neuron disease (MND).<sup>1</sup> Recently, several genes and chromosomal loci, including the progranulin (*PGRN*) gene,<sup>2,3</sup> valosin-containing protein (*VCP*) gene,<sup>4</sup> and an unidentified site at chromosome 9p,<sup>5,6</sup>

have been reported to be associated with familial FTLD-U. Ubiquitin-positive, tau-negative NCIs have also been recognized in patients with the classic type of MND, amyotrophic lateral sclerosis (ALS),<sup>7</sup> in which skein-like cytoplasmic inclusions are found in the lower motor neurons of the hypoglossal nucleus and spinal cord.<sup>8,9</sup> In both FTLD-U and ALS, understanding why these inclusions form may provide critical clues to the neurodegenerative process.

Recently, TAR DNA-binding protein of 43kDa

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Received Nov 8, 2007, and in revised form April 8, 2008. Accepted for publication April 22, 2008.

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21425

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(TDP-43), which functions in regulating transcription and alternative splicing,<sup>10,11</sup> was identified as a component of these UPIs.<sup>12-14</sup> TDP-43 appears to belong to the group of two RNA-binding domain-Glycyl RNA-binding proteins, which include the heterogeneous nuclear ribonucleoprotein (hnRNP) family and factors involved in RNA splicing and transport.<sup>15</sup> TDP-43 binds hnRNP A/B and hnRNP A1 through its C-terminal region, inhibiting premessenger RNA splicing.<sup>16</sup> Several disorders, including FTL-D-U, FTL-D-MND, and ALS are now referred to as TDP-43 proteinopathies.<sup>12-14</sup> Immunoblot analysis of the sarkosyl-insoluble fraction extracted from brains of patients afflicted with these disorders shows an abnormal TDP-43-immunoreactive band at 45kDa. The electric mobility of this band changes after dephosphorylation, suggesting that abnormal phosphorylation takes place in accumulated TDP-43.<sup>12,13</sup> However, the phosphorylation sites, responsible kinases, and pathological significance of phosphorylation are still unknown.

In this report, we demonstrate that multiple antibodies raised against TDP-43 phosphopeptides label UPIs in histological sections from FTL-D-U and ALS brains. These antibodies may offer advantages over previous antibodies used to identify these structures because they appear to be more sensitive than anti-ubiquitin antibodies and, unlike commercially available anti-TDP-43 antibodies, do not stain normal neuronal nuclei. In addition, these antibodies specifically recog-

nize abnormal TDP-43 species on immunoblots of sarkosyl-insoluble fractions extracted from FTL-D-U and ALS brains. Furthermore, we show that the multiple phosphorylation epitopes identified in aggregated TDP-43 are generated by casein kinase-1 (CK1), and that oligomerization or fibril formation of TDP-43 is promoted by phosphorylation with CK1 *in vitro*. These results suggest that phosphorylated TDP-43 is a critical component of UPIs in FTL-D-U and ALS, and that phosphorylation of TDP-43 by CK1 may be involved in the accumulation of the protein.

## Subjects and Methods

### Materials

Human brain tissue was obtained from the Brain Donation Program at Sun Health Research Institute (Sun City, AZ), Aichi Medical University (Japan), Jichi Medical University (Japan), National Shimofusa Mental Hospital (Japan), and Tokyo Metropolitan Matsuzawa Hospital (Japan). Small blocks of brain tissue were dissected at autopsy and frozen rapidly at -70 to 80°C or fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 days. Brain tissue from sporadic FTL-D-U, familial FTL-D-U with PGRN mutations (mPGRN), sporadic ALS, and sporadic FTL-D-MND was compared with brain tissue from Alzheimer's disease (AD) and neurologically normal control subjects. The age, sex, brain regions examined, diagnosis, and histopathological subtyping for these cases are given in Table 1. Neuropathological diagnoses of FTL-D-U, FTL-D-MND, ALS, and AD were made in accordance with published guidelines.<sup>1,9,17-19</sup>

**Table 1. Subjects, Brain Regions, Pathological Diagnosis, and Subtypes Examined**

Case No.	Age (yr)	Sex	Region	Diagnosis	Subtype
1	67	M	Hip, T, F	FTLD-U (sporadic)	1
2	59	M	Hip, T	FTLD-U (sporadic)	1
3	68	F	Hip, T	FTLD-U (sporadic)	1
4	49	F	T	FTLD-MND	2
5	76	M	Prec	FTLD-MND	2
6	66	M	F, SC	ALS	2
7	70	M	Prec, SC	ALS	2
8	69	M	Prec	ALS	2
9	53	M	Hip, T, F	FTLD-U (mPGRN)	3
10	56	F	Hip, T, F	FTLD-U (mPGRN)	3
11	54	M	Hip, T, F	FTLD-U (mPGRN)	3
12	68	F	Hip, T	AD	—
13	83	F	Hip, T	AD	—
14	65	M	Hip, T	Control	—
15	72	M	Hip, T	Control	—

Hip = hippocampus; T = temporal; F = frontal; FTL-D-U = frontotemporal lobar degeneration with ubiquitinated inclusions; MND = motor neuron disease; Prec = precentral; SC = spinal cord; ALS = amyotrophic lateral sclerosis; mPGRN = mutations of progranulin gene; AD = Alzheimer's disease.



Although none of the three ALS cases had a documented history of dementia, all had immunohistochemical evidence of pathology in the neocortex.

#### Preparation of Antibodies

Immunogens consisted of 39 synthetic phosphopeptides representing 36 of the 64 Ser/Thr/Tyr sites in the human TDP-43 molecule. All peptides were conjugated at the amino or carboxyl terminal by a cysteine linkage to synthetic thyroglobulin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as a coupling reagent.<sup>20</sup> The rabbit antisera were purified by obtaining flow-through fractions from a Toyopearl AF-Tresyl-650M (TOSOH, Tokyo, Japan) or SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL) precoated with the nonphosphorylated synthetic peptide. The specificities of the antibodies were verified by enzyme-linked immunosorbent assay and immunoblot. A phosphorylation-independent rabbit polyclonal antibody to TDP-43 was also produced using a C-terminal peptide of TDP-43 (405-414) as immunogen.

#### Immunohistochemistry

After cryoprotection in 15% sucrose in 0.01M phosphate-buffered saline (pH 7.4), paraformaldehyde-fixed tissue blocks were cut on a freezing microtome at 30 $\mu$ m thickness. The free-floating sections were immunostained with an anti-ubiquitin antibody (DF2, a gift from Dr Mori, 1:200),<sup>21</sup> a commercially obtained phosphorylation-independent anti-TDP-43 antibody (10782-1-AP; ProteinTech Group, Chicago, IL; 1:2,000), and a panel of phosphorylation-dependent anti-TDP-43 antibodies including pS409/410, using methods previously described.<sup>13</sup> Double-labeled immunofluorescence was performed using fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies; sections were examined with a confocal laser microscope (LSM5 PASCAL; Carl Zeiss Microimaging GmbH, Jena, Germany).

#### Immunoelectron Microscopy

Tissue blocks of ALS lumbar spinal cord were fixed in paraformaldehyde and embedded in LR White Resin (London Resin, Reading, United Kingdom). Ultrathin sections were incubated with pS409/410 (1:1,000), and the immunoreaction products were probed using colloidal gold particles (1:10 dilution; BBInternational, Cardiff, United Kingdom) according to a standard immunogold-based postembedding electron microscopic procedure.<sup>22</sup>

#### Immunoblotting

Sarkosyl-insoluble, urea-soluble fractions were extracted from the frontal and the temporal regions of control, FTLD-U, and ALS brains as previously described.<sup>23</sup> The samples before (-) and after (+) the treatment with lambda protein phosphatase (APase) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins in the gel were then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 3% gelatin in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (50mM Tris-HCl, pH 7.5, 150mM NaCl), membranes were incubated overnight with the primary antibody.

ies. After incubation with an appropriate biotinylated secondary antibody, labeling was detected as described previously.<sup>13,23</sup>

#### In Vitro Phosphorylation and Fibrillization of TDP-43

Human TDP-43 complementary DNA were subcloned into pRK172 expression vectors and transformed into *Escherichia coli* BL21(DE3). For in vitro phosphorylation, crude extracts from *E. coli* that expressed human TDP-43 were used to prepare partially purified TDP-43 using heparin-Toyopearl column chromatography and elution with 0.5M NaCl. The elutes were phosphorylated with CK1 (10,000U/ml; New England Biolabs, Beverly, MA), casein kinase-2 (CK2) (10,000U/ml; New England Biolabs), or glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) (10,000U/ml; New England Biolabs) at 30°C for 14 hours. To study fibrillization, we incubated partially purified TDP-43 aliquots in 30mM Tris-HCl at pH 7.5 containing 4mM magnesium chloride, 2mM ATP, with or without CK1 (10,000U/ml) at 30°C for 3 days. A few drops of reaction solution was then applied to a carbon-coated copper grid and allowed to air-dry. The grid was placed on a drop of blocking solution (10mg/ml bovine serum albumin in phosphate-buffered saline) for 10 minutes and then placed on a drop of primary antibody (pS409/410, 1:200) for 2 hours at room temperature. After washing with 10mg/ml bovine serum albumin in phosphate-buffered saline, the grid was placed on a drop of the secondary antibody conjugated to 10nm colloidal gold particles (1:50; Sigma, St. Louis, MO) for 1 hour at room temperature. Finally, after another round of washing, the grid was negatively stained with 2% sodium phosphotungstate and examined with the electron microscopy (JEM-1230; JEOL, Akishime, Japan).

#### Results

##### *Multiple Sites within TDP-43 Are Abnormally Phosphorylated in Frontotemporal Lobar Degeneration with Ubiquitinated Inclusions and Amyotrophic Lateral Sclerosis*

There are multiple potential phosphorylation sites within human TDP-43, including 41 serine (Ser), 15 threonine (Thr), and 8 tyrosine (Tyr) residues. To identify the critical phosphorylation sites of TDP-43, we raised antibodies against 39 different synthetic phosphopeptides, representing 36 of 64 candidate phosphorylation sites (Table 2). The major strategy was to choose Ser and Thr residues that cover known protein kinase consensus phosphorylation motifs, including R-X-pSer/Thr for protein kinase A, pSer/Thr-X-X-Ser/Thr for CK1, pSer/Thr-X-X-E/D for CK2, and pSer/Thr-X-X-X-Ser for GSK3 and CK1. In addition, Ser/Thr residues in C-terminal region of TDP-43 were chosen because they are analogous to abnormal phosphorylation sites found in tau or  $\alpha$ -synuclein.

Of the generated antibodies, pS379, pS403/404, pS409, pS410, and pS409/410 intensely immunostained the UPs in FTLD-U and ALS, and demonstrated, on immunoblots of sarkosyl-insoluble fractions



**Table 2. Antigen Peptides for Immunization of Rabbits**

	Site	Antigen Peptide
1	pT8	EYIRVT(p)EDENDEC
2	pS20	PIEIPS(p)EDDGTC
3	pS29	GTVLLS(p)TVTAC
4	pT88	CNYPKDNKRKMDDET(p)D
5	pS91	CDETDAS(p)SAVKVKR
6	pS92	CDETDASS(p)AVKVKR
7	pS91/92	DETDAS(p)S(p)AVKVC
8	pT103	CKRAVQKT(p)SDLIVLG
9	pS104	CKRAVQKTS(p)DLIVLG
10	pT116	PWKTT(p)EQDLKEC
11	pT141	KKDLKT(p)GHSKGC
12	pT153	CGFVRFT(p)EYETQVK
13	pS180	CKLPNS(p)KQSQDE
14	pS183	CPNSKQS(p)QDEPLR
15	pS190	CKQSQDEPLRS(p)RK
16	pT199	CT(p)EDMTEDLE
17	pT203	RCTEDMT(p)EDELRL
18	pT233	CRAFADVT(p)FADDQ
19	pS254	IIKGIS(p)VKISC
20	pS258	CISVHIS(p)NAEPK
21	pS266	CPKHNS(p)NRQLER
22	pS273	CRQLERS(p)GRFGGN
23	pS292	CGFGNS(p)RGGGA
24	pS305	CNNQGS(p)NMGGG
25	pS317	CFGAFS(p)INPAM
26	pS332	CAALQS(p)SWGMM
27	pS350	CQSGPS(p)GNNQN
28	pS375	GNNSYS(p)GSNSGC
29	pS379	CSGSNS(p)GAAIG
30	pS389	CGWGSAS(p)NAGS
31	pS393	CASNAGS(p)GSGF
32	pS395	CAGSGS(p)GFNGG
33	pS403	CGFNGGFGS(p)SMD
34	pS404	CNGGFGSS(p)MDSK
35	pS403/ 404	CNGGFGS(p)S(p)MDSK
36	pS407	CGSSMDS(p)KSSGW
37	pS409	CMDSKS(p)SGWGM
38	pS410	CMDSKSS(p)GWGM
39	pS409/ 410	CMDSKS(p)S(p)GWGM

extracted from brains of the FTLU and ALS cases, an abnormal band of 45kDa but not the 43kDa band corresponding to normal TDP-43. The results suggest that these sites are phosphorylated in the abnormal aggregates of TDP-43 present in FTLU and ALS.

#### *Immunohistochemical Characterization of Phosphorylated TDP-43*

Immunohistochemical staining showed that five of the phosphorylation-specific anti-TDP-43 antibodies identified UPIs in both FTLU and ALS brains. Of the phosphorylation-specific antibodies, the immunoreactivity of pS409/410 was particularly robust (Fig 1). In comparison, the commercially obtained, phosphorylation-independent, anti-TDP-43 antibody labeled NCIs, DNs, and neuronal nuclei in the dentate gyrus (see Fig 1A) and the temporal cortex (see Fig 1B) of the sporadic FTLU cases, and skein-like inclusions and neuronal nuclei in the spinal cord of ALS cases (see Fig 1C). It was particularly difficult to distinguish the staining of NCIs from that of neuronal nuclei in the dentate gyrus of the sporadic FTLU cases (see Fig 1A). By contrast, NCIs and DNs were unambiguously identified by the phosphorylation-specific antibody pS409/410, with no nuclear staining (see Figs 1D, E). Similarly, pS409/410 clearly labeled skein-like (see Fig 1F) and glial inclusions (see Fig 1G) in the spinal cord, and NCIs in the frontal and precentral cortices of the FTLU-MND and ALS cases (see Fig 1H). Glial inclusions in the frontal and precentral regions of the FTLU-MND and ALS cases were also immunopositive (data not shown). In the cases of familial FTLU with PGRN mutations, pS409/410 intensely stained NCIs, DNs, and NIIs in the cerebral cortex (see Figs 1I-K) and abundant positive structures in the cerebral white matter (see Fig 1L). The results of double-immunofluorescence staining showed that pS409/410 identified more NCIs than the ubiquitin antibody (see Figs 1M-O).

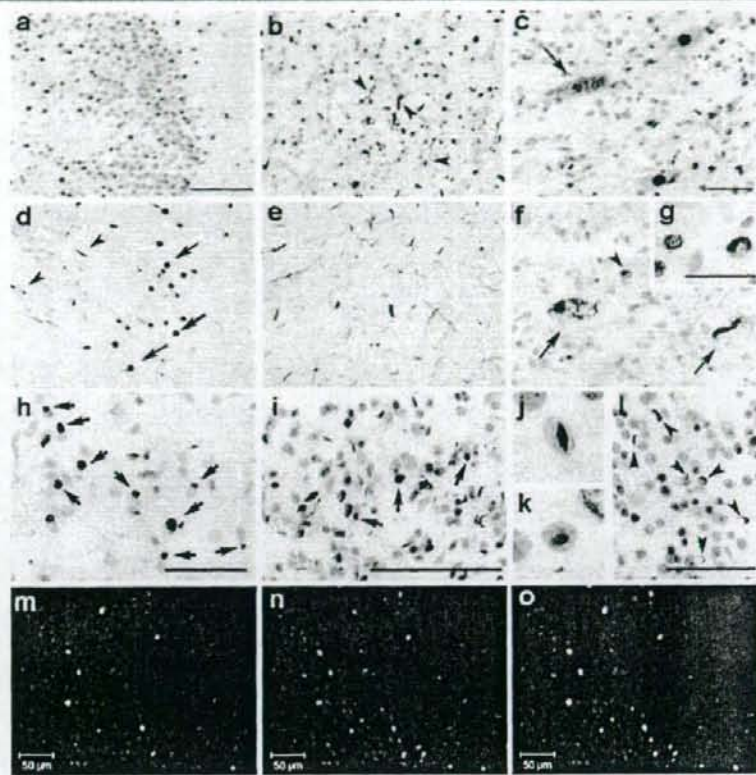
Based on morphological aspects, TDP-43 proteinopathies have been classified into four subtypes.<sup>24</sup> Type 1 is characterized by DNIs with few NCIs and no NIIs, type 2 has numerous NCIs with few DNIs and no NIIs, type 3 has numerous NCIs and DNIs and an occasional NII, and type 4 has numerous NIIs and DNIs with few NCIs. Type 4 is specific for familial FTLU with mutations of valosin-containing protein (VCP) gene. In this study, using the commercial anti-TDP-43 antibody, all of the sporadic FTLU cases showed type 1 pathology, all FTLU-MND and ALS cases showed type 2 pathology, and all FTLU with mPGRN showed type 3 pathology, in agreement with previous reports.<sup>24,25</sup>

The staining patterns obtained with our phosphorylation-dependent antibodies were similar to those seen

with the commercial phosphorylation-independent antibody, suggesting that all of the inclusion types previously described contain phosphorylated TDP-43. Similar staining patterns were obtained using pS379 (Figs 2A–C), pS403/404 (see Figs 2D–F), pS409 (see

Figs 2G–I), and pS410 (see Figs 2J–L). Preabsorption of the antibodies with phosphopeptide immunogens abolished the labeling of these structures (data not shown).

Immunoelectron microscopic examination of the



**Fig 1.** Immunohistochemical comparison of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) brains using the phosphorylation-independent anti-TAR DNA-binding protein of 43kDa (TDP-43) antibody (ProteinTech) (A–C) and the phosphorylation-dependent anti-TDP-43 antibody (pS409/410) (D–L), in the dentate gyrus (A, D) and temporal cortex (B, E) of the sporadic FTLD-U cases, in the lumbar spinal cord (C, F, G) and the frontal cortex (H) of the ALS cases, and in the frontal cortex (I–K) and the frontal white matter (L) of the familial FTLD-U cases with progranulin (PGRN) mutations. (A) Because most of the nuclei of dentate gyrus granular neurons are immunopositive with the phosphorylation-independent antibody, it is difficult to identify neuronal cytoplasmic inclusions (NCIs). (B) TDP-43-positive dystrophic neurites (DNs) are recognizable (arrowheads) in addition to the nuclei. (C) The black arrow indicates a cell with skein-like inclusions. White arrows and arrowheads indicate the normal nuclei of anterior horn cells and glial nuclei, respectively. Photomicrographs (D–F) illustrate the corresponding areas to (A–C), respectively. Note the absence of nuclear staining in (D–F) with the phosphorylation-dependent antibody pS409/410. (D) NCIs (arrows) and DNs (arrowheads) are clearly seen. (E) More abundant DNs are seen than in (B). (F) Arrows indicate skein-like inclusions; arrowheads indicate glial inclusions. (G, insert) Glial inclusions at a higher magnification. (H) NCIs in the frontal cortices of the ALS case are immunopositive. In the cases with PGRN mutations, pS409/410 clearly stains NCIs (arrows), DNs (I), and neuronal intranuclear inclusions (NIIs) (J, K) in the superficial cortical layers, and abundant immunopositive structures in the white matter (L, arrowheads), with no nuclear staining. Sections are counterstained with hematoxylin to show nuclei in (C, F–L). (M–O) Antiubiquitin (DF2) and pS409/410 double-label immunofluorescence histochemistry of the dentate gyrus in the FTLD-U case. Only some of the pS409/410-positive NCIs are also ubiquitin positive. (M) DF2; (N) pS409/410; (O) merge. Cell nuclei are stained with TO-PRO-3 (Invitrogen, Tokyo, Japan), producing a blue color. Scale bars = 100 $\mu$ m (A, B, D, E, I); 50 $\mu$ m (C, F, H, L); 25 $\mu$ m (G); 10 $\mu$ m (J, K).



spinal cord motoneuron inclusions of an ALS patient with the pS409/410 antibody showed immunopositive abnormal fibers of 15nm in diameter (Figs 3A, B).

#### Immunoblot Analysis of Phosphorylated TDP-43

Immunoblot analyses of sarkosyl-insoluble fractions extracted from the brains of control, AD, FTLD-U, and ALS cases with the phosphorylation-independent TDP-43 antibody (ProteinTech) always showed a band of 43kDa and also showed an additional 45kDa band that was present only in FTLD-U and ALS cases, as described previously<sup>12,13</sup> (Fig 4A). The phosphorylation-dependent antibodies specific for pS409/410 (see Fig 4B), pS409 (see Fig 4C), pS410 (see Fig 4D), pS403/404 (see Fig 4E), and pS379 (see Fig 4F) did not recognize the normal 43kDa band, showing a single band at approximately 45kDa, several smaller fragments at approximately 25kDa, and indistinct smears in FTLD-U and ALS cases but not in control and AD cases (see Figs 4B–F). The intensity of the approximately 25kDa fragments tended to be greater than that of the 45kDa band in FTLD-U (see Figs 4B–E) and in ALS (see Figs 4B, D). As for the immunohistochemical findings, the antibody to pS409/410 showed the most intense labeling (see Fig 4B). All of the immunoreactive bands were completely abolished by dephosphory-

lation, which was performed with lambda protein phosphatase (8,000U/ml; New England Biolabs) at 30°C for 2 hours.

#### Immunoblot Distinction between Clinicopathological Subtypes of TDP-43

To investigate the biochemical basis of the different TDP-43 clinicopathological subtypes, we have carefully compared the results of immunoblots of the sarkosyl-insoluble, urea-soluble fractions from cerebral cortex of sporadic FTLD-U, FTLD-MND, ALS, and mPGRN cases. The results showed that the band patterns of the 18 to 26kDa fragments differed between clinicopathological subtypes (Figs 5A, B). Sporadic FTLD-U cases showed 2 major bands at 23 and 24kDa, and 2 minor bands at 18 and 19kDa, whereas FTLD-MND and ALS cases showed 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 23kDa band is the most intense in sporadic FTLD-U, whereas a 24kDa band is the most intense in FTLD-MND and ALS. Furthermore, the band pattern of mPGRN cases was not distinctive but intermediate between FTLD-U, FTLD-MND, and ALS cases.

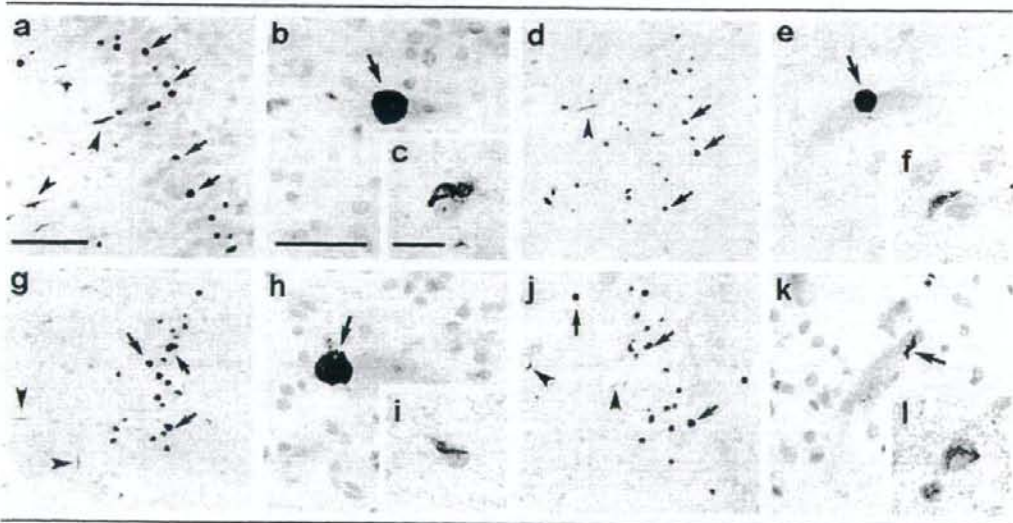


Fig 2. Immunohistochemistry of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) brains and amyotrophic lateral sclerosis (ALS) spinal cords using the phosphorylation-dependent anti-TAR DNA-binding protein of 43kDa (TDP-43) antibodies specific for pS379 (A–C), pS403/404 (D–F), pS409 (G–I), and pS410 (J–L). These antibodies recognize neuronal cytoplasmic inclusions (NCIs) (arrows in A, D, G, J) and dystrophic neurites (DNs) (arrowheads in A, D, G, J) in the dentate gyrus of the sporadic FTLD-U cases and motoneuronal round inclusions (arrow in B, E, H), skein-like inclusion (K, arrow), and glial inclusions (C, F, I, L) in the lumbar spinal cord of the ALS cases. Note the absence of nuclear staining. Sections are counterstained with hematoxylin to show nuclei in (A–C, E, F, H, I, K, L). Scale bars = 100µm (A, D, G, J); 25µm (B, E, H, K); 12.5µm (C, F, I, L).



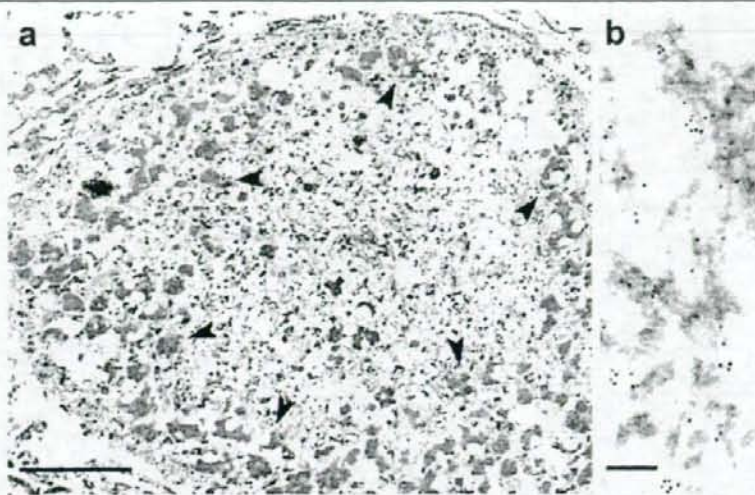


Fig 3. (A) A low-power immunoelectron micrograph of a phosphorylated TAR DNA-binding protein of 43kDa (TDP-43)-positive motoneuronal inclusion in the spinal cord of an amyotrophic lateral sclerosis (ALS) patient. The irregularly shaped structure surrounded by lipofuscins (arrowheads) is the inclusion. (B) At higher magnification, abnormal filaments of 15nm in diameter are immunopositive. Immunoreaction with pS409/410, probed with immunogold particles (diameter 10nm), appears as black dots. Bars = 5µm (A); 500nm (B).

#### Phosphorylation Epitopes Are Generated by Casein Kinase-1

To investigate the kinase responsible for the abnormal phosphorylation of TDP-43, we treated recombinant TDP-43 *in vitro* with CK1, CK2, and GSK3β. Immunoblot analyses of the recombinant TDP-43 showed that phosphorylation by CK1 caused a reduction in gel mobility of TDP-43 to approximately 45kDa and strong immunoreactivity to the phosphorylation-specific antibodies (Fig 6A). TDP-43 phosphorylated by CK2 was only weakly immunoreactive for these antibodies (see Fig 6A), and that phosphorylated by GSK3β was negative (data not shown). Kinase activity capable of generating the approximately 45kDa TDP-43 with pS409/410 epitopes was also detected in crude rat brain extracted with a high concentration (10–20mM) of MgCl<sub>2</sub> (data not shown). This kinase activity was not inhibited by the CK2 inhibitor heparin, suggesting that CK1 may be the major kinase in brain extract. Interestingly, increased levels of sodium dodecyl sulfate-stable TDP-43 oligomers were observed after phosphorylation by CK1 (see Fig 6B). Furthermore, based on immunoelectron microscopic analysis, recombinant TDP-43 phosphorylated by CK1 formed abundant filaments when applied on a carbon-coated copper grid (see Fig 6C), whereas nonphosphorylated recombinant TDP-43 formed few filaments (data not shown).

#### Discussion

We show here that antibodies generated to multiple TDP-43 phosphorylation sites stain the pathological structures in FTL-D-U and ALS. These structures include NCIs, NIIs, and DNIs in the cerebral cortex and hippocampus, as well as skein-like, round, and glial inclusions in the spinal cord. The phosphorylation-dependent antibodies stain these structures more extensively than an anti-ubiquitin antibody and do not stain normal neuronal nuclei. Furthermore, on immunoelectron microscopy, the phosphorylation-dependent antibodies label abnormal filaments in the motoneuronal inclusion of the ALS case, although these findings may not be the same as for other types of cytoplasmic and intranuclear inclusions.<sup>26</sup> Immunoblot analysis of sarkosyl-insoluble fractions from FTL-D-U and ALS brains shows that these antibodies specifically stain abnormal TDP-43 species. These findings are therefore analogous to previous discoveries of phosphorylation-specific epitopes for tau and α-synuclein in tauopathies and α-synucleinopathies.<sup>27–29</sup>

At least five sites on TDP-43 are phosphorylated (Ser 379, Ser 403/404, Ser 409/410) in subjects with FTL-D-U and ALS. These results suggest that abnormal phosphorylation takes place mainly near the carboxyl (C)-terminal region of TDP-43. This again is similar to tauopathies and synucleinopathies,<sup>27,28</sup> where multiple Ser residues in the C-terminal region, including

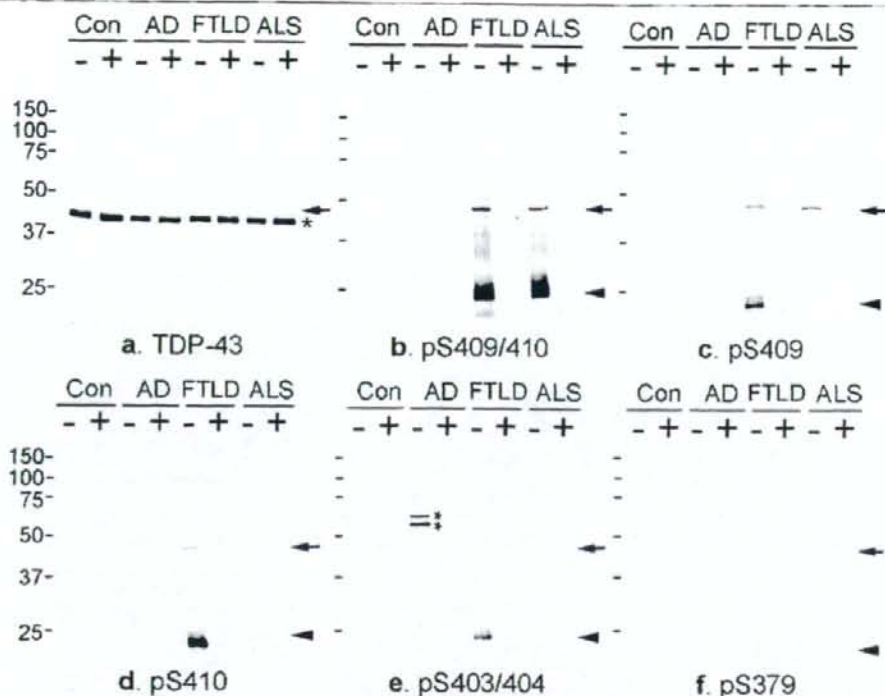


Fig 4. (A) Immunoblot analyses of sarkosyl-insoluble, urea-soluble fractions from control, Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD; frontotemporal lobar degeneration with ubiquitinated inclusions [FTLD-U]), and amyotrophic lateral sclerosis (ALS) brains with phosphorylation-independent anti-TAR DNA-binding protein of 43kDa (TDP-43) antibody (Protein-Tech) (A) and phosphorylation-dependent anti-TDP-43 antibodies specific for pS409/410 (B), pS409 (C), pS410 (D), pS403/404 (E), and pS379 (F) before (-) and after (+) the treatment with lambda protein phosphatase ( $\lambda$ PPase). (A) With the phosphorylation-independent antibody, a positive band of 43kDa is commonly seen (asterisk), whereas an additional band of 45kDa is observed only in FTLD and ALS (arrow), the labeling of which is abolished after dephosphorylation. (B-F) The phosphorylation-dependent antibodies specifically label the approximately 45kDa band (arrow) and the approximately 25kDa fragment (arrowhead), as well as a smear, only in FTLD and ALS. These immunoreactivities are abolished after dephosphorylation. Normal 43kDa TDP-43 in control and diseased brains is not stained by these phosphorylation-dependent antibodies. The two bands recognized by the antibody specific for pS403/404 in AD (E, double asterisk) disappear after dephosphorylation, suggesting a cross-reaction of the antibody to other phosphorylated proteins.

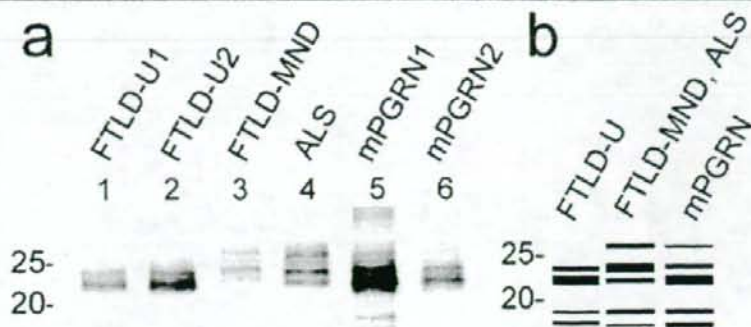
Ser422 in tau and C-terminal Ser129 in  $\alpha$ -synuclein, are abnormally phosphorylated. It has been established that hyperphosphorylated tau and  $\alpha$ -synuclein represent the earliest detectable molecular change in the brain in these neurodegenerative diseases.<sup>29,30</sup> Thus, the results of this study suggest that abnormally phosphorylated TDP-43 is a critical component of UPIs in FTLD-U and ALS.

There is a close relation between the pathological subtypes of TDP-43 proteinopathy and the immunoblot pattern of C-terminal fragments of phosphorylated TDP-43. These findings confirm and extend Sampathu and colleagues<sup>31</sup> and Neumann and coworkers<sup>12</sup> previous reports that showed C-terminal fragment compo-

sition varied between cases with type 1 and 2 pathology. Furthermore, we have shown that cases with type 3 pathology have a band pattern that is mixed or intermediate. These results parallel our earlier findings of differing C-terminal tau fragments in progressive supranuclear palsy and corticobasal degeneration, despite identical composition of tau isoforms.<sup>32</sup> Taken together, these results suggest that elucidating the mechanism of C-terminal fragment origination may shed light on the pathogenesis of several neurodegenerative disorders involving TDP-43 proteinopathy and tauopathy.

These phosphorylation-specific antibodies are a new and powerful tool for the investigation of TDP-43 pro-





**Fig 5.** A relation between the clinicopathological subtypes of TAR DNA-binding protein of 43kDa (TDP-43) proteinopathies and the band pattern of the C-terminal fragments of phosphorylated TDP-43. (A) Immunoblots of the sarkosyl-insoluble, urea-soluble fractions from sporadic frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U), FTLD-motor neuron disease (MND), amyotrophic lateral sclerosis (ALS), and progranulin mutations (mPGRN) cases with the pS409/410 antibody. The samples are loaded on 15% polyacrylamide gel. Sporadic FTLD-U cases (lanes 1, 2) show a band pattern with 2 major bands at 23 and 24kDa, and 2 minor bands at 18 and 19kDa. A band of 24kDa is weaker than that of 23kDa, and a 19kDa band is weaker than an 18kDa band. FTLD-MND (lane 3) and ALS (lane 4) cases show a pattern with 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 24kDa band is the most intense, and an 18kDa band is weaker than a 19kDa band. mPGRN (lanes 5, 6) cases show 3 major bands at 23, 24, and 26kDa, and 2 minor bands at 18 and 19kDa. A 23kDa band is the most intense, and a band of 18kDa and that of 19kDa show similar intensity. The band pattern of mPGRN cases is therefore a composite of that seen in FTLD-U, FTLD-MND, and ALS. (B) Schematic diagram of the band pattern of the C-terminal fragments of phosphorylated TDP-43.

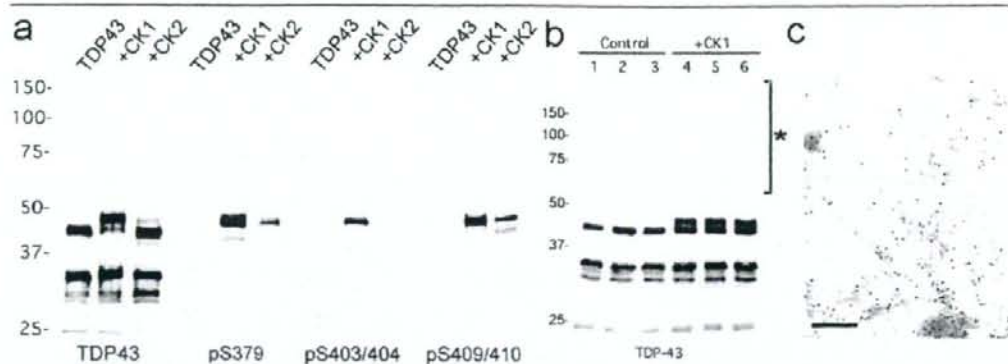
teinopathies. Because phosphorylation-dependent antibodies to TDP-43 react only with abnormally deposited TDP-43, they offer advantages over existing commercially available antibodies for the pathological diagnosis and subtyping of TDP-43 proteinopathies. In addition, and again in analogy with tauopathies, these antibodies may be useful for detecting abnormal TDP-43 in biological fluids such as cerebrospinal fluid.<sup>33</sup>

The results suggest that CK1 is involved in the abnormal phosphorylation and accumulation of TDP-43. In this study, the treatment of recombinant TDP-43 by CK1 generates the same phosphorylation epitopes that are recognized by phosphorylation-dependent antibodies. In addition, phosphorylation at these epitopes facilitates filament formation. In comparison, several protein kinases have been reported to be responsible for phosphorylating tau and  $\alpha$ -synuclein. They include, for tau phosphorylation,<sup>34–37</sup> GSK3 $\beta$ , cyclin-dependent kinase 5, mitogen-activated protein kinase, and mitogen-activated protein/microtubule affinity-regulating kinase, and for  $\alpha$ -synuclein phosphorylation,<sup>38–40</sup> CK1, CK2, and G-protein-coupled receptor kinase 5.

The pathological significance of phosphorylation of TDP-43 is not clear. It is well known that protein phosphorylation plays an important role in regulating

transcription and pre-messenger RNA splicing. Several splicing factors including hnRNPs, small nuclear ribonucleoproteins, and serine/arginine-rich protein family are known to be phosphorylated *in vivo*. Various kinases including CK1 have been implicated in phosphorylating these factors.<sup>41–43</sup> Phosphorylation of these factors modulates protein-protein and protein-RNA interactions, and affects their subcellular localization and physiological functions.<sup>41</sup> For instance, Habelhah and colleagues<sup>44</sup> showed that phosphorylation of hnRNP-K by extracellular-signal-regulated kinase results in its cytoplasmic accumulation and also inhibits messenger RNA translation. van der Hoven van Oordt and co-authors reported that stress-induced activation of the mitogen-activated protein kinase kinase<sub>3/6</sub>-p38 pathway causes hyperphosphorylation and cytoplasmic accumulation of hnRNP A1, affecting alternative splicing regulation.<sup>45</sup> Thus, phosphorylation of TDP-43 may lead to its cytoplasmic accumulation and influence various physiological functions. Currently, however, it is unclear whether TDP-43 is physiologically phosphorylated in brain. Although in HeLa cells, Ser91 and Ser92 of TDP-43 were reported to be phosphorylated,<sup>46</sup> the antibody specific to pS91/pS92 we made in this study did not stain any structures in normal brains (data not shown). Despite the normal nuclear location of TDP-43, none of our five phosphorylation-





**Fig. 6.** (A) Immunoblot analyses of recombinant TAR DNA-binding protein of 43kDa (TDP-43) phosphorylated *in vitro*. The crude extract from *E. coli* that expressed human TDP-43 is treated with casein kinase-1 (CK1) and CK2 at 30°C for 14 hours, and probed with a phosphorylation-independent antibody against a C-terminal peptide of TDP-43 (405–414), and with phosphorylation-dependent antibodies pS379, pS403/404, and pS409/410. Phosphorylation by CK1 causes the mobility shift to approximately 45kDa and induction of intense immunoreactivity to the phosphorylation-dependent antibodies. (B) Immunoblot analyses of recombinant TDP-43 phosphorylated by CK1. The recombinant TDP-43, which is partially purified by heparin-Toyopearl column chromatography, is incubated with (lanes 4–6) or without (lanes 1–3) CK1 in the presence of adenosine triphosphate at 37°C for 14 hours, and probed with the phosphorylation-independent TDP-43 antibody (ProteinTech). Results in three independent, representative experiments are shown. Note the sodium dodecyl sulfate (SDS)-stable TDP-43 oligomers at approximately 100 to 200kDa (asterisk) are detected after phosphorylation by CK1. (C) Positive immunolabeling by pS409/410 of filaments assembled from recombinant TDP-43 phosphorylated by CK1 (10nm colloidal gold). Scale bar = 200nm.

dependent antibodies stained normal nuclei, suggesting that phosphorylation of these sites is a disease-specific phenomenon.

Our *in vitro* studies suggest that phosphorylation of TDP-43 facilitates the formation of sodium dodecyl sulfate-stable oligomers and filaments of TDP-43. These abnormal structures may be neurotoxic, as suggested previously for tauopathies and  $\alpha$ -synucleinopathies.<sup>30</sup> Thus, abnormal phosphorylation of TDP-43 may be pathological through either a loss of function or a toxic gain of function, or both, leading to the characteristic neuronal degeneration and clinical syndromes.

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research on Priority Areas—Research on Pathomechanisms of Brain Disorders, M.H. (20023038); a Grant-in-Aid for Scientific Research, M.H. (18300117); Grants-in-Aid for Scientific Research, T.A. (19591024), T.N. (19590299)); Grants-in-Aid from the Research Committee of CNS Degenerative Diseases, the Ministry of Health, Labour and Welfare of Japan, I.N. and M.Y. (20261501); the Brain Donation Program at Sun Health Research Institute is supported by the NIH (National Institute on Aging, P30 AG19610), Arizona Alzheimer's Disease Core Center, the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, and 05-901), and the Prescott Family Initiative of the Michael J. Fox Foundation for Parkinson's Research; F.B. and E.B. were supported by Telethon Onlus Foundation, Italy (GGP06147) and by a European Community Grant (EURASNET-LSHG-CT-2005-518238).

We thank H. Kondo and Y. Izumiya for their excellent technical assistance.

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## Inverse Ocular Bobbing in a Patient With Encephalitis Associated With Antibodies to the N-methyl-D-aspartate Receptor

**A** 30-YEAR-OLD WOMAN presented with headache, fever, disorientation, and recent memory disturbance. Brain magnetic resonance imaging showed fluid-attenuated inversion recovery hyperintense abnormalities in both hippocampi, without abnormal findings in other areas of the brain or brainstem.<sup>1</sup> The patient subsequently developed tonic convulsions, restlessness, anxiety, and hypoventilation that led to the use of sedation and mechanical ventilation. While in the intensive care unit, inverse ocular bobbing and skew deviation were transiently observed (Figure) (a video is available at <http://www.archneurology.com>). Antibodies to NR1/NR2 heteromers of the N-methyl-D-aspartate receptor were identified in her serum and cerebrospinal fluid. After immunotherapy and removal of an ovarian teratoma, all symptoms started to improve and the patient was able to return to her job 1 year later.

### COMMENT

Inverse ocular bobbing, or ocular dipping, consists of a slow, spontaneous downward eye movement with fast return to midposition. In

 Video available online at [www.archneurology.com](http://www.archneurology.com)

contrast to ocular bobbing, which is most often attributed to large, destructive pontine lesions, ocular dipping may be observed in anoxic coma or following prolonged status epilepticus and is thought to be a marker of diffuse, rather than focal, brain damage.<sup>2</sup> The encephalitis associated with antibodies to the N-methyl-D-aspartate receptor of-



**Figure.** The position of the patient's eyes showed skew deviation when the inverse ocular bobbing resolved.

ten associates with central hypoventilation and, much less frequently, with vertical gaze paresis or cranial nerve dysfunction, all suggesting involvement of the brainstem.<sup>3</sup> In the current case with head magnetic resonance images that did not show any signal abnormality in the brainstem, the association of ocular dipping with hypoventilation and skew deviation suggests that the diffuse encephalopathy in addition to involvement of the brainstem ocular pathways were responsible for the abnormal ocular movement.

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Dalmau. Drafting of the manuscript: Shimazaki. Critical revision of the manuscript for important intellectual content: Shimazaki, Morita, Nakano, and Dalmau. Administrative, technical, and material support: Shimazaki. Study supervision: Morita, Nakano, and Dalmau.

**Financial Disclosure:** None reported. **Funding/Support:** This work was partially supported by a Grant-in-Aid for Scientific Research (C) (20591009 to Dr Shimazaki) of The Ministry of Education, Culture, Sports, Science and Technology in Japan.

**Additional Information:** Video is available at <http://www.archneurology.com>.

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