calcium.

In addition to physical anomalies, widespread aberrant high signal lesions of cerebral white matter, basal ganglia and brainstem may imply neurodegeneration of the corticospinal tract, which might be caused by some aberrant gap junctions between cells. Mutant Cx43 may have some correlation between the expression of a CNS phenotype and peripheral nerves. To date, no pathology and no postmortem data have been reported, which could help in the search for the gene product responsible for ODDD.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research (C) to M. Ikeda (23591231), K. Okamoto (20591017) and Y. Fujita (21591108) and by grants from the Ministry of Health, Labour, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K. Okamoto, and also supported by Grants-in-Aid from the Research Committee of CNS Degenerative Diseases, the Ministry of Health, Labour and Welfare of Japan to K. Okamoto.

References

1. Reisner SH, Kott E, Bornstein B, Salinger H, Kaplan I, Gordin RJ. Oculodentodigital dysplasia. Am J Dis Child **118**: 600-607, 1969.
2. Patton MA, Laurence KM. Three new cases of oculodentodigital (ODD) syndrome: development of the facial phenotype. J Med Genet **22**: 386-389, 1985.
3. Fára M, Horák I, Hrivnáková J, Kapras J, Nová M, Stloukalová M. Oculodentodigital dysplasia. Acta Chir Plast **19**: 110-122, 1977.
4. Loddenkemper T, Grote K, Evers S, Oelerich M, Stögbauser F. Neurological manifestations of the oculodentodigital dysplasia syndrome. J Neurol **249**: 584-595, 2002.
5. Paznekas WA, Boyadjiev SA, Shapiro RE, et al. Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. Am J Hum Genet **72**: 408-418, 2003.
6. Joss SK, Ghazawy S, Tomkins S, Ahmed M, Bradbury J, Sheridan E. Variable expression of neurological phenotype in autosomal recessive oculodentodigital dysplasia of two sibs and review of the literature. Eur J Pediatr **167**: 341-345, 2008.
7. Frasson M, Calixto N, Cronemberger S, de Aguiar RA, Leão LL, de Aguiar MJ. Oculodentodigital dysplasia: study of ophthalmological and clinical manifestations in three boys with probably autosomal recessive inheritance. Ophthalmic Genet **25**: 227-236, 2004.
8. Richardson RJ, Donnai D, Meire F, Dixon M. Expression of Gja1 correlates with the phenotype observed in oculodentodigital syndrome/type III syndactyly. J Med Genet **41**: 60-67, 2004.
9. Kjaer KW, Hansen L, Eiberg H, Leicht P, Optz JM, Tommerup N. Novel Connexin 43 (GJA1) mutation causes oculo-dento-digital dysplasia with curly hair. Am J Med Genet A **127**: 152-157, 2004.
10. Pizzuti A, Flex E, Mingarelli R, Salpietro C, Zelante L, Dallapiccola B. A homozygous GJA1 gene mutation causes a Hallermann-Streiff/ODDD spectrum phenotype. Hum Mutat **23**: 286, 2004.
11. van Steensel MA, Spruijt L, van der Burgt I, et al. A 2-bp deletion in the GJA1 gene is associated with oculo-dento-digital dysplasia with palmoplantar keratoderma. Am J Med Genet A **132**: 171-174, 2005.
12. Vitiello C, D'Adamo P, Gentile F, Vingolo EM, Gasparini P, Banfi S. A novel GJA1 mutation causes oculodentodigital dysplasia without syndactyly. Am J Med Genet A **133A**: 58-60, 2005.
13. Paznekas WA, Karczeski B, Vermeer S, et al. GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. Human Mutation **30**(Suppl): 724-733, 2009.
14. Ikeda M, Abe K, Aoki M, et al. Variable clinical symptoms in familial amyotrophic lateral sclerosis with a novel point mutation in the Cu/Zn superoxide dismutase gene. Neurology **45**: 2038-2042, 1995.
15. Ikeda M, Sharma V, Sumi SM, et al. The clinical phenotype of two missense mutations in the presenilin I gene in Japanese patients. Ann Neurol **40**: 912-917, 1996.
16. Richardson R, Donnai D, Meire F, Dixon MJ. Expression of Gja1 correlates with the phenotype observed in oculodentodigital syndrome/type III syndactyly. J Med Genet **41**: 60-67, 2004.
17. Beyer EC, Steinberg TH. Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. J Biol Chem **266**: 7971-7974, 1991.
18. Cottrell GT, Wu Y, Burt JM. Cx40 and Cx43 expression ratio influences heteromeric/heterotypic gap junction channel properties. Am J Physiol Cell Physiol **282**: C1469-C1482, 2002.
19. Cottrell GT, Burt JM. Functional consequences of heterogeneous gap junction channel formation and its influence in health and disease. Biochim Biophys Acta **1711**: 126-141, 2005.
20. Trosko JE, Madhukar BV, Chang CC. Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. Life Sci **53**: 1-19, 1993.
21. Norton KK, Carey JC, Gutmann DH. Oculodentodigital dysplasia with cerebral white matter abnormalities in a two-generation family. Am J Med Genet **57**: 458-461, 1995.
22. Audry D, Dumas R, Nivelon A, Audry F. Neurological manifestations of oculodentodigital dysplasia: a case report. Rev Otoneuroophthalmol **53**: 209-214, 1981 (in French).
23. Ginsberg LE, Jewett T, Grub R, McLean WT. Oculodental digital dysplasia: neuroimaging in a kindred. Neuroradiology **38**: 84-86, 1996.
24. Mambetisaeva ET, Gire V, Evans WH. Multiple connexin expression in peripheral nerve, Schwann cells, and Schwannoma cells. J Neurosci Res **57**: 166-175, 1999.
25. Taylor RA, Simon EM, Marks HG, Scherer SS. The CNS phenotype of X-linked Charcot-Marie-Tooth disease: more than a peripheral problem. Neurology **61**: 1475-1478, 2003.
26. Laird DW. Closing the gap on autosomal dominant connexin-26 and connexin-43 mutants linked to human disease. J Biol Chem **283**: 2997-3001, 2008.
27. Mazzeo A, Di Leo R, Toscano A, et al. Charcot-Marie-Tooth type X: unusual phenotype of a novel CX32 mutation. Eur J Neurol **15**: 1140-1142, 2008.
28. Kassubek J, Bretschneider V, Sperfeld AD. Corticospinal tract MRI hyperintensity in X-linked Charcot-Marie-Tooth Disease. J Clin Neurosci **12**: 588-589, 2005.
29. Roscoe W, Veitch GI, Gong XQ, et al. Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. J Biol Chem **280**: 11458-11466, 2005.
30. Locke D, Harris AL. Connexin channels and phospholipids: association and modulation. BMC Biol **7**: 52-75, 2009.
31. Peracchia C, Sotkis A, Wang XG, Peracchia LL, Persechini A. Calmodulin directly gates gap junction channels. J Biol Chem **275**: 26220-26224, 2000.

NEUROPATHOLOGY

Neuropathology 2013; ••, ••–••

doi:10.1111/neup.12010

Original Article

Reduced expression of BTBD10 in anterior horn cells with Golgi fragmentation and pTDP-43-positive inclusions in patients with sporadic amyotrophic lateral sclerosis

Natsumi Furuta,[1] Kouki Makioka,[1] Yukio Fujita,[1] Masaki Ikeda,[1] Masamitsu Takatama,[2] Masaaki Matsuoka[3] and Koichi Okamoto[1]

[1]Department of Neurology, Gunma University Graduate School of Medicine, [2]Department of Internal Medicine, Geriatrics Research Institute and Hospital, Gunma and [3]Department of Pharmacology, Tokyo Medical University, Tokyo, Japan

Overexpression of BTBD10 (BTB/POZ domain-containing protein 10) suppresses G93A-superoxide dismutase 1 (SOD1)-induced motor neuron death in a cell-based amyotrophic lateral sclerosis (ALS) model. In the present study, paraffin sections of spinal cords from 13 patients with sporadic ALS and 10 with non-ALS disorders were immunostained using a polyclonal anti-BTBD10 antibody. Reduced BTBD10 expression in the anterior horn cells was more frequent in spinal cords from ALS patients than in cords from patients with non-ALS disorders. We further investigated the relationship between the level of BTBD10 immunoreactivity and the morphology of the Golgi apparatus (GA) and the presence of phosphorylated TAR-DNA-binding protein 43 (pTDP-43). Mirror sections of spinal cords from five sporadic ALS cases were immunostained with antibodies against BTBD10 and trans-Golgi-network (TGN)-46 or pTDP-43. Whereas 89.7–96.5% of the neurons with normal BTBD10 immunoreactivity showed normal GA morphology and no pTDP-43 cytoplasmic aggregates, 86.2–94.3% of the neurons with reduced BTBD10 expression showed GA fragmentation and abnormal pTDP-43 aggregates. These findings suggest that reduced BTBD10 expression is closely linked to the pathogenesis of sporadic ALS.

Key words: ALS, BTBD10, Golgi apparatus, pathology, pTDP-43.

Correspondence: Natsumi Furuta, MD, Department of Neurology, Gunma University Graduate School of Medicine, Gunma 371-8511, Japan. Email: n-furuta@gunma-u.ac.jp

Received 15 October 2012; revised 16 November 2012 and accepted 25 November 2012.

INTRODUCTION

BTBD10 (BTB/POZ domain-containing protein 10) activates Akt by inhibiting the protein phosphatase 2A-mediated dephosphorylation and inactivation of Akt.[1,2] Akt family proteins, which are composed of Akt1, Akt2 and Akt3 in mammalian cells, mediate the primary intracellular critical prosurvival signal by phosphorylating many target proteins. They play a central role in the control of cell survival, growth, proliferation, and apoptosis throughout the body. Akt malfunction can cause a variety of complex diseases, including type-2 diabetes and cancer.[3,4] BTBD10 was first cloned by Chen *et al.*[5] It was found to be downregulated in gliomas[5] and identified as a molecule that interacts with Akt3.[1,2] BTBD10 equally binds to any Akt.[1]

The autosomal-recessive familial amyotrophic lateral sclerosis (ALS)-linked gene 2 (ALS2) product, alsin long form (LF), is involved in intracellular vesicle/membrane trafficking and endosome dynamics. Loss of alsin LF function contributes to motor neuron death.[6] In addition, alsin LF inhibits familial ALS-related Cu/Zn superoxide dismutase 1 (SOD1) mutant-induced neurotoxicity through activation of the prosurvival signal cascade mediated by Rac1/PI3K/Akt3. Akt3 is the major downstream mediator for alsin LF-mediated neuroprotection.[7]

Overexpression of BTBD10 inhibits G93A-SOD1-induced motor neuron death. In agreement with BTBD10-mediated upregulation of the Akt phosphorylation levels, enforced expression of BTBD10 leads to the suppression of mutant SOD-1-induced neuronal death *in vitro*.[1] In a recent study, Nawa *et al.* further suggested that BTBD10 expression is reduced in the anterior horn cells of the

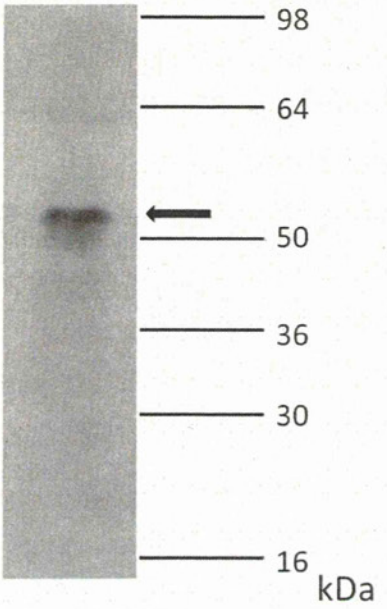

Fig. 1 Anti-BTBD10 (BTB/POZ domain-containing protein 10) antibody was used in immunoblot analysis of lysates from whole normal human brain. The antibody revealed an immunoreactive band around 54 kDa (arrow).

spinal cord in sporadic ALS patients.[8] These findings suggest that BTBD10 is closely linked to motor neuron death. However, whether the reduced expression of BTBD10 is linked to human ALS pathogenesis remains insufficiently defined.

The Golgi apparatus (GA) is frequently fragmented in the anterior horn cells of patients with sporadic ALS; that is, the GA loses its normal network-like configuration, which is replaced by disconnected small elements.[9,10] In addition, TAR-DNA-binding protein 43 (TDP-43) is a major disease marker in ALS and frontotemporal lobar degeneration with TDP-43.[11-13] Using specific antibodies to phosphorylated TDP-43 (pTDP-43), Hasegawa *et al.* found immunoreactivity in abnormal inclusions, but not nuclei, which are the normal physiological site for TDP-43 localization.[14]

In the present study, we used immunohistological methods to investigate the relationship between the reduced BTBD10 expression and fragmentation of the GA, and the presence of pTDP-43 aggregates in the anterior horn cells of spinal cords from patients with sporadic ALS.

MATERIALS AND METHODS

We examined the spinal cords of 13 patients with sporadic ALS (age at death: 56–75 years, mean: 64.3 years; 7 men and 6 women) and 10 patients diagnosed with various kinds of non-ALS disorders (age at death: 64–96 years, mean: 81.0 years; 8 men and 2 women). None of the patients had a family history of ALS. Autopsy samples were obtained from patients of Gunma University Hospital and Geriatric Research Hospital, japan, under established procedures after obtaining informed consent from the family of each patient. ALS patients were definitively diagnosed based on clinical and light microscopic findings. Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin.

Five-micrometer-thick transverse paraffin sections of the lumbar spinal cords were prepared for HE staining and immunohistochemical analysis using a rabbit polyclonal anti-BTBD10 antibody (1:1000) generated in our laboratory. The rabbit polyclonal antibody was generated by immunization with a synthetic peptide, CPSGNSDLDP-DAQNPML, corresponding to the C-terminal 16-amino-acid peptide sequence of human BTBD10, and conjugated to keyhole limpet hemocyanin (Technical Keystone Craft, Takasaki, Japan).

The anti-BTBD10-antibody was used in immunoblot analysis of lysates from whole normal human brain (Novus Biologicals, Littleton, CO, USA) to investigate its specifi-

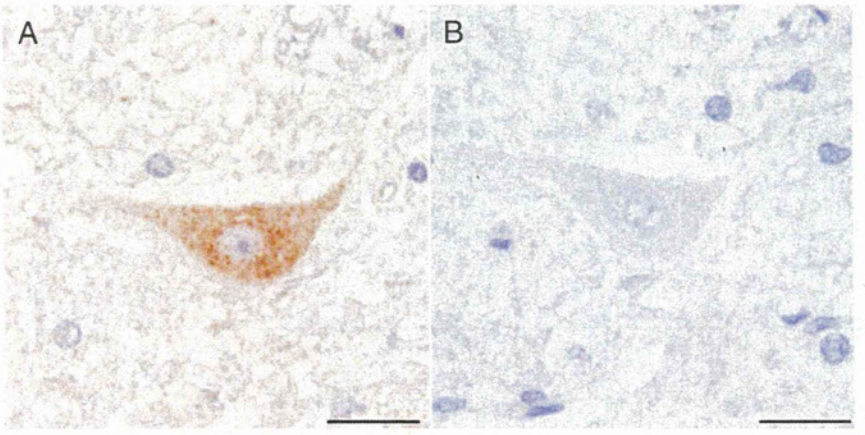

Fig. 2 BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity. Immunostaining for BTBD10 (A) and for BTBD10 preabsorbed with BTBD10 recombinant protein (B). BTBD10 immunostaining was obviously specific. Scale bar: 20 µm.

city. The immunoblotting revealed a single band at around 54 kDa (Fig. 1). Antibody specificity was also analyzed by adsorption tests using immunohistochemistry. A mirror section was immunostained after preadsorption of anti-BTBD10 antibody with BTBD10 recombinant protein. BTBD10-positive staining was not present as a result of the preadsorption (Fig. 2), indicating the fidelity of the immunoreactivity.

To enhance staining in the immunohistochemical analysis, the samples were autoclaved (121°C, 10 min). After endogenous peroxidase activity was quenched in 0.3% H_2O_2 (30 min), nonspecific binding sites on the sections were blocked with normal horse serum for 30 min at room temperature, then incubated with the primary antibody at 4°C overnight, washed in PBS for 30 min, incubated with the secondary antibody provided in a Histofine SAB-PO kit (Nichirei, Tokyo, Japan), and washed in PBS for 30 min. Finally, immunoreactivity was visualized using an avidin–biotin–peroxidase method. Sections were examined using an Olympus BX50 microscope.

After immunostaining the anterior horn cells of lumbar spinal cords from both sporadic ALS and non-ALS

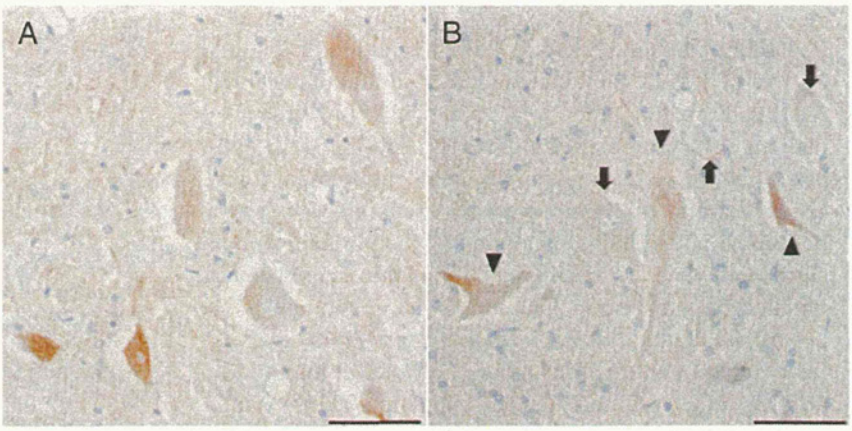

Fig. 3 Immunostaining with anti-BTBD10 (BTB/POZ domain-containing protein 10) antibody. In control cases (A), BTBD10-positive small granular cytoplasmic immunostaining was observed diffusely in the anterior horn cells. In amyotrophic lateral sclerosis (ALS) cases (B), the number of BTBD10-positive neurons was significantly lower in the anterior horns (arrow head). Neurons with reduced BTBD10 immunoreactivity are shown with arrows. The reduction in BTBD10 immunoreactivity was observed more frequently in large neurons than in small neurons. Bars, A, B: 30 μm.

Table 1 The spinal cords in 13 sporadic ALS patients and 10 non-ALS patients were immunostained with antibody against BTBD10.

Case	Sex	Age at death (years)	Diagnosis	Duration of disease (months)	Respirator	Reduced BTBD10/All remaining neurons (%)
1	M	75	SALS	7	(−)	71/136 (52.2%)
2	F	69	SALS	15	(−)	65/135 (48.1%)
3	M	62	SALS	24	(−)	89/166 (53.6%)
4	F	56	SALS	25	(−)	77/148 (52.0%)
5	M	64	SALS	29	(−)	84/141 (59.5%)
6	F	68	SALS	30	(−)	86/171 (50.3%)
7	M	61	SALS	30	(−)	60/116 (51.7%)
8	F	60	SALS	33	5 days	55/105 (52.4%)
9	F	70	SALS	36	(−)	80/153 (52.3%)
10	M	57	SALS	48	(−)	47/93 (50.5%)
11	F	64	SALS	84	(−)	67/122 (54.9%)
12	M	65	SALS	96	31 months	54/97 (55.7%)
13	M	66	SALS	108	(−)	53/129 (42.1%)
14	M	90	AD			9/189 (4.8%)
15	M	82	Pneumoniae			11/221 (5.0%)
16	M	96	Colon carcinoma			11/224 (4.9%)
17	F	81	AD			14/274 (5.1%)
18	M	65	Pneumoniae			11/223 (4.9%)
19	M	91	Pneumoniae			9/206 (4.4%)
20	M	71	AMI			11/227 (4.8%)
21	F	86	Ileus			13/223 (5.8%)
22	M	75	RA			8/201 (4.0%)
23	M	64	Gastric carcinoma			12/207 (5.8%)

Reduction in BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity was observed more frequently in the anterior horn cells of patients with sporadic amyotrophic lateral sclerosis (SALS). There was no apparent relationship between the disease duration and the proportion of neurons with reduced BTBD10 immunoreactivity. AD, Alzheimer disease; AMI, acute myocardial infarction; F, female; M, male; RA, rheumatoid arthritis.

patients using the anti-BTBD10 antibody, the numbers of neurons with normal and reduced BTBD10 immunoreactivity were counted.

BTBD10 was found to be markedly expressed in neurons in the spinal anterior horns and only minimally expressed in astrocytes. BTBD10-positive motor neurons were defined as neurons with staining intensity stronger than that of the background neuropil of surrounding motor neurons (Fig. 3A). Most of the BTBD10-positive motor neurons were slightly positive. Other neurons with reduced staining were defined as neurons with reduced BTBD10 immunoreactivity (Fig. 3B). We investigated both large and small nucleated neurons in the anterior horn cells.

To investigate further the relationship between BTBD10 immunoreactivity and fragmentation of the GA or the pTDP-43 immunoreactivity, we immunostained 3-μm-thick mirror sections of lumbar spinal cords from five patients with sporadic ALS using the anti-BTBD10 antibody (1:1000) and rabbit polyclonal anti-human trans-Golgi-network (TGN)-46 antibody (1:1000)[15] or rabbit polyclonal anti-pTDP-43 antibody (1:1000).[16] These three antibodies were generated in our laboratory. To detect immunoreactivity with the anti-TGN-46 antibody, the peroxidase–antibody complex was visualized using a VIP Substrate kit (Vector, Tokyo, Japan). To detect immunoreactivity with the anti-BTBD10 and anti-pTDP-43 antibodies, diaminobenzidine was used as the chromogen. Subsequently, micrographs of the sections were obtained.

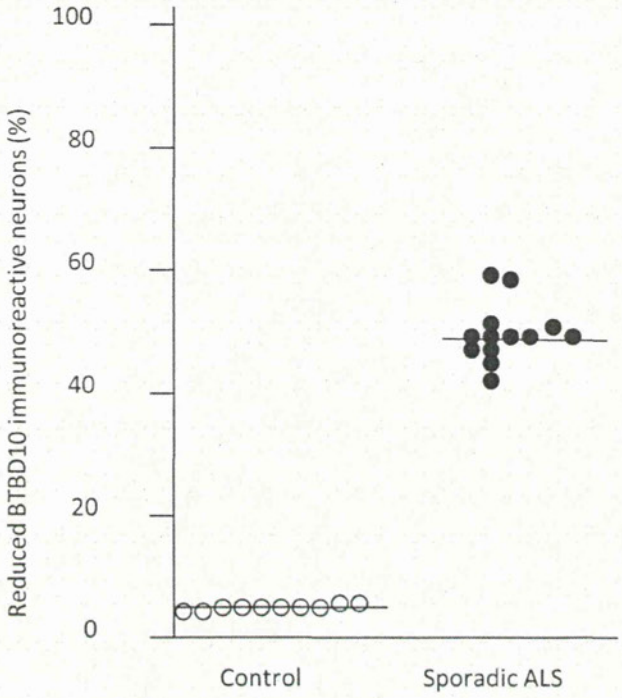

Fig. 4 Percentages of neurons with reduced BTBD10 (BTB/POZ domain-containing protein 10) immunoreactivity. The median percentage of neurons with reduced BTBD10 immunoreactivity in all samples (control ($n = 10$), sporadic amyotrophic lateral sclerosis (ALS) ($n = 13$)) was calculated. The average of the ratio of neurons with reduced BTBD10 immunoreactivity is indicated as a crossbar. The percentage of reduced BTBD10 immunoreactivity neurons was significantly higher in sporadic ALS ($P < 0.001$ by χ^2 test).

RESULTS

In sporadic ALS patients, approximately half (range, 42.1–59.5%) of the remaining anterior horn cells were identified as neurons with reduced BTBD10 immunoreactivity. Reduced BTBD10 immunoreactivity was more frequent in large neurons than in small neurons (Fig. 3). Bunina bodies and skein-like inclusions were BTBD10 negative. In contrast, in the non-ALS control cases, a reduction in BTBD10 immunoreactivity was identified in only approximately 5% (range, 4.4–5.8%) of the anterior horn cells. The level of BTBD10 was significantly decreased in the motor neurons of the spinal cords from sporadic ALS patients ($P < 0.001$ by χ^2 test). However, we found no correlation between the ratio of neurons with reduced BTBD10 immunoreactivity to the number of all remaining neurons and the clinical course of the disease (age at onset and duration of illness) (Table 1; Fig. 4).

Next, we examined the relationship between reduced BTBD10 immunoreactivity and fragmentation of the GA. Immunohistochemical analysis of mirror sections of lumbar spinal cord tissue from patients with sporadic ALS showed that approximately 90% (range, 89.8–92.5%) of BTBD10-positive neurons had normal GA morphology and most neurons with reduced BTBD10 immunoreactivity showed fragmentation of the GA (range, 92.9–94.3%) (Table 2; Figs 5A and 6A). There were few neurons with reduced BTBD10 immunoreactivity, but normal GA morphology (range, 5.7–7.1%) and normally stained BTBD10-positive neurons with GA fragmentation (range, 7.5–13.8%) (Fig. 6A). The percentage of normal GA morphology was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity. The percentage of GA fragmentation was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons ($P < 0.001$ by χ^2 test) (Fig. 6A).

Finally, we examined the relationship between reduced BTBD10 expression and pTDP-43 immunoreactivity in the anterior horn cells. Immunohistochemical analysis of mirror sections of lumbar cord tissue from patients with sporadic ALS showed that approximately 90% (range, 89.7–93.1%) of BTBD10-positive neurons had no pTDP-43-positive cytoplasmic aggregates. Almost all (range, 93.8–96.5%) neurons with reduced BTBD10 immunoreactivity had pTDP-43-positive cytoplasmic aggregates (Table 2;

Table 2 Immunohistochemical analysis of mirror sections revealed that almost all neurons with reduced BTBD10 immunoreactivity had a fragmented GA and pTDP-43 immunoreactive aggregates, and almost all neurons with normal BTBD10 immunoreactivity had normal GA morphology and no abnormal pTDP-43-positive aggregates

Case	Sex	Age at death (years)	Duration of disease (months)	Normal GA/normal BTBD10 (%)	GA fragmentations/reduced BTBD10 (%)	Negative pTDP-43/normal BTBD10 (%)	Positive pTDP-43/reduced BTBD10 (%)
1	M	79	5	44/49 (89.8%)	57/61 (93.4%)	45/49 (91.8%)	58/61 (95.1%)
2	M	75	15	55/62 (88.7%)	82/87 (94.3%)	57/62 (91.9%)	83/87 (95.4%)
3	M	64	29	50/58 (86.2%)	79/85 (92.9%)	52/58 (89.7%)	82/85 (96.5%)
4	M	61	30	49/53 (92.5%)	54/58 (93.1%)	50/53 (93.1%)	55/58 (94.8%)
5	F	64	84	49/54 (90.7%)	61/65 (93.8%)	50/54 (92.6%)	61/65 (93.8%)

BTBD10, BTB/POZ domain-containing protein 10; F, female; GA, Golgi apparatus; M, male; pTDP-43, phosphorylated TAR DNA-binding protein of 43 kDa.

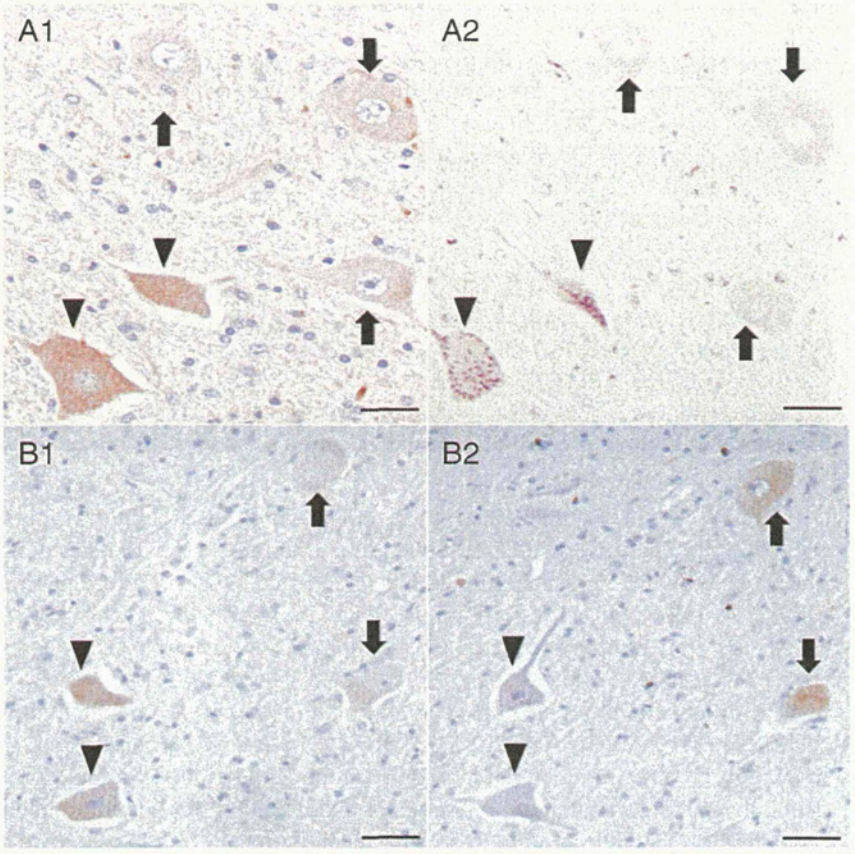

Fig. 5 Mirror sections of the anterior horns in amyotrophic lateral sclerosis (ALS) cases. Immunostaining with anti-BTBD10 (BTB/POZ domain-containing protein 10) (A1, B1), anti-trans-Golgi-network (TGN)-46 antibodies (A2), and anti-phosphorylated TAR-DNA-binding protein 43 (pTDP-43) (B2). All arrows and arrowheads indicate neurons. In most neurons, those with reduced BTBD10 immunoreactivity (arrows in A1 and B1) showed fragmentation of the GA (A2, arrow) and pTDP-43-positive inclusions (B2, arrow), whereas neurons with normal BTBD10 immunoreactivity (arrow heads in A1 and B1) had normal Golgi apparatus (GA) morphology (A2, arrow head) and no pTDP-43-positive aggregates (B2, arrow head). Bars, A: 30 μm, B: 50 μm.

Figs 5B and 6B). We observed a few (range, 3.5–6.2%) neurons with reduced BTBD10 immunoreactivity, but without pTDP-43-positive cytoplasmic aggregates and a few (range, 6.9–10.3%) normal BTBD10-positive neurons with pTDP-43-positive cytoplasmic aggregates (Fig. 6B). The percentage of pTDP-43-negative neurons was significantly higher in the BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity. The percentage of pTDP-43-positive neurons was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons ($P < 0.001$ by χ^2 test) (Fig. 6B).

DISCUSSION

A recent study showed that the expression of BTBD10 was reduced in 44.6% of motor neurons in spinal cords from ALS patients, but only in 18.7% of motor neurons in spinal cords from control cases.[8] In the present study, we generated a new anti-BTBD10 antibody and confirmed that the expression of BTBD10 is markedly reduced in the anterior horn cells of spinal cords from patients with sporadic ALS compared with cords from control cases. These findings suggest that BTBD10 expression may decline during the process of neurodegeneration.

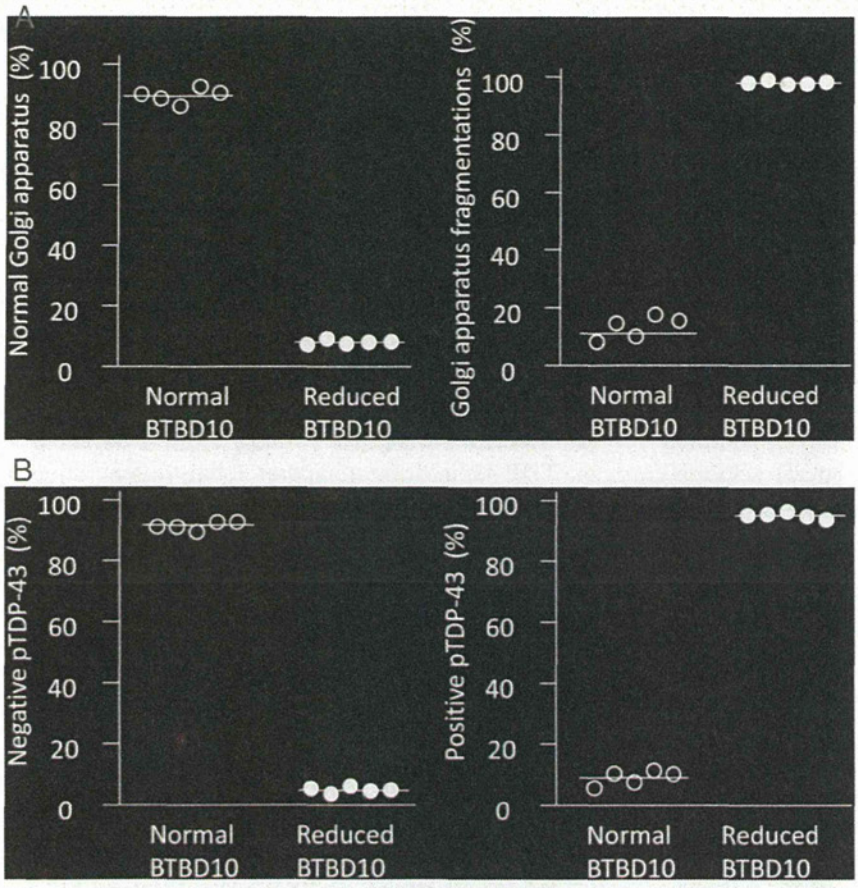

Fig. 6 Comparison of BTBD10 (BTB/POZ domain-containing protein 10) and Golgi apparatus (GA)/phosphorylated TAR-DNA-binding protein 43 (pTDP-43) immunoreactivity. (A) The percentage of cells with normal GA morphology was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity (left side). The percentage of cells with GA fragmentation was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons (right side) ($P < 0.001$ by χ^2 test). Each crossbar indicates the average. (B) The percentage of pTDP-43-negative neurons was significantly higher in BTBD10-positive neurons than in neurons with reduced BTBD10 immunoreactivity (left side). The percentage of pTDP-43-positive neurons was significantly higher in neurons with reduced BTBD10 immunoreactivity compared with BTBD10-positive neurons (right side) ($P < 0.001$ by χ^2 test). Each crossbar indicates the average.

TDP-43 was originally cloned as a human protein capable of binding to TAR DNA of the human immunodeficiency virus 1 long terminal repeat lesion. Physiologically, TDP-43 regulates a variety of RNA metabolisms.[17] Consistent with its function as an RNA-binding protein, TDP-43 associates with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins such as hnRNP A2/B1, hnRNP A1, hnRNP C1/C2 and hnRNP A3.[18–20] The interaction of TDP-43 with hnRNPs is dependent on its C-terminal glycine-rich domain.[18,21] Dysregulation of TDP-43 accounts for neurodegeneration in most ALS cases.[22] We suggest that reduced BTBD10 expression may serve as a common mechanism underlying the pathogenesis of ALS based on the findings from the present study that the appearance of pTDP-43-positive aggregates correlated strongly with reduced BTBD10 immunoreactivity.

In our previous study, we demonstrated that the majority of neurons with pTDP-43-positive cytoplasmic aggregates had GA fragmentation, using TGN-46 immunostaining.[23] In the present study, we found that the majority of neurons with reduced BTBD10 expression simultaneously had both GA fragmentation and pTDP-43-positive cytoplasmic aggregates. The GA plays a key role in the transportation, processing and targeting of numerous proteins destined for secretion, the plasma membrane and lysosomes.[24,25] In neurons, the GA is involved in the axoplasmic flow of numerous endogenous proteins and of exogenous macromolecules transported by orthograde, retrograde, and transsynaptic routes.[26–28] Therefore, GA fragmentation will have deleterious consequences for the proper function of axons and presynaptic terminals. Biologically, GA fragmentation is thought to be an early and probably irreversible lesion in neurodegeneration, caused by a variety of mechanisms, and is not necessarily secondary to apoptosis.[29] The findings in the present study suggest that GA fragmentation has a close relationship with reduced BTBD10 immunoreactivity. Dysfunction of stathmin, a regulator of microtubule polymerization, is thought to be linked to GA fragmentation.[29] It has been suggested that the phosphorylation of stathmin is an indicator of the activation of the PI3K/Akt signal.[30] Together, these results suggest that BTBD10 contributes indirectly to GA integrity by activating stathmin.

Finally, in the present study, we could not detect any relationship between the reduced BTBD10 immunoreactivity and the clinical course of the disease. Further studies are needed to determine the relationship between

BTBD10 reduction and clinical findings or course in ALS.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Health, Labour, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K. Okamoto.

REFERENCES

1. Nawa M, Kanekura K, Hashimoto Y *et al.* A novel Akt/PKB-interacting protein promotes cell adhesion and inhibits familial amyotrophic lateral sclerosis-linked mutant SOD1-induced neuronal death via inhibition of PP2A-mediated dephosphorylation of Akt/PKB. *Cell Signal* 2008; **20**: 493–505.
2. Ugi S, Imamura T, Maegawa H *et al.* Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. *Mol Cell Biol* 2004; **24**: 8778–8789.
3. Franke TF. PI3K/Akt: getting it right matters. *Oncogene* 2008; **27**: 6473–6488.
4. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; **129**: 1261–1274.
5. Chen J, Xu J, Ying K *et al.* Molecular cloning and characterization of a novel human BTB domain-containing gene, BTBD10, which is down-regulated in glioma. *Gene* 2004; **340**: 61–69.
6. Hadano S, Kunita R, Otomo A *et al.* Molecular and cellular function of ALS2/alsin: implication of membrane dynamics in neuronal development and degeneration. *Neurochem Int* 2007; **51**: 74–84.
7. Kanekura K, Hashimoto Y, Kita Y *et al.* A Rac1/phosphatidylinositol 3-kinase/Akt3 anti-apoptotic pathway, triggered by AlsinLF, the product of the *ALS2* gene, antagonizes Cu/Zn-superoxide dismutase (SOD1) mutant-induced motoneuronal cell death. *J Biol Chem* 2005; **280**: 4532–4543.
8. Nawa M, Kage-Nakadai E, Aiso S *et al.* Reduced expression of BTBD10, an Akt activator, leads to motor neuron death. *Cell Death Differ* 2012; **19**: 1398–1407.
9. Gonatas JO, Mezitis SGE, Stieber A *et al.* MG-160: a novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J Biol Chem* 1989; **264**: 646–653.
10. Stieber A, Chen Y, Weil S *et al.* The fragmented neuronal Golgi apparatus in amyotrophic lateral sclerosis includes the trans-Golgi-network: functional implications. *Acta Neuropathol* 1998; **95**: 245–253.
11. Mackenzie IR, Neumann M, Bigio EH *et al.* Nomenclature for neuropathologic subtypes of frontotemporal lobar degeneration: consensus recommendations. *Acta Neuropathol* 2009; **117**: 15–18.
12. Arai T, Hasegawa M, Akiyama H *et al.* TDP-43 is a component of ubiquitin-positive inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* 2006; **351**: 602–611.
13. Neumann M, Sampathu DM, Kwong LK *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006; **314**: 130–133.
14. Hasegawa M, Arai T, Nonaka T *et al.* Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann Neurol* 2008; **64**: 60–70.
15. Sakurai A, Okamoto K, Fujita Y *et al.* Fragmentation of the Golgi apparatus of ballooned neurons in patients with corticobasal degeneration and Creutzfeldt-Jakob disease. *Acta Neuropathol* 2000; **100**: 270–274.
16. Kadokura A, Yamazaki T, Kakuda S *et al.* Phosphorylation-dependent TDP-43 antibody detects intraneuronal dot-like structures showing morphological characters of granulivacuolar degeneration. *Neurosci Lett* 2009; **463**: 87–92.
17. Ou SH, Wu F, Harrich D *et al.* Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J Virol* 1995; **69**: 3584–3596.
18. Buratti E, Brindisi A, Giombi M *et al.* TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *J Biol Chem* 2005; **280**: 37572–37584.
19. Ling SC, Albuquerque CP, Han JS *et al.* ALS-associated mutations in TDP-43 increase its stability and promote TDP-43 complexes with FUS/TLS. *Proc Natl Acad Sci U S A* 2010; **107**: 13318–13323.
20. Freibaum BD, Chitta RK, High AA *et al.* Global analysis of TDP-43 interacting proteins reveals strong association with RNA splicing and translation machinery. *J Proteome Res* 2010; **9**: 1104–1120.
21. D'Ambrogio A, Buratti E, Stuani C *et al.* Functional mapping of the interaction between TDP-43 and hnRNP A2 in vivo. *Nucleic Acids Res* 2009; **37**: 4116–4126.
22. Lee EB, Lee VM, Trojanowski JQ. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. *Nat Rev Neurosci* 2011; **13**: 38–50.

23. Fujita Y, Mizuno Y, Takatama M *et al.* Anterior horn cells with abnormal TDP-43 immunoreactivities show fragmentation of the Golgi apparatus in ALS. *J Neurol Sci* 2008; **269**: 30–34.
24. Farquhar MG. Progress in unraveling pathways of Golgi traffic. *Annu Rev Cell Biol* 1985; **1**: 447–488.
25. Mellman I, Simons K. The Golgi complex: in vitro veritas? *Cell* 1992; **68**: 829–840.
26. Hammerschlag R, Stone GC, Bolen FA *et al.* Evidence that all newly synthesized proteins destined for fast axonal transport pass through the Golgi apparatus. *J Cell Biol* 1982; **93**: 568–575.
27. Rhodes CH, Stieber A, Gonatas NK. A quantitative electron microscopic study of the intracellular localization of wheat germ agglutinin in retinal neurons. *J Comp Neurol* 1986; **254**: 287–296.
28. Rhodes CH, Stieber A, Gonatas NK. Transneuronally transported wheat germ agglutinin labels glia as well as neurons in the rat visual system. *J Comp Neurol* 1987; **261**: 460–465.
29. Gonatas NK, Stieber A, Gonatas JO. Fragmentation of the Golgi apparatus in neurodegenerative diseases and cell death. *J Neurol Sci* 2006; **246**: 21–30.
30. Am K, Levanon K, Duraisamy S *et al.* Stathmin 1, a marker of PI3K pathway activation and regulator of microtubule dynamics, is expressed in early pelvic serous carcinomas. *Gynecol Oncol* 2011; **123**: 5–12.

Amyotrophic Lateral Sclerosis, 2012; 13: 562–566

informa
healthcare

ORIGINAL ARTICLE

Mutational analysis of familial and sporadic amyotrophic lateral sclerosis with *OPTN* mutations in Japanese population

HIROYA NARUSE[1], YUJI TAKAHASHI[1], TAMEKO KIHIRA[2], SOHEI YOSHIDA[2], YASUMASA KOKUBO[3], SHIGEKI KUZUHARA[4], HIROYUKI ISHIURA[1], MASAHARU AMAGASA[5], SHIGEO MURAYAMA[6], SHOJI TSUJI[1] & JUN GOTO[1]

[1]*Department of Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo,* [2]*Kansai University of Health Sciences, Kumatori, Osaka,* [3]*Department of Neurology, Mie University School of Medicine, Tsu, Mie,* [4]*Department of Medical Welfare, Suzuka University of Medical Science, Suzuka, Mie,* [5]*Department of Neurology and Neurosurgery, Yamagata Tokushukai Hospital, Yamagata, and* [6]*Geriatric Neuroscience (Neuropathology), Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*

Abstract

Our objective was to elucidate the genetic epidemiology of familial amyotrophic lateral sclerosis (FALS) and sporadic ALS (SALS) with *OPTN* mutations in the Japanese population. Mutational analysis of *OPTN* was conducted in 18 FALS pedigrees in whom mutations in other causative genes have been excluded and in 218 SALS patients by direct nucleotide sequence analysis. Novel non-synonymous variants identified in ALS patients were further screened in 271 controls. Results showed that although no mutations were identified in the FALS pedigrees, a novel heterozygous non-synonymous variant c.481G>A (p.V161M) was identified in one SALS patient, who originated from the southernmost part of the Kii Peninsula. The mutation was not present in 271 controls. As the clinical feature, the patient carrying V161M showed predominantly upper motor neuron signs with slow progression. This study suggests that mutations in *OPTN* are not the main cause of ALS in the Japanese population.

Key words: *Motor neuron disease, amyotrophic lateral sclerosis, OPTN mutation, genetic analysis, V161M*

Introduction

Molecular genetic research on amyotrophic lateral sclerosis (ALS) has revealed a number of causative genes for familial ALS (FALS), which include *SOD1* (1), *ALS2* (2,3), *DCTN1* (4), *VAPB* (5), *CHMP2B* (6), *ANG* (7), *TARDBP* (8), and *FUS* (9,10). These genes collectively account for approximately 30% of FALS pedigrees (11). Mutations in these genes have also been identified in some sporadic ALS (SALS) patients, suggesting mutations with reduced penetrance or *de novo* mutations (12,13). Recently, hexanucleotide repeat expansion within the *C9ORF72* gene has been reported to be associated with a large proportion of cases of ALS and frontotemporal dementia (FTD) with wider European ancestry (14–16). Mutations in *UBQLN2* were also identified to cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia (17). *OPTN*, which was previously identified as the causative gene for rare autosomal dominant familial primary open-angle glaucoma (POAG), has been reported as the causative gene for autosomal dominant and autosomal recessive FALS (18). Subsequent genetic epidemiological studies on *OPTN* mutations in different cohorts have revealed that frequencies of mutations in patients with FALS and SALS vary among cohorts, from 0% to 4.35% (pedigree frequency) in those with FALS, and from 0% to 3.54% (case frequency) in those with SALS (18–23). Further analyses on larger cohorts of various ethnic backgrounds will be necessary to establish the genetic epidemiology and clinical characteristics of ALS and the genotype-phenotype correlations of ALS with *OPTN* mutations. We conducted further mutational analysis of *OPTN* in our cohorts to establish the molecular epidemiology of ALS in patients with mutations in *OPTN*.

Correspondence: J. Goto, Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: 81 3 5800 6844. E-mail: gotoj-tky@umin.ac.jp

(Received 15 December 2011; accepted 2 April 2012)

ISSN 1748-2968 print/ISSN 1471-180X online © 2012 Informa Healthcare
DOI: 10.3109/17482968.2012.684213