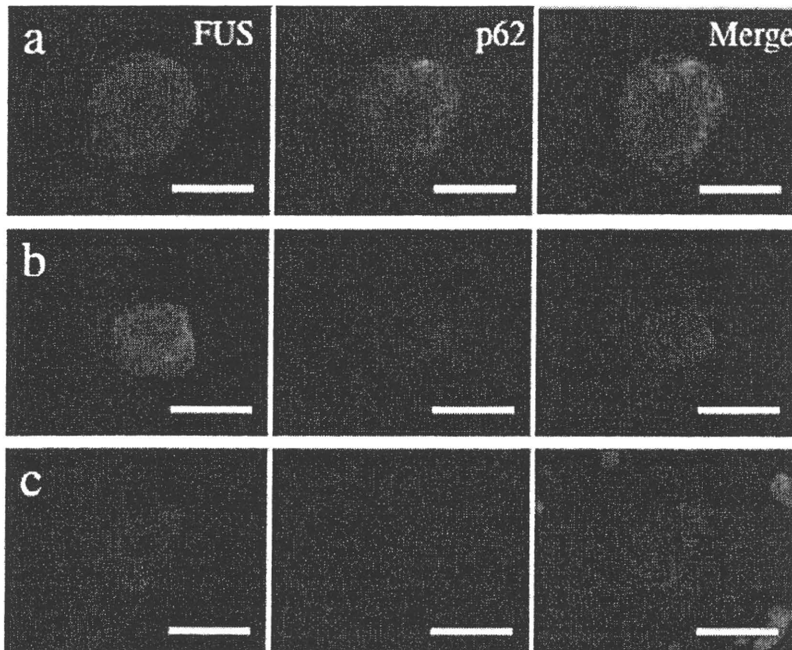


Fig. 4 Double-labeled immunofluorescence for fused in sarcoma protein (FUS) (*red*) and p62 (*green*), and merged images in the anterior horn cells. 4′-6-diamidino-2-phenylindol (DAPI) was used for nuclear counterstaining (*blue*). Neuronal inclusion was labeled for both anti-FUS and anti-p62 antibodies (a). Anti-p62 antibody labeled a part of the FUS-positive inclusion (b). The inclusion was labeled for anti-FUS antibody but not for anti-p62 antibody (c). Scale bar: 20 μm.

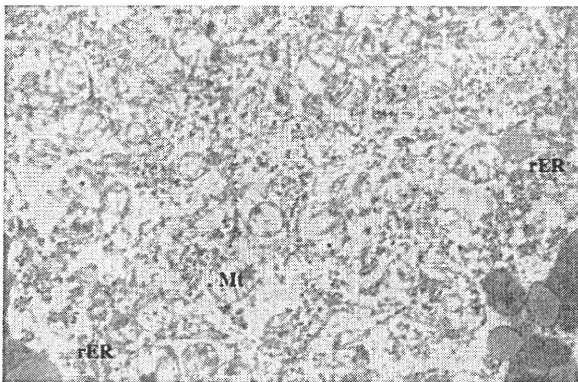


Fig. 5 Electron micrograph of a basophilic inclusion in an anterior horn cell shows accumulations of granular and filamentous materials, with mitochondria and rough endoplasmic reticulum present in the peripheral region. 6700×, Mt = mitochondria, rER = rough endoplasmic reticulum.

However, patients with BIBD do not exhibit TDP-43-positive aggregates, and the inclusions found in these patients stain negatively with anti-TDP-43 antibody.[6,7] Furthermore, although the normal nuclear staining of TDP-43 is lost in neurons exhibiting abnormal cytoplasmic TDP-43 immunoreactivity,[20] the nuclear staining of FUS was preserved in some neurons with FUS-positive inclusions in the present study. We also observed here that more FUS-positive neuronal and glial inclusions were present in some anatomical regions, and p62 labeled only parts of BIs that were entirely immunostained for FUS antibody by immunofluorescence analyses. These results suggest that BI formation and TDP-43 aggregation have different pathogenic mechanisms, and FUS may play an important role in the pathogenesis of MND with BIs.

In clinical settings, similar to FALS with mutations in the *FUS* gene, MND with BIs was previously referred to as juvenile MND because cases of adult-onset disease were rare.[9–12] Some cases of MND with BIs show atypical features of classic sporadic MND, such as autonomic dysfunction, dementia, and eye movement disorder.[9,11] On the other hand, this patient first developed symptoms at the age of 73. This is the oldest reported age of onset for MND with BIs, and the clinical features observed in this patient were indistinguishable from those of classic sporadic ALS without apparent pyramidal tract signs. Therefore, we postulate that FUS-related disorders may vary widely in their age of onset and have variable clinical features.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research (C) to 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.

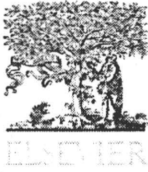
© 2010 Japanese Society of Neuropathology

REFERENCES

1. Sreedharan J, Blair IP, Tripathi VB *et al.* TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* 2008; **319**: 1668–1672.
2. Vance C, Rogelj B, Hortobágyi T *et al.* Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis Type 6. *Science* 2009; **323**: 1208–1211.
3. Kwiatkowski TJ Jr, Bosco DA, Leclerc AL *et al.* Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 2009; **323**: 1205–1208.
4. Belzil VV, Valdmanis PN, Dion PA *et al.* Mutations in FUS cause FALS and SALS in French and French Canadian populations. *Neurology* 2009; **73**: 1176–1179.
5. Chiò A, Restagno G, Brunetti M *et al.* Two Italian kindreds with familial amyotrophic lateral sclerosis due to FUS mutation. *Neurobiol Aging* 2009; **30**: 1272–1275.
6. Munoz DG, Neuumann M, Kusaka H *et al.* FUS pathology in basophilic inclusion body disease. *Acta Neuropathol* 2009; **118**: 617–627.
7. Neumann M, Roeber S, Kretzschmar HA, Rademakers R, Baker M, Mackenzie IR. Abundant FUS-immunoreactive pathology in neuronal intermediate filament inclusion disease. *Acta Neuropathol* 2009; **118**: 605–616.
8. Munoz DG. The pathology of Pick complex. In: Kertesz A, Munoz DG, eds. *Pick's Disease and Pick Complex*. New York: Wiley-Liss, 1998; 211–241.
9. Matsumoto S, Kuaka H, Murakami N, Hashizume Y, Okazaki H, Hirano A. Basophilic inclusions in sporadic juvenile amyotrophic lateral sclerosis: an immunocytochemical and ultrastructural study. *Acta Neuropathol* 1992; **83**: 579–583.
10. Nelson JS, Prensky AL. Sporadic juvenile amyotrophic lateral sclerosis. A clinical study of a case with neuronal cytoplasmic inclusions containing RNA. *Arch Neurol* 1972; **27**: 300–306.
11. Oda M, Kakogawa N, Tabuchi Y, Tanabe H. A sporadic juvenile case of the amyotrophic lateral sclerosis with neuronal intracytoplasmic inclusions. *Acta Neuropathol* 1978; **44**: 211–216.
12. Fujita Y, Okamoto K, Sakurai A *et al.* The Golgi apparatus is fragmented in spinal cord motor neurons of amyotrophic lateral sclerosis with basophilic inclusions. *Act Neuropathol* 2002; **103**: 243–247.
13. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press, 2001.
14. Kadokura A, Yamazaki T, Kaunda S *et al.* Phosphorylation-dependent TDP-43 antibody detects intraneuronal dot-like structures showing morphological characters of granulovacuolar degeneration. *Neurosci Lett* 2009; **463**: 87–92.
15. Fujita Y, Omaha E, Takatama M, Al-Sarraj S, Okamoto K. Golgi apparatus of nigral neurons with α-synuclein-positive inclusions in patients with Parkinson's disease. *Acta Neuropathol* 2006; **112**: 261–265.
16. Mizuno Y, Amati M, Takatama M, Aizawa H, Mihara B, Okamoto K. Immunoreactivities of p62, an ubiqutin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis. *J Neurol Sci* 2006; **249**: 13–18.
17. Cairns NJ, Bigio EH, Mackenzie IRA *et al.* Neuropathologic diagnosis and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. *Acta Neuropathol* 2007; **114**: 5–22.
18. 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.
19. Neumann M, Sympathy DM, Kong LK *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006; **314**: 130–133.
20. Fujita Y, Mizuno Y, Takatama M, Okamoto K. Anterior horn cells with abnormal TDP-43 immunoreactivities show fragmentation of the Golgi apparatus in ALS. *J Neurol Sci* 2008; **269**: 30–34.

© 2010 Japanese Society of Neuropathology

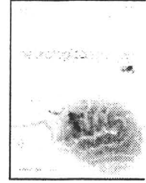
Journal of the Neurological Sciences 302 (2011) 14–18



Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns



Peripherin partially localizes in Bunina bodies in amyotrophic lateral sclerosis

Yuji Mizuno [a,*], Yukio Fujita [a], Masamitsu Takatama [b], Koichi Okamoto [a]

[a] *Department of Neurology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*
[b] *Department of Internal Medicine, Geriatrics Research Institute and Hospital, 3-26-8 Otomo-machi, Maebashi, Gunma 371-0847, Japan*

ARTICLE INFO

Article history:
Received 7 August 2010
Received in revised form 22 December 2010
Accepted 23 December 2010
Available online 15 January 2011

Keywords:
Amyotrophic lateral sclerosis
Bunina bodies
Lewy body-like inclusions
Peripherin

ABSTRACT

Peripherin is a type III intermediate filament protein expressed with low levels in spinal motor neurons. Amyotrophic lateral sclerosis (ALS) is characterized by the presence of Bunina bodies, skein-like inclusions, and Lewy body-like inclusions (LBLIs) in the remaining anterior horn cells, where the first and third structures are detected by Hematoxylin–Eosin (H & E) staining. We examined paraffin sections of lumbar spinal cords from six ALS patients, using H & E staining and immunostaining for human peripherin. The results demonstrated that there were a total of 73 anterior horn cells containing one or more Bunina bodies, and that twelve of these cells (approximately 16.4%) demonstrated peripherin-positive Bunina bodies. In fact, some part of chain-like Bunina bodies showed peripherin-positive reaction, although there were a much higher number of non-immunoreacitive Bunina bodies in each neuron. LBLIs were clearly immunostained for peripherin corresponding to the core, while some of them showed different types of immunoreactivities due to oblique cutting of inclusions. Our findings suggest that although the mechanisms underlying peripherin co-localization in Bunina bodies are unknown, peripherin could be involved in forming these inclusions. Furthermore, following cystatin C and transferrin, peripherin is the third most prevalent protein that partially localizes in Bunina bodies.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Peripherin is an intermediate filament protein (IF), a type III neuronal protein of 58 kD, that is capable of self-assembling to form homopolymeric filamentous networks like the other members of this group, vimentin, desmin, and glial fibrillary acidic protein (GFAP) [1,2]. Peripherin can co-assemble with neurofilaments (NFs) [3], and expresses predominantly in neurons of the peripheral nervous system and also in those of the central nervous system including spinal motor neurons [4]. Mutations of the gene encoding IF proteins such as keratin [5], GFAP [6], desmin [7] and the low-molecular weight subunit of NF [8] have been linked to skin disorders, Alexander's disease, myopathy and Charcot–Marie–Tooth type 2E, respectively. Many IF protein diseases are characterized by the presence of intracytoplasmic IF protein inclusions.

In addition to known genes causing familial amyotrophic lateral sclerosis (ALS) such as copper–zinc superoxide dismutase 1 (SOD1) [9], ALS2 [10], angiogenin [11], transactive response DNA-binding protein of 43 kDa (TDP-43) [12], fused in sarcoma/translated in liposarcoma (FUS) [13,14], and optineurin [15], mutation of peripherin gene or its abnormal expression could contribute to the development of ALS. Peripherin gene mutations are identified in sporadic ALS patients [16–18], causing peripherin aggregations in the remaining motor neurons. Disruption of the IF network and formation of cytoplasmic aggregation could be a contributing factor to facilitate the degeneration of anterior horn cells. Peripherin expression is increased in large dorsal root ganglion neurons after neuronal injury [19], in spinal motor neurons after sciatic nerve crush [19], in neurons after cerebral ischemia [20], and in remaining motor neurons of ALS spinal cords [21], while peripherin is expressed at low levels in the spinal cord under normal conditions.

Peripherin is observed in Lewy body-like inclusions (LBLIs) (also called round inclusions) and spheroids occurring in the proximal axons of ALS motor neurons [22]. Skein-like inclusions, that do not express peripherin [23], show positive reaction for ubiquitin [24], p62 [25] and TDP-43 [26,27], which are often detected in the remaining anterior horn cells of the majority of ALS patients. In contract to these structures, Bunina bodies [28] are small, granular eosinophilic inclusions measuring 2–4 μm in diameter. We have previously shown that Bunina bodies are immunoreactive for cystatin C [29] and transferrin [30]. The mechanism underlying the formation of Bunina bodies remains uncertain and it is unknown why Bunina bodies show a wider variety of immunoreactions than other aggregates of ALS such as skein-like inclusions and LBLIs. Little attention has been paid to peripherin expression on one of the abnormal structures, Bunina bodies. In this study, we examined the eosinophilic structures of Bunina bodies to see if they showed peripherin expression. Immunohistochemical analysis demonstrated that although peripherin was rarely observed, it was partially localized in Bunina bodies.

* Corresponding author. Tel.: +81 27 220 8064; fax: +81 27 220 8068.
E-mail address: mizunoy@med.gunma-u.ac.jp (Y. Mizuno).

0022-510X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2010.12.023

2. Materials and methods

We examined a total of 6 lumber spinal cord samples from sporadic ALS patients (average age: 66.0 years old, 3 males, 3 females). Spinal cord tissues were all obtained from the institute and our university. In all cases, the autopsies were performed in accordance with established procedures and the samples were used in this study after obtaining informed consent from the family of each patient. All patients were definitively diagnosed based on clinical and light microscopic findings. Spinal cords were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS) (pH 7.4) and embedded in paraffin. Five-μm-thick transverse paraffin sections were prepared for Hematoxylin–Eosin (H & E) staining and then immunohistochemistry, which was carried out using a rabbit polyclonal anti-peripherin antibody (1:3000, AB1530; Chemicon International, Inc.). For enhancement, the samples were autoclaved for 5 min before reaction with the antibody. Sections were blocked in normal horse serum for 30 min at room temperature, then labeled with the first antibody at 4 °C overnight, washed in PBS for 30 min, incubated with the second antibody provided in the Histofine SAB-PO kit (Nichirei, Tokyo, Japan), washed in PBS for 30 min, and finally visualized by the avidin–biotin–peroxidase method. Observation was performed using an Olympus BX50 microscope.

Since the Bunina bodies and LBLIs are demonstrated by H & E staining, this process was initially performed to observe where these structures were present. After we photographed the sections, we removed the cover glasses from the slides in xylene, decolorized the specimens in alcohol, and then performed immunohistochemistry with anti-peripherin antibody.

Specificity of the peripherin staining was confirmed by preabsorption of the antibody for 1 h at 4 °C with peripherin recombinant protein (H00005630-P01; Abnova, Taipei, Taiwan).

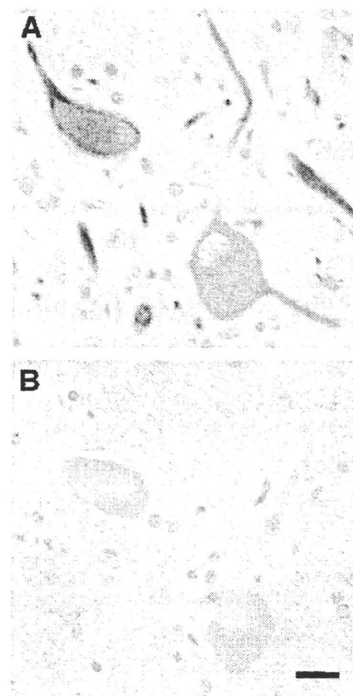


Fig. 1. Peripherin immunoreactivitiy. A: Immunostaining for peripherin. B: Immunostaining for peripherin preabsorbed with peripherin recombinant protein. Peripherin immunostaining was obviously specific. Scale bar: 20 μm.

3. Results

In general, the cytoplasm and axonal spheroid showed heterogeneously peripherin-positive immunoreactivities, although the intensities differed in each anterior horn cell, ranging from weak staining to strong staining (Fig. 1A). To determine whether the peripherin-positive reactions were specific, a serial section was immunostained after preabsorption of anti-peripherin antibody with peripherin recombinant protein. The finding showed that peripherin-positive staining disappeared (Fig. 1B), indicating that the reaction was true.

H & E staining was initially performed to search for the eosinophilic inclusions in lumbar spinal cords of ALS patients. These inclusions correspond to Bunina bodies or LBLIs. There were a total of 487 remaining anterior horn cells in 6 patients with ALS and 73 of these cells contained eosinophilic Bunina bodies. Although two of six ALS patients retained a large number of anterior horn cells containing Bunina bodies, the remaining four patients showed a very small number of such cells. H & E staining demonstrated that there were several different types of eosinophilic inclusions such as the solitary pattern (arrows in Fig. 2A and C) and the grouped or chain-like pattern (double arrows in Fig. 2A, C, and E). After photographing the Bunina bodies, immunohistochemistry for peripherin was performed on the same section. Peripherin immunoreactivities were rarely detectable at the same location as Bunina bodies. As the number of anterior horn cells with peripherin-positive Bunina bodies was 12 among 73 neurons containing Bunina bodies, the ratio was only 16.4 % and indicated low levels. Furthermore, in most cases, single and chain-like Bunina bodies were completely peripherin-negative (double arrows in Fig. 2B and single arrow in 2D); however, some parts of a few chain-like Bunina bodies showed positive reactions for peripherin (double arrows in Fig. 2D and F). Predicting whether each Bunina body would be immunoreactive for peripherin was impossible before the immunohistochemical analysis for peripherin was done.

Seven LBLIs were detected in this study. Five of them were variably immunostained with anti-peripherin antibody. Similarly to immunoreactivities of Bunina bodies, the staining pattern depended on each inclusion. One LBLI showed strong round peripherin reaction (Fig. 3B) in the center, corresponding to core, and was surrounded by a halo with no peripherin reaction (Fig. 3B). The other LBLIs were also peripherin-positive. One showed strong staining at the core (Fig. 3D) and one showed partial staining at the core, demonstrating a stick-like pattern (Fig. 3F). All of these patterns were caused by diagonal cutting of LBLIs. In addition, Bunina bodies were sometimes simultaneously present with LBLIs in the same cytoplasm (arrows in Fig. 3A and C) but no immunoreactivities were seen for peripherin in Fig. 3B and D (arrows).

4. Discussion

ALS is pathologically characterized by the presence of several intracytoplasmic inclusions/bodies. One group of neuronal aggregates shows skein-like inclusions and LBLIs, all of which are immunoreactive for ubiquitin [24], p62 [25] and TDP-43 [26,27], while Bunina bodies are different from the former two aggregates because of no staining for these proteins. Bunina bodies are visualized with H & E staining as small, round or oval, and eosinophilic inclusions. Sometimes, they form clusters and the number of Bunina bodies varies in each anterior horn cell. To date, only two proteins have been demonstrated, cystatin C [29], which is a member of a super family of protease inhibitors, and transferrin [30], which is an iron-binding plasma protein. Differing from cystatin C and transferrin, which show positive reactions in almost all Bunina bodies, peripherin appears to be the third but less detectable protein in Bunina bodies, suggesting a different role of peripherin in the aggregates compared to those of the other two proteins. Little attention has been paid to the association of Bunina bodies with peripherin expression in ALS. Otherwise, detection of peripherin-positive Bunina bodies has probably been passed over. In this study, we investigated the association between eosinopilic structures like Bunina bodies and LBLIs and immunoreactive peripherin of ALS spinal cords.

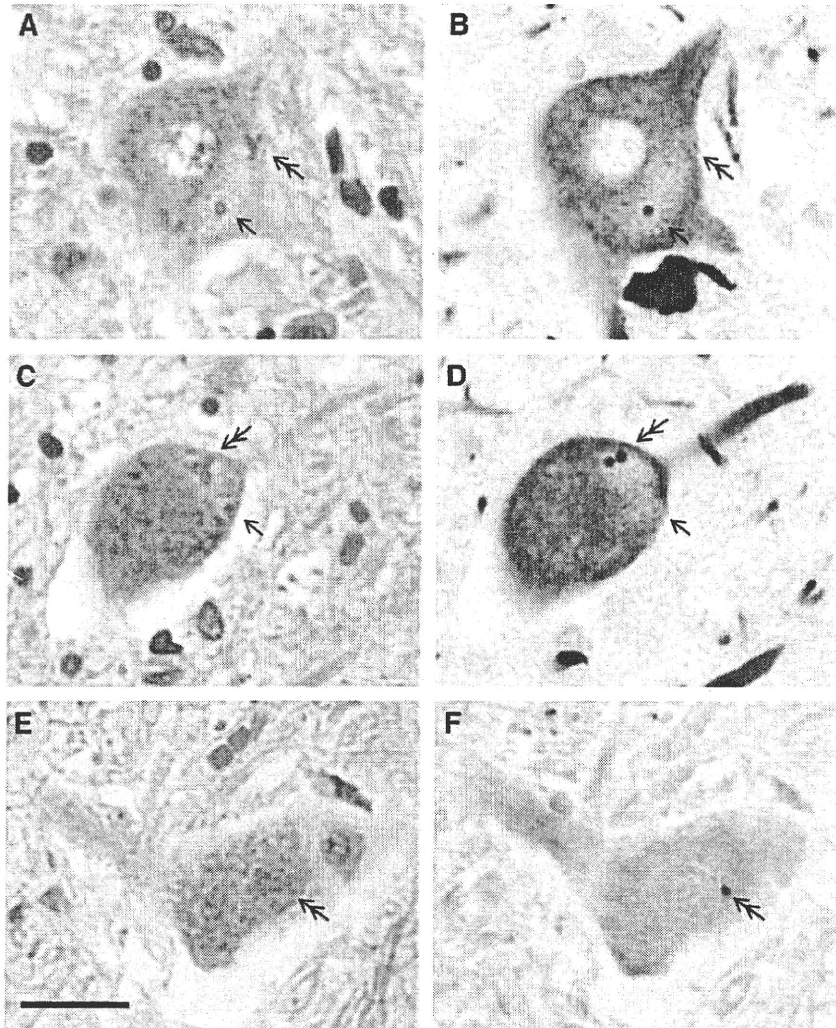


Fig. 2. Peripherin immunoreactivity in Bunina bodies with single and chain-like clusters. A, C, E: H & E staining. B, D, F: Immunostaining for peripherin. The presence of Bunina bodies was confirmed with H & E staining (A, C, and E), and the same section was examined with anti-peripherin antibody (B, D, and F). Arrows in A and C showed positive and negative reactions, respectively (B and D), while double arrows corresponding to chain-like Bunina bodies (A, C, and E) were negatively (B) and partially positively immunostained (D and F), respectively. Scale bar: 20 μm.

The variety of peripherin intensities is seen among the cytoplasm, which may be influenced by up-regulation or down-regulation of certain proteins. Peripherin expression is up-regulated after neuronal injury [19], damage to the sciatic nerve [19], and cerebral ischemia [31], and also induced by proinflammatory cytokines interleukin-6 [32] and leukemia inhibitory factor [33], suggesting that up-regulation of peripherin is associated with neuronal regeneration. Taking these findings into account, it is no wonder that over-expression of peripherin could be occurred in the cytoplasm of ALS spinal cord. Furthermore, peripherin knockout mice show 60 % and 50 % increase of alpha-internexin and vimentin expressions in the spinal cord, respectively [34], all of which are IF proteins. It is possible that these IF proteins mutually compensate for the deficiency of peripherin. In this study, we preliminarily examined the mirror sections to determine whether expression between peripherin and phosphorylated TDP-43 (pTDP-43) [35] was either proportional or reciprocal. The strong intensities of pTDP-43 in the cytoplasm mainly reflected the presence of skein-like inclusions. Variably different types of immunostaining between the two proteins were observed, showing strong reactions for both proteins, strong for peripherin and weak for pTDP-43, weak for peripherin and strong for pTDP-43 and so on. At present, no conclusions have been drawn because the intensities of the two proteins were so complicated. However, there would be some proteins that regulate peripherin expression.

Based on our findings, an immunoreactive protein was obviously present within some parts of the Bunina bodies using anti-peripherin antibody. Although this should really reflect peripherin itself, there may be another protein that cross-reacts with the antibody. Both possibilities are conceivable but there is a question as to why the peripherin staining occurs partially on each Bunina body. As the distribution of cystatin C [29] and transferrin [30] matches that of eosinophilic Bunina bodies, the third protein, peripherin, may have a unique property compared to those of cystatin C and transferrin. The partial reaction for peripherin may have several explanations. The first theory is that eosinophilic inclusions are not composed of common proteins. Some of these inclusions contain extra peripherin, while others do not have it. In this case, peripherin reaction would be partial. The second theory is that expression of alternatively spliced variant of peripherin may lead to a decrease in normal peripherin expression within the Bunina bodies. Three peripherin isoforms, Per 58, Per 56, and Per 61, have been identified, in which Per 58 is the most predominant isoform in wild-type mice [36]. Although the functions of these isoforms are not yet known, Per 61 is detected in motor neurons of

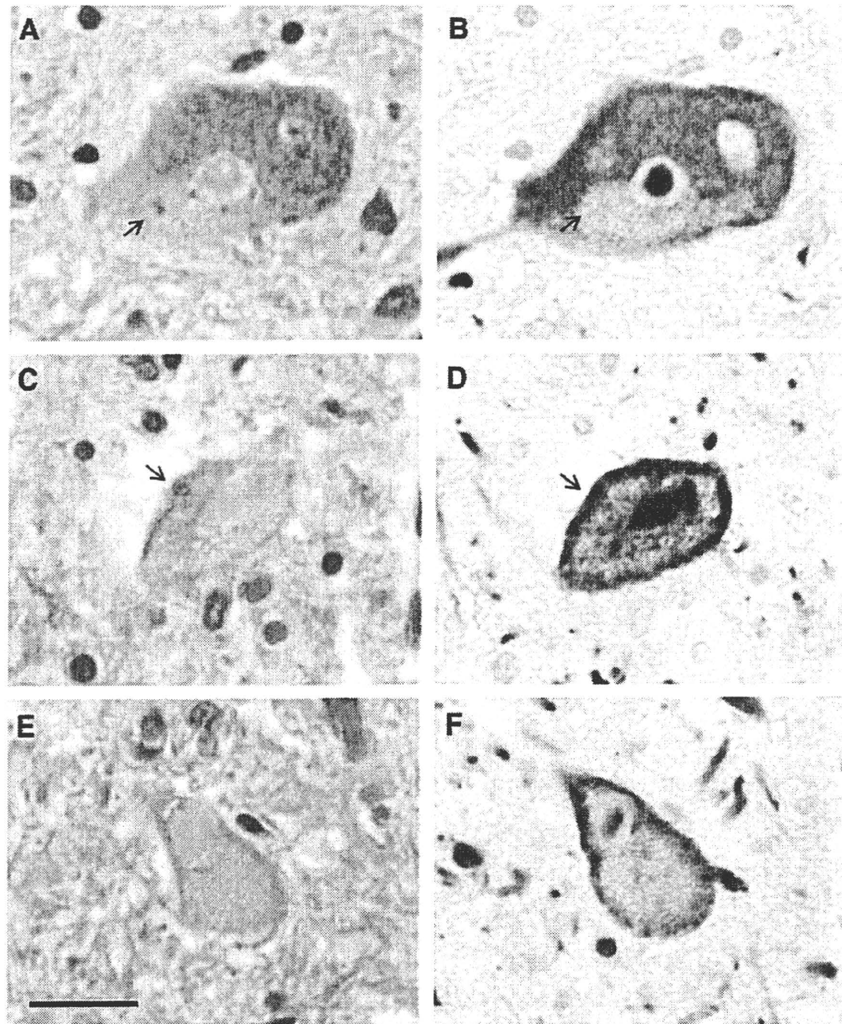


Fig. 3. Peripherin immunoreactivity in Lewy body-like inclusions. A, C, E: H & E staining. B, D, F: Immunostaining for peripherin. The presence of Lewy body-like inclusions was confirmed with H & E staining (A, C, and E), and then the same section was examined with anti-peripherin antibody (B, D, and F). The core with an obviously round shape showed strong reaction, surrounded by a halo with no peripherin reaction (B). The other shapes of LBLIs caused by different directions of inclusions were variably immunostained with antibody (D and F). Bunina bodies with LBLI in the same cytoplasm (arrow in A and C) showed negative immunoreactivities (B and D). Scale bar: 20 μm.

transgenic mice expressing mutant $SOD1^{G37R}$, while it is not observed in those of wild-type or peripherin transgenic mice [21]. An aberrant splice transcript similar to Per 61 in mice was also detected in humans and designated Per 28, and found to be up-regulated at both messenger RNA (mRNA) and protein levels in ALS [37]. These findings suggest that abnormal types of peripherin contribute to degeneration of motor neurons through neurotoxicity. In each Bunina body, the ratio of these isoforms may become unbalanced and mixed irregularly, making reduction in the levels of normal peripherin isoform in ALS degenerative neurons. Supposing that normally spliced peripherin had originally been present in Bunina bodies, the antibody used in this study would not have always recognized peripherin. Alternatively spliced isoforms are also described for other IF proteins including GFAR and synemin [8]. The third theory is as follows; typical Bunina bodies consist of amorphous electron-dense material surrounded by tubular and vesicular structures, occasionally demonstrating a few clear central areas on electron microscopic analysis [28]. The clear central area has been found to be solitarily present within electron-dense amorphous materials and contains 10 nm filaments. Antibodies against phosphorylated and non-phosphorylated neurofilaments did not detect Bunina bodies in our study (data not shown). Could these 10 nm filaments react or cross-react with anti-peripherin antibody? Based on our findings, the size and shape of peripherin immunoreactivities were apparently the same as those of eosinophilic Bunina bodies, suggesting that this possibility would be less likely. The fourth theory is that a combination of peripherin with other proteins under ALS conditions could mask the reactive epitope within Bunina bodies, resulting in a missing reaction with the antibody.

In summary, we showed for the first time that in addition to cystatin C and transferrin, peripherin is partially localized in Bunina bodies. The mechanism by which peripherin accumulates in these inclusions remains unknown. It is thought that systemic derangements of IF proteins may play some roles in the pathogenesis of neurodegenerative diseases like ALS. Detailed examination of how the inclusions are formed, how the neurons survive by forming inclusions, and what effects the inclusions have on the remaining neurons could contribute to clarifying the pathogenesis of ALS.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to Y.M., Y.F., and K.O., and by grants 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.O.

References

[1] Cui C, Stambrook PJ, Parysek LM. Peripherin assembles into homopolymers in SW13 cells. J Cell Sci 1995;108:3279–84.

[2] Ho CL, Chin SS, Carnevale K, Liem RK. Translation initiation and assembly of peripherin in cultured cells. Eur J Cell Biol 1995;68:103–12.

[3] Parysek LM, McReynolds MA, Goldman RD, Ley CA. Some neural intermediate filaments contain both peripherin and the neurofilament proteins. J Neurosci Res 1991;30:80–91.

[4] Parysek LM, Goldman RD. Distribution of a novel 57 kDa intermediate filament (IF) protein in the nervous system. J Neurosci 1988;8:555–63.

[5] Smith F. The molecular genetics of keratin disorders. Am J Clin Dermatol 2003;4:347–64.

[6] Brenner M, Johnson AB, Boespflug-Tanguy O, Rodriguez D, Goldman JE, Messing A. Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. Nat Genet 2001;27:117–20.

[7] Dalakas MC, Park KY, Semino-Mora C, Lee HS, Sivakumar K, Goldfarb LG. Desmin myopathy, a skeletal myopathy with cardiomyopathy caused by mutations in the desmin gene. N Engl J Med 200; 342: 770–80.

[8] Izmiryan A, Peltekian E, Paulin D, Li ZL, Xue ZG. Synemin isoforms in astroglial and neuronal cells from human central nervous system. Neurochem Res 2010;35:881–7.

[9] Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993;362:59–62.

[10] Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci 2006;7:710–23.

[11] Fernández-Santiago R, Hoenig S, Lichtner P, Sperfeld AD, Sharma M, Berg D, et al. Identification of novel Angiogenin (ANG) gene missense variants in German patients with amyotrophic lateral sclerosis. J Neurol 2009;256:1337–42.

[12] Lagier-Tourenne C, Cleveland DW. Rethinking ALS: the FUS about TDP-43. Cell 2009;136:1001–4.

[13] Kwiatkowski Jr TJ, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, et al. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science 2009;323:1205–8.

[14] Vance C, Rogelj B, Hortobágyi T, De Vos KJ, Nishimura AL, Sreedharan J, et al. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 2009;323:1208–11.

[15] Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, et al. Mutations of optineurin in amyotrophic lateral sclerosis. Nature 2010;465:223–6.

[16] Corrado L, Carlomagno Y, Falasco L, Mellone S, Godi M, Cova E, Cereda C, Testa L, Mazzini L, D'Alfonso S. A novel peripherin gene (PRPH) mutation identified in one sporadic amyotrophic lateral sclerosis patient. Neurobiol Aging 2010; in press.

[17] Gros-Louis F, Larivière R, Gowing G, Laurent S, Camu W, Bouchard JP, et al. A frameshift deletion in peripherin gene associated with amyotrophic lateral sclerosis. J Biol Chem 2004;279:45951–6.

[18] Leung CL, He CZ, Kaufmann P, Chin SS, Naini A, Liem RK, et al. A pathogenic peripherin gene mutation in a patient with amyotrophic lateral sclerosis. Brain Pathol 2004;14:290–6.

[19] Troy CM, Muma NA, Greene LA, Price DL, Shelanski ML. Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. Brain Res 1990;529:232–8.

[20] Mersiyanova IV, Perepelov AV, Polyakov AV, Sitnikov VF, Dadali EL, Oparin RB, et al. A new variant of Charcot–Marie–Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene. Am J Hum Genet 2000;67:37–46.

[21] Robertson J, Doroudchi MM, Nguyen MD, Durham HD, Strong MJ, Shaw G, et al. A neurotoxic peripherin splice variant in a mouse model of ALS. J Cell Biol 2003;160:939–49.

[22] Migheli A, Pezzulo T, Attanasio A, Schiffer D. Peripherin immunoreactive structures in amyotrophic lateral sclerosis. Lab Invest 1993;68:185–91.

[23] He CZ, Hays AP. Expression of peripherin in ubiquinated inclusions of amyotrophic lateral sclerosis. J Neurol Sci 2004;217:47–54.

[24] Leigh PN, Whitwell H, Garofalo O, Buller J, Swash M, Martin JE, et al. Ubiquitin-immunoreactive intraneuronal inclusions in amyotrophic lateral sclerosis. Morphology, distribution, and specificity. Brain 1991;114:775–88.

[25] Mizuno Y, Amari M, Takatama M, Aizawa H, Mihara B, Okamoto K. Immunoreactivities of p62, an ubiquitin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis. J Neurol Sci 2006;249:13–8.

[26] Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 2006;351:602–11.

[27] Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 2006;314:130–3.

[28] Okamoto K, Mizuno Y, Fujita Y. Bunina bodies in amyotrophic lateral sclerosis. Neuropathology 2008;28:109–15.

[29] Okamoto K, Hirai S, Amari M, Watanabe M, Sakurai A. Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. Neurosci Lett 1993;162:125–8.

[30] Mizuno Y, Amari M, Takatama M, Aizawa H, Mihara B, Okamoto K. Transferrin localizes in Bunina bodies in amyotrophic lateral sclerosis. Acta Neuropathol 2006;112:597–603.

[31] Beaulieu JM, Kriz J, Julien JP. Induction of peripherin expression in subsets of brain neurons after lesion injury or cerebral ischemia. Brain Res 2002;946:153–61.

[32] Sterneck E, Kaplan DR, Johnson PF. Interleukin-6 induces expression of peripherin and cooperates with Trk receptor signaling to promote neuronal differentiation in PC12 cells. J Neurochem 1996;67:1365–74.

[33] Lecomte MJ, Basseville M, Landon F, Karpov V, Fauquet M. Transcriptional activation of the mouse peripherin gene by leukemia inhibitory factor: involvement of STAT proteins. J Neurochem 1998;70:971–82.

[34] Larivière RC, Ribeiro-da-Silva A, Julien JP. Reduced number of unmyelinated sensory axons in peripherin null mice. J Neurochem 2002;81:525–32.

[35] Kadokura A, Yamazaki T, Kakuda S, Makioka K, Lemere CA, Fujita Y, et al. Phosphorylation-dependent TDP-43 antibody detects intraneuronal dot-like structures showing morphological characters of granulovacuolar degeneration. Neurosci Lett 2009;463:87–92.

[36] Landon F, Wolff A, de Néchaud B. Mouse peripherin isoforms. Biol Cell 2000;92:397–407.

[37] Xiao S, Tjostheim S, Sanelli T, McLean JR, Horne P, Fan Y, et al. An aggregate-inducing peripherin isoform generated through intron retention is upregulated in amyotrophic lateral sclerosis and associated with disease pathology. J Neurosci 2008;28:1833–40.

NEUROPATHOLOGY

Neuropathology 2011; ••, ••–••

doi:10.1111/j.1440-1789.2011.01199.x

Original Article

Optineurin in neurodegenerative diseases

Tenshi Osawa,[1] Yuji Mizuno,[1] Yukio Fujita,[1] Masamitsu Takatama,[3] Yoichi Nakazato[2] and Koichi Okamoto[1]

Departments of [1]Neurology and [2]Pathology, Gunma University Graduate School of Medicine and [3]Department of Internal Medicine, Geriatrics Research Institute and Hospital, Maebashi, Gunma, Japan

Optineurin is a gene associated with normal tension glaucoma and primary open-angle glaucoma, one of the major causes of irreversible bilateral blindness. Recently, mutations in the gene encoding optineurin were found in patients with amyotrophic lateral sclerosis (ALS). Immunohistochemical analysis showed aggregation of optineurin in skein-like inclusions and round hyaline inclusions in the spinal cord, suggesting that optineurin appears to be a more general marker for ALS. However, our detailed examinations demonstrated that optineurin was found not only in ALS-associated pathological structures, but also in ubiquitin-positive intraneuronal inclusions in ALS with dementia, basophilic inclusions in the basophilic type of ALS, neurofibrillary tangles and dystrophic neurites in Alzheimer's disease, Lewy bodies and Lewy neurites in Parkinson's disease, ballooned neurons in Creutzfeldt-Jakob disease, glial cytoplasmic inclusions in multiple system atrophy, and Pick bodies in Pick disease. With respect to optineurin-positive basophilic inclusions, these structures showed variable immunoreactivities for ubiquitin; some structures were obviously ubiquitin-positive, while others were negative for the protein, suggesting that optineurin expression was not always associated with the expression of ubiquitin. This study indicates that optineurin is widely distributed in neurodegenerative conditions; however, its significance is obscure.

Key words: amyotrophic lateral sclerosis, glaucoma, neurodegenerative diseases, optineruin, ubiquitin.

Correspondence: Yuji Mizuno, MD, PhD, Department of Neurology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Email: mizunoy@med.gunma-u.ac.jp

Received 7 October 2010; revised and accepted 20 December 2010.

© 2011 Japanese Society of Neuropathology

INTRODUCTION

Optineurin is a cytoplasmic protein ubiquitously expressed in retina, brain, heart, skeletal muscle, placenta and kidney, although it was initially shown to be a Golgi-localized protein.[1,2] Its gene spans a ~37 kb genomic region and is located on chromosome 10p15-14. The gene consists of a total of 16 exons containing three non-coding exons in the 5′ region and 13 exons coding for 577 amino acid proteins. Optineurin contains several putative domains including one bZIP motif, two leucine zippers, multiple coiled-coil motifs, ubiquitin-binding domain, and a C-terminal C2H2 type of zinc finger.[1,2]

Several interacting partners with optineurin are identified such as GTPase molecule Rab8,[3] transcription factor III[4], metabotropic glutamate receptor 1a,[5] Huntingtin,[6] myosin VI[7], ring finger protein 11,[8] and serine/threonine kinase receptor-interacting protein 1 (RIP1),[1] indicating multiple cellular functions for optineruin. In addition, optineurin translocates to the nucleus on apoptotic stimuli, which is mediated by Rab8. The interaction of optineurin with myosin VI would be implicative of a role in vesicular trafficking between Golgi apparatus and plasma membrane.[7] Optineurin was recently shown to negatively regulate tumor necrosis factor α (TNF-α)-induced activation of transcriptional factor NF-kB via binding with polyubiquitinated RIP[9]. NF-kB is involved in protecting cells from apoptosis by inducing many anti-apoptotic genes.[10]

Glaucoma is one of the leading causes of irreversible blindness characterized by progressive loss of retinal ganglion cells, degeneration in the optic nerve head, and defect of visual field. The most common form of glaucoma is primary open angle glaucoma (POAG)[11] and is frequently associated with elevated intraocular pressure. However, there is a subtype of POAG without elevated intraocular pressure, normal tension glaucoma (NTG). According to Iwase *et al.*, > 90% of POAG patients were diagnosed as NTG.[12] Glaucoma is genetically heterogeneous and caused by several susceptible genes and environmental factors.

At present, a total of 14 chromosomal loci designated as GLC1A to GLC1N have been reported as POAG,[13,14] one of which includes optineurin (GLC1E).[11,15,16] Mutation of the gene for optineurin is associated with some forms of glaucoma.

Recently Maruyama *et al.* showed that patients with amyotrophic lateral sclerosis (ALS) have mutations of the gene encoding for optineurin: a homozygous exon 5 deletion, a homozygous Q398X nonsense mutation, and a heterozygous E478G missense mutation.[17] Furthermore, the pathological structures of skein-like inclusions and round hyaline inclusions immunoreactive for ubiquitin and TDP-43 were also optineurin-positive.[17] They suggested that optineurin was a more general marker for inclusions in various types of ALS and was different from SOD1 and TDP-43.[17]

In this study, we examined the unique structures in several neurodegenerative diseases such as ALS and ALS with dementia and basophilic inclusions, Alzheimer's disease (AD), Parkinson's disease (PD), Creutzfeldt-Jakob disease (CJD), multiple system atrophy (MSA) and Pick disease, to see if comparatively disease-specific bodies or inclusions were immunoreactive to optineurin.

METHODS

We examined a total of 18 patients with neurodegenerative diseases, including three sporadic ALS cases, three sporadic ALS cases with dementia, one sporadic ALS case with basophilic inclusions, two AD cases, four PD cases, two CJD cases, two MSA cases and one case of Pick disease. Brain tissues were all obtained from the Geriatrics Research Institute and Gunma University, Japan. In all cases, the autopsies were performed in accordance with established procedures and the samples were used in this study after obtaining informed consent from the family of each patient. All patients were definitively diagnosed based on clinical and light microscopic findings. Tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin. Five-μm-thick paraffin sections were prepared for immunohistochemistry, which was carried out using a polyclonal anti-human optineurin antibody (1:200; code no. 10837-1-AP, ProteinTech Group, Chicago, IL, US) or a rabbit polyclonal anti-ubiquitin antibody (1:2000; code no. Z0458, DAKO, Glostrup, Denmark). For enhancement, autoclave treatment for 5 min was performed before reaction with the antibody for optineurin. Sections were blocked in a solution supplied by Histofine SAB-PO kit (Nichirei, Tokyo, Japan) for 30 min at room temperature, then labeled with the first antibody at 4°C overnight, washed in PBS for 30 min, incubated with the second antibody provided by Histofine SAB-PO kit, washed in PBS for 30 min, and finally visualized by the avidin-biotin-peroxidase method. Observation was performed using an Olympus BX50 microscope (Olympus, Tokyo, Japan).

Since basophilic inclusions are demonstrated by HE staining, this process was initially performed to observe where these structures were present. After we photographed the sections, we removed the cover glasses from the slides in xylene, decolorized the specimens in alcohol, and then performed immunohistochemistry with anti-optineurin antibody.

RESULTS

We started by examining anterior horn cells of spinal cords in patients with ALS (Fig. 1). Although Bunina bodies were optineurin-negative (data not shown), skein-like inclusions were clearly immunostained with antibody for optineurin (Fig. 1A), as previously reported by Maruyama *et al.*[17] Concerning the round hyaline inclusions, the peripheral portions of these inclusions showed patchy reaction with anti-optineurin antibody, while the center was weakly immunostained (Fig. 1B). Some of the neurons in the hippocampal granular cell layer in patients with ALS with dementia were immunoreactive for optineurin (Fig. 1C), which are often immunostained with anti-ubiquitin[18] and anti-vacuole-creating protein[19] antibodies forming crescent or circular immunoreactivities. The cortical neurons in those cases also contained cytoplasmic optineurin-positive inclusions (data not shown). In another type of ALS demonstrating basophilic inclusions, the aggregates were markedly immunostained by the antibody (Fig. 1D) and some of the glia also included optineurin-positive cytoplasmic aggregates (insert in Fig. 1D).

In AD cases (Fig. 2), senile plaque, neurofibrillary tangles and granulovacuolar degeneration are the major structures and these were immunohistochemically examined. Anti-optineurin antibody labeled neurofibrillary tangles (Fig. 2A). In addition, some dystrophic neurites in senile plaque, which are observed as swollen processes, were positive for optineurin (Fig. 2B). However, granulovacuolar degeneration frequently detected in hippocampal pyramidal neurons of Sommer's sector was not stained with anti-optineurin antibody (data not shown). Lewy bodies and Lewy neurites in the midbrain were also examined in patients with PD (Fig. 2). The peripheral portion of Lewy bodies showed a strong reaction to anti-optineurin antibody, while the center was weakly immunostained (Fig. 2C). Lewy neurites were also positive for optineurin (Fig. 2D).

Examination of the ballooned neurons in the temporal lobe of patients with CJD showed the disappearance of Nissl bodies as well as swollen cytoplasm with peripheral

© 2011 Japanese Society of Neuropathology

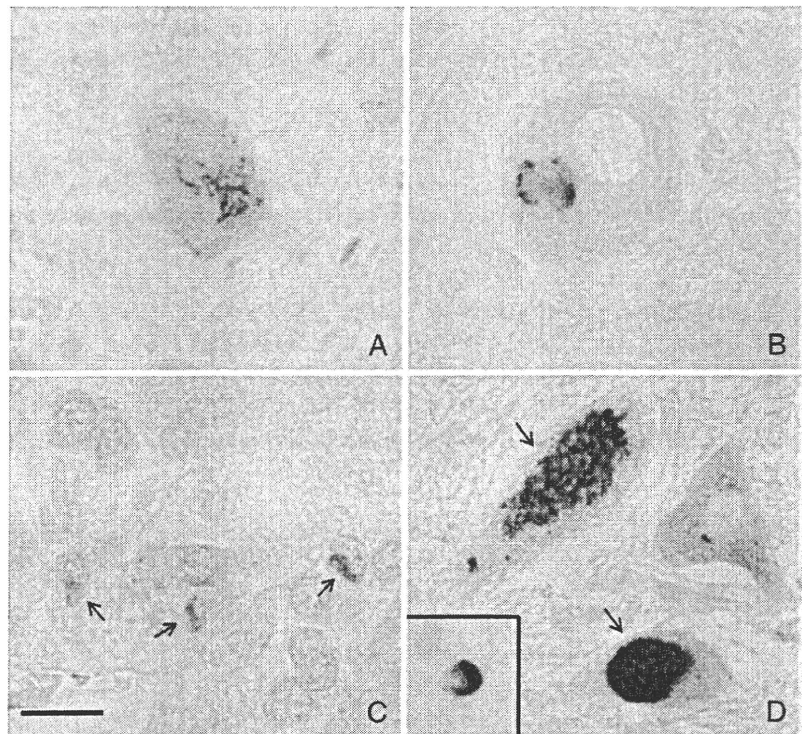


Fig. 1 Optineurin immunoreactivitiy for several pathological structures in patients with different types of amyotrophic lateral sclerosis (ALS). A–D. Immunostaining for optineurin. Skein-like inclusions were optineurin-positive (A) and round hyaline inclusions were irregularly immunoreactive for optineurin around the rim (B) with the center being weakly immunostained. In patients with ALS with dementia, some of the intraneuronal inclusions of the hippocampal granular cell layer were positively immunostained (C; arrows). In patients with ALS with basophilic inclusions, these aggregates showed obvious immunostaining (D; arrows) and some of the glia, probably oligodendrocytes, also included optineurin-positive aggregates (D; insert). Scale bar: 20 μm.

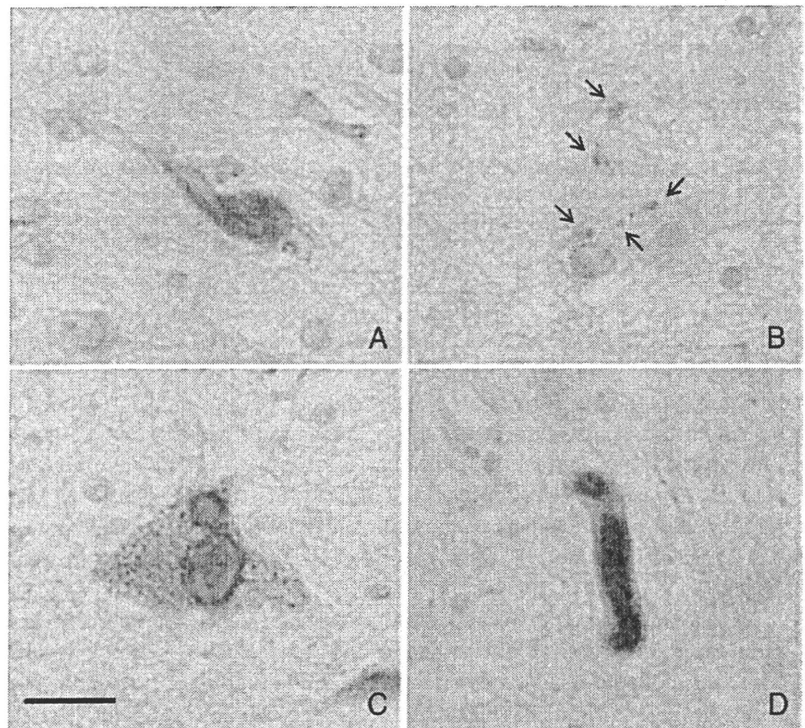


Fig. 2 Optineurin immunoreactivity for several pathological structures in patients with Alzheimer's disease (AD) and Parkinson's disease (PD). A–D. Immunostaining for optineurin. A, B: AD. C, D: PD. Neurofibrillary tangles (A) and dystrophic neurites (B; arrows) were optineurin-positive, and Lewy bodies (especially periphery) (C) and Lewy neurites (D) were positive for optineurin. Scale bar: 20 μm.

© 2011 Japanese Society of Neuropathology

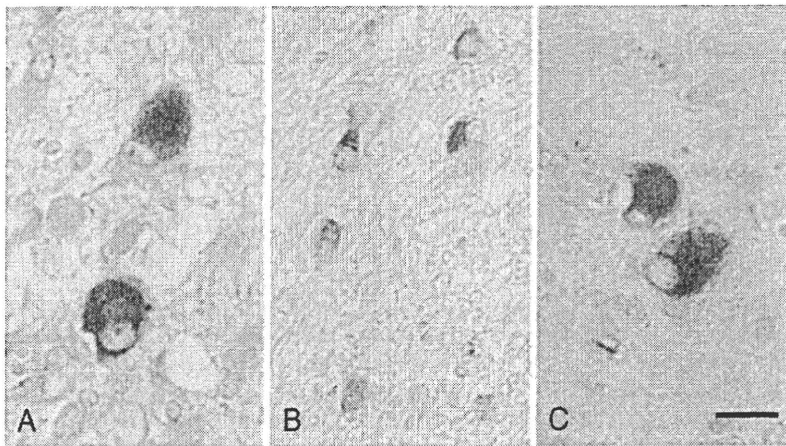


Fig. 3 Optineurin immunoreactivities of other pathological structures. A: Ballooned neurons in Creutzfeldt-Jakob disease. B: Glial cytoplasmic inclusions in multiple system atrophy. C: Pick bodies. Scale bar: 20 μm.

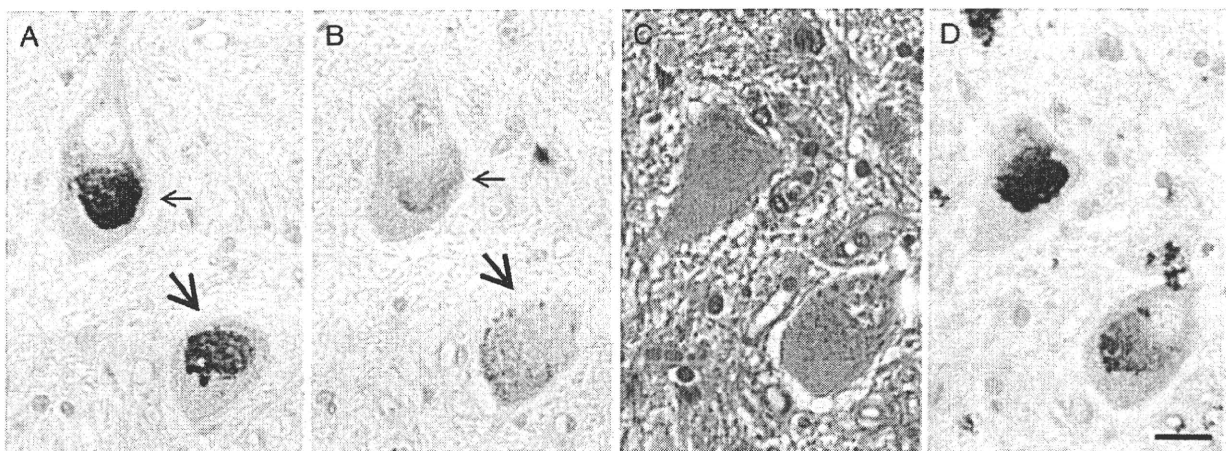


Fig. 4 Optineurin immunoreactivity of basophilic inclusions. A, D: Immunostaining for optineurin. B: Immunostaining for ubiquitin. C: HE staining. A–C: Serial sections. C, D: Same section. Some of the basophilic inclusions (C) showed obvious immunostaining for both optineurin and ubiquitin (A, B; small arrow), while others were optineurin-positive but ubiquitin-negative (A, B; large arrow). Panel C shows a serial section of B stained with HE to confirm the presence of basophilic inclusions, while Panel D shows positive immunoreactivities for optineurin in the same section (D). Scale bar: 20 μm.

nuclei. The cytoplasm of ballooned neurons was well immunostained with anti-optineurin antibody (Fig. 3A). Furthermore, the cytoplasm of the oligodendroglia in the brainstem from patients with MSA was immunoreactive with antibody for optineurin, indicating that glial cytoplasmic inclusions were optineurin-positive (Fig. 3B). In Pick diseases, Pick bodies characterized by slightly basophilic staining by HE were positive for optineurin (Fig. 3C).

With respect to optineurin-positive basophilic inclusions, three serial sections were prepared and then stained with anti-optineurin antibody (Fig. 4A), anti-ubiquitin antibody (Fig. 4B), and HE (Fig. 4C) to determine whether basophilic inclusions showed ubiquitin expression. First, when a structure showing basophilic inclusions could be confirmed with HE staining (Fig. 4C), the same section was immunostained with the antibody for optineurin to determine whether basophilic inclusions showed positive immunoreaction for optineurin (Fig. 4D). Second, the next serial section was examined using anti-ubiquitin antibody to determine whether basophilic inclusions were ubiquitin-positive (Fig. 4B). The third serial section was immunostained with anti-optineurin antibody (Fig. 4A) to confirm whether basophilic inclusions were still present. Our findings indicated that basophilic inclusions were immunoreactive for optineurin (Fig. 4A,D), while basophilic inclusions showed variable immunostainings for ubiquitin, ranging from negative (large arrow in Fig. 4B) to positive (small arrow in Fig. 4B) and from weak to strong (data now shown). In total, five of nine basophilic inclusions were ubiquitin-negative but optineurin-positive and four were positive for both.

© 2011 Japanese Society of Neuropathology

DISCUSSION

In this study, we confirmed that optineurin immunoreactivities are more widely distributed among other neurodegenerative diseases than previously reported.[17] Since both intraneurocytoplasmic and intraglial inclusions/bodies are common pathological features among these neurodegenerative diseases, we focused on disease-characteristic pathologies, such as senile plaques and neurofibrillary tangles in AD, Lewy bodies and Lewy neurites in PD, ballooned neurons in CJD, glial cytoplasmic inclusions in MSA, and Pick bodies in Pick disease, in addition to skein-like inclusions and round hyaline inclusions in ALS, neurons in the hippocampal granular cell layer from ALS with dementia, and basophilic inclusions in basophilic type of ALS. Our findings showed that optineurin immunoreactivities were more widely detected in a variety of neurodegenerative diseases in addition to ALS cases and that optineurin was not only specific for ALS but also for other diseases. It remains unclear why optineurin was found in the variety of pathological structures in neurodegenerative diseases. We could not deny that optineurin might be just bystanders to the various inclusions. The aggregation of optineurin, which is generally found to be ubiquitin-positive, may be the common process involved in neurodegeneration and cell death. It is possible that optineurin is just secondarily entrapped in the pathological structures or in ubiquitin. To investigate this possibility, we focused on the basophilic inclusions. Since variable levels of ubiquitin were present in the basophilic inclusions, we investigated whether there was some relationship between the expression of optineurin and ubiquitin. The results indicated that nine of the basophilic inclusions we examined were all optineurin-positive, and four of these were ubiquitin-positive, while the others were negative for ubiquitin, showing a wider presence of optineurin than of ubiquitin. Although the role of optineurin remains unknown, our study obviously showed that optineurin could be an aggregation-prone protein in the affected neurons and glia.

The level of optineurin is variable under certain conditions of stress. High levels of oxidative stress lead to overexpression of optineurin in NIH 3T3 cells, protecting these cells from cell death.[20] However, overexpression of normal optineurin sensitizes RGC-5 cells to TNF-α-induced cell death. In contrast, normally overexpressed optineurin reduces TNF-α-induced cell death in HeLa cells.[21] Our findings suggest that the expression of optineurin could be upregulated in pathological conditions such as ALS, AD, PD, CJD, MSA and Pick diseases. There are lots of associated factors modulating expression of optineurin, making interpretation more complicated. However, understanding the molecular mechanism that regulates the level of optineurin is a very important step in the detailed investigation of optineurin.

In conclusion, we demonstrated that optineurin-positive structures are more common to various neurodegenerative diseases, including cases of not only ALS but also AD, PD, CJD, MSA and Pick disease. As such aggregates with optineurin are generally shown to be ubiquitin-, p62-[22] and TDP-43-positive, elucidation of the relevance to these proteins would be the next step. Detailed examination of how the inclusions are formed, how these cells survive by forming the inclusions, and what effects the inclusions have on the remaining neurons, could contribute to clarifying the pathogenesis of neurodegenerative diseases.

© 2011 Japanese Society of Neuropathology

ACKNOWLEGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to Y.M., Y.F., and K.O., and by grants 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.O.

REFERENCES

1. Chalasani ML, Swarup G, Balasubramanian D. Optineurin and its mutants: molecules associated with some forms of glaucoma. *Ophthalmic Res* 2009; **42**: 176–184.
2. Ray K, Mookherjee S. Molecular complexity of primary open angle glaucoma: current concepts. *J Genet* 2009; **88**: 451–467.
3. Chibalina MV, Roberts RC, Arden SD, Kendrick-Jones J, Buss F. Rab8-optineurin-myosin VI: analysis of interactions and functions in the secretory pathway. *Methods Enzymol* 2008; **438**: 11–24.
4. Moreland RJ, Dresser ME, Rodgers JS *et al.* Identification of a transcription factor IIIA-interacting protein. *Nucleic Acids Res* 2000; **28**: 1986–1993.
5. Anborgh PH, Godin C, Pampillo M *et al.* Inhibition of metabotropic glutamate receptor signaling by the huntingtin-binding protein optineurin. *J Biol Chem* 2005; **280**: 34840–34848.
6. Hattula K, Peränen J. FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis. *Curr Biol* 2000; **10**: 1603–1606.
7. Sahlender DA, Roberts RC, Arden SD *et al.* Optineurin links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis. *J Cell Biol* 2005; **169**: 285–295.
8. Colland F, Jacq X, Trouplin V *et al.* Functional proteomics mapping of a human signaling pathway. *Genome Res* 2004; **14**: 1324–1332.

9. Zhu G, Wu CJ, Zhao Y, Ashwell JD. Optineurin negatively regulates TNFalpha- induced NF-kappaB activation by competing with NEMO for ubiquitinated RIP. *Curr Biol* 2007; **17**: 1438–1443.
10. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003; **10**: 45–65.
11. Rezaie T, Child A, Hitchings R *et al.* Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science* 2002; **295**: 1077–1079.
12. Iwase A, Suzuki Y, Araie M *et al.* The prevalence of primary open-angle glaucoma in Japanese: the Tajimi Study. *Ophthalmology* 2004; **111**: 1641–1648.
13. Wang DY, Fan BJ, Chua JK *et al.* A genome-wide scan maps a novel juvenile-onset primary open-angle glaucoma locus to 15q. *Invest Ophthalmol Vis Sci* 2006; **47**: 5315–5321.
14. Wiggs JL. Genetic etiologies of glaucoma. *Arch Ophthalmol* 2007; **125**: 30–37.
15. Allingham RR, Liu Y, Rhee DJ. The genetics of primary open-angle glaucoma: a review. *Exp Eye Res* 2009; **88**: 837–844.
16. Sarfarazi M, Child A, Stoilova D *et al.* Localization of the fourth locus (GLC1E) for adult-onset primary open-angle glaucoma to the 10p15-p14 region. *Am J Hum Genet* 1998; **62**: 641–652.
17. Maruyama H, Morino H, Ito H *et al.* Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 2010; **465**: 223–226.
18. Okamoto K, Murakami N, Kusaka H *et al.* Ubiquitin-positive intraneuronal inclusions in the extramotor cortices of presenile dementia patients with motor neuron disease. *J Neurol* 1992; **239**: 426–430.
19. Mizuno Y, Hori S, Kakizuka A, Okamoto K. Vacuole-creating protein in neurodegenerative diseases in humans. *Neurosci Lett* 2003; **343**: 77–80.
20. De Marco N, Buono M, Troise F, Diez-Roux G. Optineurin increases cell survival and translocates to the nucleus in a Rab8-dependent manner upon an apoptotic stimulus. *J Biol Chem* 2006; **281**: 16147–16156.
21. Chalasani ML, Radha V, Gupta V, Agarwal N, Balasubramanian D, Swarup G. A glaucoma-associated mutant of optineurin selectively induces death of retinal ganglion cells which is inhibited by antioxidants. *Invest Ophthalmol Vis Sci* 2007; **48**: 1607–1614.
22. Mizuno Y, Amari M, Takatama M, Aizawa H, Mihara B, Okamoto K. Immunoreactivites of p62, an ubiquitin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis. *J Neurol Sci* 2006; **249**: 13–18.

© 2011 Japanese Society of Neuropathology

NEUROPATHOLOGY

Neuropathology 2010; **30**, 189–193 doi:10.1111/j.1440-1789.2009.01088.x

Symposium: Advances in amyotrophic lateral sclerosis research

Pathology of protein synthesis and degradation systems in ALS

Koichi Okamoto, Yukio Fujita and Yuji Mizuno

Department of Neurology, Gunma University Graduate School of Medicine, Maebashi, Japan

Protein synthesis and degradation systems in neurons are among the major subjects of study in neurobiology. These systems are believed to be the main pathways involved in ALS; however, the essential pathomechanisms that underlie this disease remain obscure. In addition to the ubiquitin-proteasomal and autophagic systems, several cytoplasmic organelles are also involved in ALS. Here, we present our data and discuss the main morphological abnormalities detected in the anterior horn cells of ALS patients.

Key words: ALS, amyotrophic lateral sclerosis, Golgi apparatus, pathology, review.

INTRODUCTION

Postmortem and experimental neuropathological studies have provided clues to the pathogenesis of amyotrophic lateral sclerosis (ALS). The hallmarks of the neuropathological findings in ALS are significant motor neuron loss, Bunina bodies, and the abnormal accumulation of insoluble ubiquitinated cytoplasmic inclusions in the lower motor neurons, which include skein-like and round inclusions. However, the characteristic cytopathological features of motor neurons in ALS remain obscure. The processes of neuronal degeneration in motor neurons are complex; although several genetic mutations are involved in motor neuron injury in familial ALS, less is known about the genetic and environmental factors that contribute to sporadic ALS. There may be a complex interplay between multiple pathogenic processes, which include misfolded protein aggregates, TDP-43 abnormalities, increased oxidative stress, mitochondrial dysfunction, ribosomal dysfunction, disturbance of intracellular trafficking, dysfunction of the ubiquitin–proteasomal and autophagic systems, increased glutamate excitotoxicity, and impaired axonal transport. Many papers and reviews have been published on the pathomechanisms of ALS;[1–8] there is increasing evidence that non-neuronal cells in the vicinity of motor neurons may also contribute to neuronal degeneration[9] and that a programmed mechanism of cell death resembling apoptosis is responsible for motor neuron degeneration in ALS.[10] Here, we present our data and discuss the main morphological abnormalities seen in the anterior horn cells of ALS patients.

FRAGMENTATION OF THE GOLGI APPARATUS

The Golgi apparatus (GA) plays a role in the transport, processing, and targeting of numerous proteins destined for secretion, the plasma membrane, and lysosomes.[11] In neurons, the GA is involved in the axoplasmic flow of endogenous proteins and of exogenous macromolecules transported by orthograde, retrograde, and trans-synaptic routes. Lesions of the neuronal GA undoubtedly have detrimental consequences for the function of axons and presynaptic terminals.

Deparaffinized sections from ALS patients, which included 16 cases of sporadic ALS, two cases of juvenile ALS with basophilic inclusions, and three cases of familial ALS with SOD1 mutations and posterior column involvement, as well as five cases of X-linked spinal and bulbar muscular atrophy (SBMA), were immunostained with a polyclonal anti-MG160 antibody, which recognizes a sialoglycoprotein of the medial cisternae of the GA, and with a polyclonal anti-TGN46 antibody, which recognizes an intrinsic membrane protein of the *trans*-Golgi network (TGN). These antibodies were used as markers for the GA.[12] We also immunostained motor neurons with the monoclonal anti-1C2 antibody, which recognizes a polyglutamine stretch, and with a polyclonal anti-SOD1 (Cu,

Correspondence: Koichi Okamoto, MD, PhD, Department of Neurology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi 371-8511, Japan. Email: okamotok@med.gunma-u.ac.jp

Received 29 October 2009 and accepted 10 November 2009; published online 19 January 2010.

© 2010 Japanese Society of Neuropathology

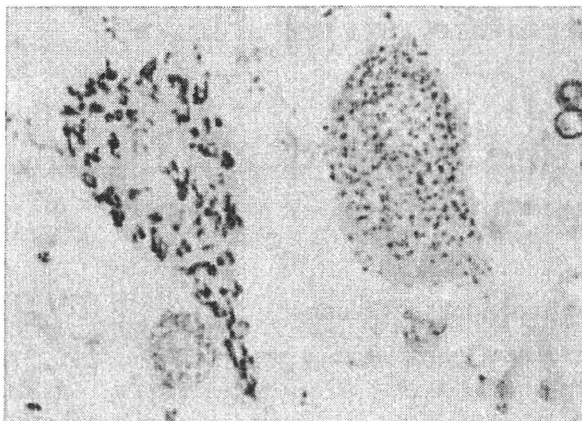


Fig. 1 The *trans*-Golgi network (TGN)46 immunostaining of anterior horn cells of a sporadic ALS patient. The Golgi apparatus (GA) in the neuron shown on the left had a normal network appearance, whereas the GA in the neuron shown on the right was fragmented. ×200.

Zn) antibody. Fragmentation of the GA was frequently observed in the motor neurons of patients with sporadic ALS (Fig. 1), familial ALS with posterior column involvement, and juvenile ALS with basophilic inclusions.[12] A decrease in the size of the GA was observed in numerous anterior horn cells of SBMA samples; however, fragmentation of the GA was also observed in a few neurons in these patients.[13] The variable frequency of fragmented GA between SBMA and ALS adds to the pathogenetic differences in the mode of neurodegeneration in these two motor neuron diseases. Fragmentation of the GA is not specific to ALS, because similar findings have also been observed in olivary hypertrophic neurons and ballooned neurons in patients with corticobasal degeneration and CJD.[14,15] Experimental evidence of cell injury induced by a variety of agents indicates that fragmentation of the GA is associated with defects of membrane flow and protein transport.[11] We showed that the GA of motor neurons was frequently fragmented in patients with ALS and that the majority of motor neurons that contained cytoplasmic inclusions exhibited GA fragmentation. Furthermore, fragmentation of the GA is an early neuropathological change in transgenic mice expressing the G93A mutation of the SOD1 gene.[11,12] These results suggest that fragmentation of the GA may be related to the neuronal degeneration in ALS patients; therefore, further examination of the significance of GA fragmentation in ALS is required to answer this question.

TDP-43 AND GOLGI FRAGMENTATION

We investigated the relationship between TDP-43 immunoreactivity and fragmentation of the GA in ALS.[16] We divided the anterior horn cells of ALS patients into four subtypes, according to the observed differences in TDP-43 immunoreactivity, and found that all of neurons with normal nuclear staining exhibited normal GA profiles. In contrast, GA fragmentation was detected in neurons that displayed abnormal TDP-43 cytoplasmic immunoreactivity. These results suggest that abnormal TDP-43 immunoreactivity is associated with dysfunction of the secretory pathway in motor neurons.

OXIDATIVE AND ENDOPLASMIC RETICULUM STRESS

The effects of oxidative stress within neurons may be cumulative, and cellular injury caused by free radicals is a major potential cause of the age-related deterioration observed in these cells.[2] However, it remains to be determined whether oxidative stress is a cause, a consequence, or an epiphenomenon of the pathological processes.[3] This phenomenon has been studied mainly in SOD1-related familial ALS; however, the roles of oxidative stress in sporadic ALS remain obscure. Recent studies using autopsy ALS samples and mutant SOD1 transgenic mice suggest that endoplasmic reticulum (ER) stress-related toxicity may be a relevant ALS pathomechanism.[17] Urushitani *et al.*[18] suggested that the ER–Golgi pathway is a predominant cellular site of aggregation of mutant SOD1. Oyanagi *et al.*[19] reported that the rough endoplasmic reticulum (rER) in shrunken anterior horn cells was irregularly distended and exhibited detachment of the ribosomes. Atkin *et al.*[20] showed that a full unfolded protein response, which included the expression of stress sensor kinases, chaperones, and apoptotic mediators, is present in spinal cord samples of sporadic ALS patients.

Our previous studies of human olivary hypertrophy or pseudohypertrophy revealed that fundamental changes occur in neurons in which the rER exhibits electron-dense granules.[21] Furthermore, increased expression of alpha B crystalline was also observed in the early stages of disease.[22] This transneuronal mechanism may be a form of human stress response; however, there are no reports of similar electron-dense rER granules in the motor neurons of ALS patients. Recently, Sasaki *et al.*[23] reported the presence of electron-dense or amorphous granules in distended rER in anterior horn cells of sporadic ALS patients, which is suggestive of an ER stress mechanism.

TDP-43 and fused in sarcoma (FUS) were recently identified as ALS genes. These genes are involved in the regulation of transcription and in RNA splicing and transport, respectively. This suggests that a common mechanism may underlie motor neuron degeneration.[6,7,24]

© 2010 Japanese Society of Neuropathology

MITOCHONDRIAL DYSFUNCTION

The main functions of mitochondria include the generation of intracellular ATP and the buffering of intracellular calcium. Morphological mitochondrial alterations were reported in ALS motor neurons.[25,26]

EXCITOTOXICITY

Excitotoxicity mediated by ionotropic glutamate receptors has been regarded as a principal cause of neuronal cell death in ALS.[5] Recently, glial involvement has been shown to be essential for ALS-related motoneuronal death. Sasabe *et al.*[27] showed that the N-methyl-D-aspartate (NMDA) receptor coagonist D-serine is a glia-derived enhancer of glutamate toxicity in ALS motoneurons. Therefore, glutamate toxicity enhanced by D-serine that is overproduced in glia has been proposed as a novel mechanism underlying ALS motoneuronal death. Thus, this mechanism may be regarded as a potential therapeutic target for ALS.

BUNINA BODIES

The pathological aggregation of cytoplasmic constituents in the form of a variety of inclusions may play a role in the pathogenesis of neuronal death. The inclusions observed in anterior horn cells include Bunina bodies, which are small eosinophilic intracytoplasmic inclusions present in the remaining lower motor neurons, as well as skein-like, round, and basophilic types. Bunina bodies are generally considered to be specific pathological hallmarks of ALS.[28,29] Bunina bodies consist of amorphous electron-dense material surrounded by tubular and vesicular structures on electron microscopy. Although the nature and significance of Bunina bodies in ALS remain unclear, these bodies may represent abnormal accumulation of unknown proteinous materials. At present, only two proteins have been shown to be present in Bunina bodies: cystatin C[30] and transferrin.[31] The use of antibodies against ubiquitin, neurofilament, tau, alpha-tubulin, beta-tubulin, microtubule-associated proteins, actin, myosin, desmin, synaptophysin, amyloid precursor protein, GFAP, alpha-synuclein, p62, and TDP-43 failed to demonstrate their presence in Bunina bodies. Cystatin C, which has a molecular weight of 13,260 Da, is an inhibitor of lysosomal cysteine proteinases and is present at low concentration in various extracellular fluids in humans; it is also present within human cortical neurons. The main physiological function of cystatin C is the local regulation of cysteine proteinases. Many small cystatin C-immunostained granules were scattered in almost all neurons and their dendrites in a non-ALS spinal cord sample. Bunina bodies were clearly labeled with an anti-cystatin C antibody. The cytoplasmic and dendritic cystatin C-positive granules were reduced in number and size in neurons that contained Bunina bodies compared with normal-appearing neurons. Lewy body-like/round, skein-like, and spheroid inclusions were not labeled with anti-cystatin C antibody. There seems to be a consensus on the hypothesis that Bunina bodies consist of amorphous electron-dense material surrounded by tubular and vesicular structures with a few central clear areas containing cytoplasmic components. Compared with the routine electron microscopic features of Bunina bodies, cystatin C immunoperoxidase products appeared to be localized mainly in the tubular and vesicular structures in the periphery of the bodies. We speculate that amorphous material may develop around the tubular and vesicular structures in the early stage of Bunina body formation.[29] These deposits may increase with the proliferation of vesicles and tubules, which may become embedded in cellular organelles and may be engulfed in some areas. These structures are devoid of ribosome attachment; therefore, it is suggested that they originate from smooth ER or from the Golgi apparatus. However, they were immunohistologically negative for Golgi and autophagic markers. Additional studies are needed to clarify the relationship between Bunina bodies and cellular organelles.

Transferrin, which is an iron-binding protein, plays an important role in the transport and delivery of circulating ferric iron to tissues. Mizuno *et al.*[31] used antibodies against human transferrin to examine transverse paraffin sections of lumbar spinal cords from 12 ALS cases, which included two ALS cases with dementia and two ALS cases with basophilic inclusions. Their results demonstrated that transferrin was localized in Bunina bodies and in some basophilic inclusions. In contrast, skein-like, Lewy body-like, and round inclusions did not exhibit obviously detectable transferrin immunoreactivity. These findings suggest that although the mechanisms underlying transferrin accumulation in Bunina bodies and basophilic inclusions remain unknown, transferrin may be involved in the formation of these inclusions.

SKEIN-LIKE INCLUSIONS

Skein-like inclusions comprise bundles of ubiquitinated filaments that are detected in most sporadic ALS cases. Skein-like inclusions may result from ubiquitin aggregation with another unknown protein that is abnormally accumulated within the cytoplasm. Ubiquitin is involved in extralysosomal protein degradation by the proteasome; therefore, the nonlysosomal degradation system may be related to the degenerative processes in ALS. Ubiquitination may play a protective role within cells by postponing cell death.[32]

© 2010 Japanese Society of Neuropathology

Van Welsem *et al.*[33] investigated the relationship between three lesions (motor neuronal loss, Bunina bodies, and skein-like inclusions) in the cervical and lumbar anterior horns and hypoglossal nuclei of 20 ALS patients and nine control samples using a quantitative light microscopy study. Immunohistochemistry using anti-cystatin C and anti-ubiquitin antibodies was performed to detect Bunina bodies and skein-like inclusions, respectively. A significant correlation was found between the severity of neuronal loss and the proportion of skein-like inclusion-containing neurons in the spinal cord, whereas no correlation was found for Bunina bodies. These authors therefore propose that Bunina bodies and skein-like inclusions participate in two different steps of the cascade that leads to neuronal loss.

AUTOPHAGY AND LYSOSOMES

Autophagy is a major degradation pathway for intracytosolic aggregate-prone proteins. In addition, it acts as a constitutive self-degradative process involved both in the basal turnover of cellular components and in the response to nutrient starvation. Fornai *et al.*[34] found that defective autophagy was a final common pathway in the genesis of ALS. The number of secondary lysosomes was increased in anterior horn cells in several sporadic ALS patients;[35] therefore, lysosomes may also play an important role in degenerative processes. We have previously performed immunohistochemical studies using six lysosome-related polyclonal antibodies against cathepsins B, D, H, L, and LPG 120 and ATP synthase subunit C.[36] The number of cathepsin D- and ATP synthase C-positive granules was decreased in anterior horn cells of sporadic ALS patients, whereas LPG 120-positive neurons were increased in ALS patients compared with non-ALS patients. Wootz *et al.*[37] suggest that the changes in protein levels and distribution of cathepsin D and cystatin B and C indicate a role for these proteins in the degeneration of motor neurons in SOD1 transgenic mice.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid 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. Shaw PJ. Molecular and cellular pathways of neurodegeneration in motor neurone disease. *J Neurol Neurosurg Psychiatry* 2005; **76**: 1046–1057.
2. Kabashi E, Valdmanis PN, Dion P, Rouleau GA. Oxidized/misfolded superoxide dismutase-1: the cause of all amyotrophic lateral sclerosis? *Ann Neurol* 2007; **62**: 553–559.
3. Shibata N, Kobayashi M. The role for oxidative stress in neurodegenerative diseases. *Brain Nerve* 2008; **60**: 157–170 (in Japanense).
4. Ström AL, Gal J, Shi P, Kasarskis EJ, Hayward LJ, Zhu H. Retrograde axonal transport and motor neuron disease. *J Neurochem* 2008; **106**: 495–505.
5. Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Ann Neurol* 2009; **65** (Suppl): S3–S9.
6. Neumann M. Molecular neuropathology of TDP-43 proteinopathies. *Int J Mol Aci* 2009; **10**: 232–246.
7. Lagier-Tourenne C, Cleveland DW. Rethinking ALS: the FUS about TDP-43. *Cell* 2009; **136**: 1001–1004.
8. Kanekura K, Suzuki H, Aiso S, Matsuoka M. ER stress and unfolded protein response in amyotrophic lateral sclerosis. *Mol Neurobil* 2009; **39**: 81–89.
9. Van Den B, Robberecht W. Crosstalk between astrocytes and motor neurons: what is the message? *Exp Neurol* 2008; **211**: 1–6.
10. Sathasivam S, Shaw PJ. Apoptosis in amyotrophic lateral sclerosis – what is the evidence? *Lancet Neurol* 2005; **4**: 500–509.
11. Gonatas NK, Stieber A, Gonatas JO. Fragmentation of the Golgi apparatus in neurodegenerative diseases and cell death. *J Neurol Sci* 2006; **246**: 21–30.
12. Fujita Y, Okamoto K. Golgi apparatus of the motor neurons in patients with amyotrophic lateral sclerosis and in mice models of amyotrophic lateral sclerosis. *Neuropathology* 2005; **25**: 388–394.
13. Yaguchi M, Hashizume Y, Yoshida M, Gonatas NK, Okamoto K. Reduction of the size of the Golgi apparatus of spinal anterior horn cells in patients with X-linked spinal and bulbar muscular atrophy. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2003; **4**: 17–21.
14. Takamine K, Okamoto K, Fujita Y, Sakurai A, Takatama M, Gonatas NK. The involvement of the neuronal Golgi apparatus and trans-Golgi network in the human olivary hypertrophy. *J Neurol Sci* 2000; **182**: 45–50.
15. Sakurai A, Okamoto K, Fujita Y *et al.* Fragmentation of the Golgi apparatus of the ballooned neurons in patients with corticobasal degeneration and Creutzfeldt-Jakob disease. *Acta Neuropathol* 2000; **100**: 270–274.
16. Fujita Y, Mizuno Y, Takatama M, Okamoto K. Anterior horn cells with abnormal TDP-43 immunoreactivities show fragmentation of the Golgi apparatus in ALS. *J Neurol Sci* 2008; **269**: 30–34.

© 2010 Japanese Society of Neuropathology

17. Nishitoh H, Kadowaki H, Nagai A *et al.* ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev* 2008; **22**: 1451–1464.
18. Urushitani M, Ezzi SA, Matsuo A, Tooyama I, Julien J-P. The endoplasmic reticulum-Golgi pathway is a target for trasnslocation and aggeregation of mutant superoxide dismutase linked to ALS. *FASREB J* 2008; **22**: 2476–2487.
19. Oyanagi K, Yamazki M, Takahashi H *et al.* Spinal anterior horn cells in sporadic amyotrophic lateral sclerosis show ribosomal detachment from, and ciscternal distention of rough endoplasmic reticulum. *Neuropathol App Neurobiol* 2008; **34**: 650–658.
20. Atkin JD, Farg MA, Walker AK, McLean C, Tomas D, Horne MK. Endoplasmic reticulum stress and induction of unfolded protein response in human sporadic amyotrophic lateral sclerosis. *Neurobil Dis* 2008; **30**: 400–407.
21. Okamoto K, Hirai S, Iizuka T, Watanabe M. Fundamental morphological changes in human olivary hypertrophy. *Acta Pathol Jpn* 1992; **42**: 408–413.
22. Fukushima K, Mizuno Y, Takatama M, Okamoto K. Increased neuronal expression of alpha B-crystallin in human olivary hypertrophy. *Neuropathology* 2006; **26**: 196–200.
23. Sasaki S, Takeda T, Honma A, Uchiyama S. Rough endoplasmic reticulum alterations in the anterior horn neurons of the spinal cord in sporadic amyotrophic lateral sclerosis. *Neuropathology* 2009; **29** (Suppl): 110 (Abstract, in Japanese).
24. Vance C, Rogelj B, Hortobágyi T *et al.* Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 2009; **323**: 1208–1210.
25. Xu Z, Jung C, Higgins C, Levine J, Kong J. Mitochondrial degeneration in amyotrophic lateral sclerosis. *J Bioenerg Bionmem* 2004; **36**: 395–399.
26. Sasaki S, Iwata M. Mitochondrial alterations in the spinal cord of patients with sporadic amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 2007; **66**: 10–16.
27. Sasabe J, Chiba T, Yamada M *et al.* D-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. *EMBO J* 2007; **26**: 4149–4159.
28. Rowland LP. T.J. Bunina, Asao Hirano, and the post mortem cellular diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler* 2009; **10**: 74–78.
29. Okamoto K, Mizuno Y, Fujita Y. Bunina bodies in amyotrophic lateral sclerosis. *Neuropathology* 2008; **28**: 109–115.
30. Okamoto K, Hirai S, Amari M *et al.* Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. *Neurosci Lett* 1993; **162**: 125–128.
31. Mizuno Y, Amari M, Takatama M *et al.* Transferrin localizes in Bunina bodies in amyotrophic lateral sclerosis. *Acta Neuropathol* 2006; **112**: 597–603.
32. Shimura H, Schlossmacher MG, Hattori N *et al.* Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 2001; **293**: 263–269.
33. Van Welsem ME, Hogenhuis JA, Meininger V, Metsaars WP, Hauw J-J, Seilhean D. The relationship between Bunina bodies, skein-like inclusions and neuronal loss in amyotrophic lateral sclerosis. *Acta Neuropathol* 2002; **103**: 583–589.
34. Fornai F, Lonone P, Ferrucci M, Lenzi P, Isidoro C, Ruggieri S, Paparelli A. Autophagy and amyotrophic lateral scleosis: the multiple role of lithium. *Autophagy* 2008; **16**: 527–530.
35. Okamoto K, Watanabe M, Amari M, Hirai S. Membranous cytoplasmic bodies in the anterior horn neurons in two patients with amyotrophic lateral sclerosis. *Neuropathology* 1996; **16**: 6–9.
36. Okamoto K, Amari M, Sakurai A, Hirai S, Kominami E. Electron microscopic and immunohistochemical studies of lysosomal abnormalities in patients with amyotrophic lateral sclerosis. In 1995 Annual Report of the Research Committee of CNS Degenerative Disease, the Ministry of health and Welfare of Japan, 1996, pp. 197–200.
37. Wootz H, Weber E, Korhonen L, Lindholm D. Altered distribution and levels of cathepsin D and cystatins in amyotrophic lateral sclerosis transgenic mice: possible roles in motor neuron survival. *Neuroscience* 2006; **143**: 419–430.

© 2010 Japanese Society of Neuropathology

NEUROPATHOLOGY

Neuropathology 2010; **30**, 486–489 doi:10.1111/j.1440-1789.2010.01121.x

The 50th Anniversary of Japanese Society of Neuropathology

Memorial Symposium: Milestones in Neuropathology from Japan

Ubiquitin-positive tau-negative intraneuronal inclusions in dementia with motor neuron disease

Koichi Okamoto

Department of Neurology, Gunma University Graduate School of Medicine, Maebashi, Japan

We first reported ubiquitin-positive tau-negative intraneuronal inclusions in the hippocampal granular cell layer and entorhinal cortices in patients with amyotrophic lateral sclerosis (ALS). We then found that those inclusions occur frequently in patients with presenile dementia and motor neuron disease. The ultrastructure of the inclusions consists mainly of granules with a few filaments. In 2006, TDP-43 was identified as a major component of the inclusions specific for frontotemporal lobar degeneration and ALS. Here, we review the current knowledge regarding ubiquitin-positive tau-negative intraneuronal inclusions.

Key words: amyotrophic lateral sclerosis, frontotemporal lobar degeneration, motor neuron disease, tau, ubiquitin.

BACKGROUND

In 1964, Yuasa[1] described a patient with both neurological features typical of amyotrophic lateral sclerosis (ALS) and behavioral and psychiatric symptoms of frontotemporal dementia. However, autopsy findings were not reported. In 1985, Mitsuyama[2] reviewed the clinicopathological findings of 26 patients with presenile dementia and motor neuron disease (MND) in Japan. Pathologically, there were non-specific mild degenerative changes throughout the CNS, and he suggested the possibility of a new disease. Thereafter, we used (mainly in Japan) the term "Yuasa–Mitsuyama-type" dementia with MND to describe these patients.[3] MND and ALS were used almost synonymously.

At that time, we studied the pathological findings of senile changes in the autopsied brains from 21 patients with sporadic ALS, aged 42–81 years. Paraffin-embedded sections were examined with the Bielschowsky method and by imunohistochemical staining with antibodies directed against β-protein, tau and ubiquitin. We suggested that aged ALS patients accelerate senile plaque formation.[4] During these studies, we chanced to find ubiquitin-positive tau-negative intracytoplasmic inclusions in the hippocampal granular cells of some patients with sporadic ALS. These inclusions had not been previously reported, and similar inclusions are not found in routinely autopsied brains. Therefore, we studied their morphology and their specificity to ALS.

FIRST REPORT OF THE INCLUSIONS[5]

We studied the brains of 27 patients with clinically and pathologically confirmed sporadic ALS (aged 42–84 years), including one patient with dementia and ALS. Fifty non-ALS patients were also studied. Formalin-fixed and paraffin-embedded sections were stained with HE, KB, Nissl, phosphotungstic acid hematoxylin, Marolly, periodic acid–Schiff, Congo red, Bodian, and the modified Bielschowsky methods. Immunohistochemical studies were also performed using various antibodies, including those directed against ubiquitin, neurofilament, tau, paired helical filament (PHF), β-tubulin, β-protein, α-actin, GFAP and desmin. In seven of the 27 ALS patients, ubiquitin-positive intracytoplasmic inclusions were observed in the neurons of the hippocampal granular cell layers (Fig. 1). The inclusions formed a crescent or circular pattern around the nucleus and were seen in approximately 1–10% of the remaining granular cells. The inclusions were not seen with routine HE staining, nor did they show anilinophilia, argentophilia or congophilia. These seven ALS patients also showed similar inclusions in the small neurons of the second and third layers of the lateral part of the entorhinal cortices. The incidence of the inclusions was almost the same in the granular cell layer and the entorhinal cortex. In one patient who suffered from dementia with ALS, many ubiquitin-positive inclusions were seen in both

Correspondence: Koichi Okamoto, MD, PhD, Department of Neurology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi 371-8511, Japan. Email: okamotok@med.gunma-u.ac.jp

Received 10 February 2010; revised 9 March 2010 and accepted 10 March 2010; published online 24 May 2010.

© 2010 Japanese Society of Neuropathology

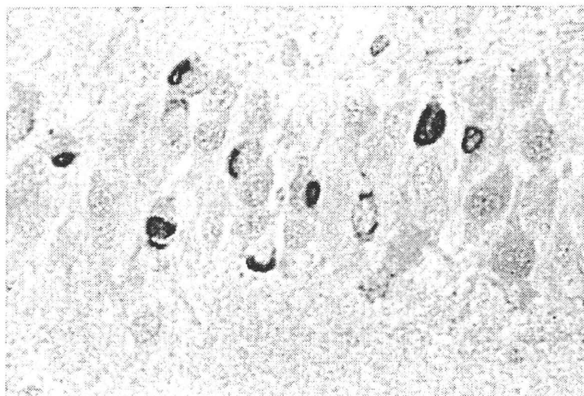


Fig. 1 Many ubiquitin-positive intraneuronal inclusions in the granular cell layer, ×200.

the hippocampal granular cells and the frontal and temporal cortices. No similar inclusions were seen in the 50 control brains. We first differentiated the inclusions from other known intracytoplasmic inclusions, such as Alzheimer neurofibrillary tangles (NFT) and Pick bodies. They did not stain for tau or PHF, and no argentophilia was observed, which excluded the possibility of NFT and Pick bodies.

Because of poor fixation and the relatively small amounts of filamentous material available, it was difficult to demonstrate clearly the fine structure of the ubiquitin-positive inclusions with a conventional electron-microscopic examination. Therefore, we performed an immunoelectron-microscopic examination, using a pre-embedding method with anti-ubiquitin antiserum. Immunoperoxidase products were seen in the cytoplasm of the hippocampal granular cells and in the small neurons of the entorhinal and frontal cortices of the ALS patient with dementia, and loosely arranged lineal filaments and granular material were also observed.

INCLUSIONS IN DEMENTIA PATIENTS WITH ALS[6]

We found no clinical or pathological differences between the seven inclusion-positive ALS patients and the 20 inclusion-negative ALS patients. However, we noticed ubiquitin-positive inclusions in many small neurons in the second layer of the frontal cortex of one patient with a history of dementia. Therefore, we studied the brains and spinal cords of 10 patients with clinically and pathologically confirmed presenile dementia and MND. All 10 patients had ubiquitin-positive tau-negative intracytoplasmic inclusions in the neurons of the hippocampal granular cell layers and in 1–14% of the remaining granular cells. No inclusions were seen in the pyramidal neurons of the hippocampus. The inclusions were frequently observed in the small neurons of the second and third layers of the entorhinal and frontal cortices. These results suggest that ubiquitin-related cytoskeletal abnormalities are common in cerebral non-motor small neurons in these patients.

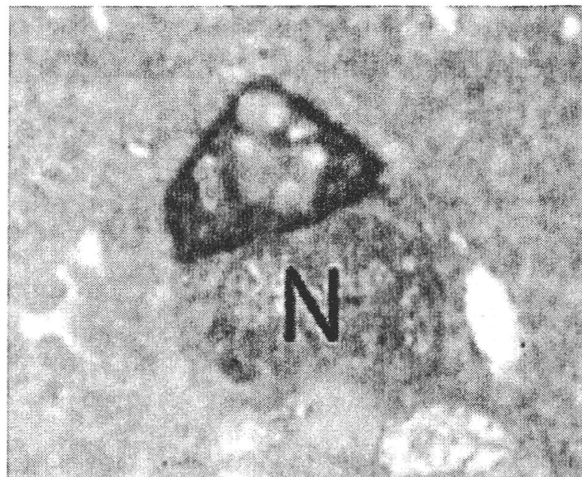


Fig. 2 Ubiquitin-immunostained semithin section of a hippocampal cortical neuron. Lipofuscin granules were negative for ubiquitin, ×400. N, nucleus.

In the following year, Wightman *et al.*[7] confirmed our findings. In 1994, the Lund and Manchester Groups proposed clinical and neuropathological criteria for frontotemporal dementia, dividing it into three subgroups: the frontal lobe degeneration type, the Pick type and the MND type.[8] The inclusions were described as a neuropathological marker of the MND type, in which "hippocampal dentate gyrus neurons show inclusions that are ubiquitin-positive but not silver or tau reactive".

In 1998, Neary *et al.*[9] proposed a consensus on the clinical diagnostic criteria for frontotemporal lobar degeneration (FTLD). However, FTLD is a heterogeneous entity, and the pathological diagnosis of FTLD includes tau-positive FTLD and tau-negative FTLD.[10] Two variants of tau-negative FTLD are FTLD with and without MND. FTLD with ubiquitin-positive tau-negative neuronal inclusions was grouped as FTLD-U.

FINE STRUCTURE OF THE INCLUSIONS

In 1996, we examined the inclusions using paired routine electron-microscopic ultrathin sections and adjacent semithin sections.[11] After the removal of the epon, the semithin sections were stained with anti-ubiquitin antiserum. In the ubiquitin-stained semithin sections, the inclusions formed a crescent or circular pattern around the nucleus (Fig. 2). The adjacent ultrathin sections were examined by electron microscopy, and there was no limiting membrane around

© 2010 Japanese Society of Neuropathology