in the brainstem and limbic system. The amygdala was the most affected structure in Kii ALS/PDC. The interaction between tau and α-synuclein might modify the pathogenesis of Kii ALS/PDC.

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REFERENCES

1. Hirano A, Malamud N, Elizan TS, et al. Amyotrophic lateral sclerosis and parkinsonism-dementia complex on Guam. Further pathologic studies. Arch Neurol 1966;15:35–51
2. Shiraki H, Yase Y. Amyotrophic lateral sclerosis in Japan. In: Vinken PJ, Bruyn GW, Klawans HL, eds. *Handbook of Clinical Neurology*. Amsterdam, the Netherlands: North Holland Publishing Company, 1975:353–419
3. Kuzuhara S, Kokubo Y, Sasaki R, et al. Familial amyotrophic lateral sclerosis and parkinsonism-dementia complex of the Kii peninsula of Japan: Clinical and neuropathological study and tau analysis. Ann Neurol 2001;49:501–11
4. Mimuro M, Kokubo Y, Kuzuhara S. Similar topographical distribution of neurofibrillary tangles in amyotrophic lateral sclerosis and parkinsonism-dementia complex in people living in the Kii peninsula of Japan suggests a single tauopathy. Acta Neuropathol 2007;113:653–58
5. Spillantini M, Schmidt M, Lee V-Y, et al. α-Synuclein in Lewy bodies. Nature 1997;388:839–40
6. Wakabayashi K, Yoshimoto M, Tsuji S, et al. α-Synuclein immunoreactivity in glial cytoplasmic inclusions in multiple system atrophy. Neurosci Lett 1998;249:180–82
7. Yamazaki M, Arai Y, Baba M, et al. α-Synuclein inclusions in amygdala in the brains of patients with the parkinsonism-dementia complex of Guam. J Neuropathol Exp Neurol 2000;59:585–91
8. Forman MS, Schmidt ML, Kasturi S, et al. Tau and α-Synuclein pathology in amygdala of parkinsonism-dementia complex patients of Guam. Am J Pathol 2002;160:1725–31
9. Sebeo J, Hof PR, Perl DP. Occurrence of alpha-synuclein pathology in the cerebellum of Guamanian patients with parkinsonism-dementia complex. Acta Neuropathol 2004;107:497–503
10. Miake H, Mizusawa H, Iwatsubo T, et al. Biochemical characterization of the core structure of alpha-synuclein filaments. J Biol Chem 2002;277: 19213–19
11. Fujiwara H, Hasegawa M, Dohmae N, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002;4:160–64
12. Hasegawa M, Fujiwara H, Nonaka T, et al. Phosphorylated α-Synuclein is ubiquitinated in α-Synuclein lesions. J Biol Chem 2002;277:49071–76
13. Hamilton RL. Lewy bodies in Alzheimer's disease: A neuropathological review of 145 cases using α-Synuclein immunohistochemistry. Brain Pathol 2000;10:378–84
14. Kazee AM, Han LY. Cortical Lewy bodies in Alzheimer's disease. Arch Pathol Lab Med 1995;119:448–53
15. Lippa CF, Fujiwara H, Mann DM, et al. Lewy bodies contain altered α-Synuclein in brains of many familial Alzheimer's disease patients with mutations in presenilin and amyloid precursor protein genes. Am J Pathol 1998;153:1365–70
16. Iseki E, Togo T, Suzuki T, et al. Dementia with Lewy bodies from the perspective of tauopathy. Acta Neuropathol 2003;105:265–70
17. Clarimon J, Molina-Porcel L, Gomez-Isla T, et al. Early-onset familial Lewy body dementia with extensive tauopathy: A clinical, genetic, and neuropathological study. J Neuropathol Exp Neurol 2009;68:73–82
18. Giasson BI, Forman MS, Higuchi M, et al. Initiation and synergistic fibrillization of tau and alpha-synuclein. Science 2003;300:636–40
19. Lippa CF, Schmidt ML, Lee VM-Y, et al. Antibodies to α-synuclein detect Lewy bodies in many Down's syndrome brains with Alzheimer's disease. Ann Neurol 1999;45:353–57
20. Hayashi S, Akasaki Y, Morimura Y, et al. An autopsy case of late infantile and juvenile neuroaxonal dystrophy with diffuse Lewy bodies and neurofibrillary tangles. Clin Neuropathol 1992;11:1–5
21. Wakabayashi K, Fukushima T, Koide R, et al. Juvenile-onset generalized neuroaxonal dystrophy (Hallervorden-Spatz disease) with diffuse neurofibrillary and Lewy body pathology. Acta Neuropathol 2000;99:331–36
22. Saito Y, Kawai M, Inoue K, et al. Widespread expression of α-Synuclein and tau immunoreactivity in Hallervorden-Spatz syndrome with protracted clinical course. J Neurol Sci 2000;177:48–59
23. Giasson BI, Forman MS, Higuchi M, et al. Initiation and synergistic fibrillization of tau and alpha-Synuclein. Science 2003;300:636–40

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Enhanced Antigen Retrieval of Amyloid β Immunohistochemistry: Re-evaluation of Amyloid β Pathology in Alzheimer Disease and Its Mouse Model

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Summary

Senile plaques, extracellular deposits of amyloid β peptide (Aβ), are one of the pathological hallmarks of Alzheimer disease (AD). As the standard immunohistochemical detection method for Aβ deposits, anti-Aβ immunohistochemistry combined with antigen retrieval (AR) by formic acid (FA) has been generally used. Here, we present a more efficient AR for Aβ antigen. On brain sections of AD and its mouse model, a double combination of either autoclave heating in EDTA buffer or digestion with proteinase K plus FA treatment reinforced Aβ immunoreactivity. A further triple combination of digestion with proteinase K (P), autoclave heating in EDTA buffer (A), and FA treatment (F), when employed in this order, gave a more enhanced immunoreactivity. Our PAF method prominently visualized various forms of Aβ deposits in AD that have not been clearly detected previously and revealed numerous minute-sized plaques both in AD and the mouse model. Quantification of Aβ loads showed that the AR effect by the PAF method was 1.86-fold (in the aged human brain) and 4.64-fold (in the mouse brain) higher than that by the FA method. Thus, the PAF method could have the potential to be the most sensitive tool so far to study Aβ pathology in AD and its mouse model. (J Histochem Cytochem 60:761–769, 2012)

Keywords

Alzheimer disease, amyloidβ, antigen retrieval, APP-SL mouse, autoclave heating, formic acid, immunohistochemistry, minute plaque, PAF method, proteinase K

Senile plaques (SPs) and neurofibrillary tangles (NFTs) are two pathological hallmarks that characterize brains afflicted with Alzheimer disease (AD). SPs are extracellular deposits of amyloid β peptide (Aβ) mainly consisting of 40 and 42 residues, which are cleavage products of the amyloid precursor proteins (APPs) (Masters et al. 1985; Kang et al. 1987; Iwatsubo et al. 1994). Aβ is a hydrophobic self-aggregating peptide, and the aggregation of soluble Aβ monomers leads to the composition of insoluble fibrillar polymers, Aβ fibrils. NFTs are intracellular aggregated bundles of a hyperphosphorylated form of the microtubule-associated protein tau (Lee et al. 1991; Ballatore et al. 2007).

Although it is not yet completely elucidated whether SPs and NFTs are the causes or the results of AD onset, the aggregation of Aβ is believed to be implicated in the upper stream of the cascade of AD pathogenesis as a pivotal player in the development of dementia: the amyloid hypothesis (Selkoe 1991; Hardy and Higgins 1992; Hardy and Selkoe 2002). Therefore, the detection of SPs or Aβ deposits with high specificity and sensitivity is essential for elucidating the roles of

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parenchymal Aβ deposition and its implication for the pathogenesis of AD as well as its pathological diagnosis. In 1987, our attempt to attain sensitive Aβ immunohistochemistry (IHC) results on formalin-fixed paraffin-embedded (FFPE) tissue sections led to the development of Aβ antigen retrieval (AR) by formic acid (FA) (Kitamoto et al. 1987). This straightforward method dramatically enhances the detection level of Aβ deposits in the AD brain, and since then, anti-AβIHC coupled with FA treatment has been the standard method in the field of Aβ pathology. There is no guarantee, however, that this method can expose all of the existing Aβ deposits without the remains, and we consider that there might be room for improvement of the AR technique. In fact, we had a chance to find irregular and larger forms of Aβ staining, known as fleecy amyloid deposits (Thal et al. 1999), which appear different from the usual SPs in the entorhinal cortex of some AD cases. These structures of Aβ aggregates stained too faintly to be recognized clearly, and we thought that the AR mediated by FA was not efficient enough to detect these Aβ structures. Thus, we tackled the development of a new AR method with a higher efficiency than the conventional FA method. We could substantially improve Aβ IHC by applying two other AR procedures prior to FA treatment. This new AR method enhanced the detection level of numerous SPs and various Aβ deposits that have not been clearly detected by the conventional method and provides a tool to uncover new aspects of Aβ pathology in AD and its mouse models.

Materials and Methods

Brain Specimens

Human brain specimens were derived from patients with AD (n=11; age range = 63–79 years), non-AD aged individuals with Aβ plaques (n=4; age range = 63–77 years), and negative controls for Aβ IHC, who have no family history of AD, including non-AD aged individuals without Aβ plaques (n=10; age range = 64–94 years) and healthy young individuals (n=6; age range = 21–38 years). For quantification of the Aβ loads, we examined a series of aged human individuals with varying degrees of the Aβ burden (n=54; age range = 69–94 years). As transgenic AD mouse models, we used the APP–Swedish/London (SL) lines 7–5 and 7–9, which overexpress human $APP_{Swe/Lon}$ harboring both the Swedish- and London-type mutations. The levels of APP, Aβ40, and Aβ42 in the brain tissues of the mice of line 7–5 are higher than the corresponding levels in the mice of line 7–9 (Shin et al. 2007). The outline of the ages of each of them is as follows: line 7–5 of APP-SL mice aged 6 months (n=3), 8 months (n=8), 9 months (n=2), 10 months (n=2), 11 months (n=1), 12 months (n=4), 13 months (n=6), 15 months (n=1), 16 months (n=5), and 18 months (n=1); line 7–9 of APP-SL mice aged 3 months (n=3), 6 months (n=3), 9 months (n=3), 12 months (n=2), 13 months (n=3), 15 months (n=1), 16 months (n=2), 18 months (n=2), 19 months (n=1), and 36 months (n=1). The fixation time of brains was 7–13 days with 20% buffered formalin in humans and 3–4 days with 10% buffered formalin in mice. The use of human brains for this work was approved by the Institutional Review Board of Tohoku University Graduate School of Medicine and Tokyo Metropolitan Geriatric Hospital & Institute of Gerontology, and all the animal experiments were done according to the Guidelines for Animal Care and Use at Otsuka Pharmaceutical Co. Ltd.

AR Procedures

FA pretreatment has been the standard AR method for Aβ IHC. In FA pretreatment, brain tissue sections were incubated in 98% FA (Wako Pure Chemical Industries; Osaka, Japan) for 5 min at room temperature. A challenging trial to largely improve the FA method was performed by combining and applying other AR methods prior to FA treatment. The other AR methods used in this study include heating that employs immersion of tissue sections in 10 mM EDTA (pH 3.0, pH 6.0, and pH 10.0) (Murayama et al. 1999), 0.05% citraconic anhydride (pH 3.0, pH 7.4, and pH 10.0) (Namimatsu et al. 2005), and 0.1 M sodium citrate (pH 3.0, pH 7.2, and pH 10.0) (Bataille et al. 2006) solutions, and distilled water (DW) (pH 3.0 adjusted with hydrochloric acid, pH 7.1, and pH 10.0 adjusted with sodium hydroxide), using an autoclave at 105C or 121C for 10 min (Shin et al. 1991) or using a microwave oven at 90C intermittently but for a total of about 10 min; the proteolytic digestion of tissue sections was performed at 37C for 30 min with 1.0 μg/ml of proteinase K (PK) (Wako Pure Chemical Industries) and 100.0 μg/ml trypsin (Wako Pure Chemical Industries) dissolved in 1.0 mM $CaCl_2$/50 mM Tris buffer (pH 7.6). After each AR treatment, these sections were washed with tap water for at least 5 min and then incubated in DW for at least 5 min.

Immunostaining

With pretreatment of various combinations of the AR methods, immunostaining was performed as described (Murayama et al. 1999; Shin et al. 2007) using the polyclonal Aβ antibody 4702 (1:1500) (Shin et al. 2007) and monoclonal Aβ antibodies 6E10 (1:2000–4000; Senetek, Maryland Heights, MO) and 4G8 (1:20,000; Senetek). The concentrations of these antibodies were optimized in consideration of both the immunoreactivity and backgrounds of IHC. Brain sections were incubated with primary antibodies in 0.1% Tween-20/Tris-buffered saline (Tris, 50 mM; NaCl, 500 mM; pH 7.6) containing 5% nonfat dried milk for about 15 hr at room temperature. To exclude nonspecific staining unrelated to these polyclonal and monoclonal antibodies, immunostaining was performed with

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omission of the antibodies but with all other procedures unchanged in some experiments. Secondary antibodies of EnVision+ system HRP-labeled polymer (Dako; Glostrup, Denmark) were used for the detection of antigen primary mouse or rabbit antibody complexes by diaminobenzidine (DAB). The incubation of the secondary antibodies was for about 1 hr at room temperature. The immunostained brain sections were counterstained with hematoxylin.

Microscopes

The photomicrographs of human and murine samples were captured by an Axiophot2 microscope (Carl Zeiss; Oberkochen, Germany) with Axio Vision version 4.6.3.0 software (Carl Zeiss). In quantification of the Aβ load in the hippocampus of the murine samples, the same system was used in order to assemble sequential micrographs into a single larger one. For measuring the Aβ load, aged human sample photomicrographs were captured by C9600 NanoZoomer (Hamamatsu Photonics; Hamamatsu, Japan) with NDP.view software (Hamamatsu Photonics) because this system is conveniently applicable for capturing and comparing the same regions from serial sections.

Measurement of the Area of Aβ Deposits

In the human brains, we selected three microscopic fields in the fusiform gyrus, which are adequately separated from each other and contain relatively higher Aβ loads. We photographed precisely the same fields in each serial section immunostained following the FA method or PK digestion (P), EDTA autoclaving (A), and FA treatment (F) (in that order; referred to as "PAF") method. All images were from regions of 1408 μm × 1874 μm. In the mouse brains, we selected the whole hippocampus, and its image was constructed from the photomicrographs of 872 μm × 1100 μm by using panorama module of AxioVision version 4.6.3 (Carl Zeiss). All the images were analyzed by ImageJ version 1.43 m (National Institutes of Health; Bethesda, MD) as follows: 1) each raw image was resolved into three images by the color deconvolution setting in hematoxylin and eosin and DAB; 2) the DAB color image among the three resolved images was selected for analysis; 3) the threshold value of the selected image was set to zero as the minimum value and at the optically optimum value set as the maximum value; 4) the thresholded areas of cerebral amyloid angiopathy and artifacts were excluded by selecting and filling them; 5) areas of the Aβ loads (%) to be measured were within a circle (diameter = 1408 μm), the center of which being in the middle of each image in the aged human brains, and within the circumscribed edge of the hippocampus drawn using the selection tools in the mouse brains; and 6) area fractions of the residual thresholded objects within these selections were measured.

Statistical Analyses

All the statistical analyses were performed with SPSS version 17.0 (SPSS; Chicago, IL).

Results

Development of the Enhanced AR Method for Aβ IHC

For the development of a more efficient Aβ AR method, our strategy was to modify and reinforce the retrieving effects of FA by applying other AR procedures prior to FA treatment. Such AR procedures included autoclave heating in EDTA buffer (the chelating autoclave method) (Murayama et al. 1999) and digestion with PK. With each of these AR procedures followed by FA treatment or with FA treatment alone, immunostaining using the polyclonal anti-Aβ 4702 antibody was performed on brain tissue sections derived from AD patients and APP-SL line 7–5 mice. In immunostaining using the 4702 antibody with no AR, almost no plaques were detected in the AD brains or only a few in the mouse brains (Suppl. Fig. S1). Thus, this 4702 antibody was conveniently used to easily evaluate the effectiveness of the Aβ AR methods.

Compared to the AR procedures above followed by FA treatment and FA treatment alone, both combinations of AR enhanced the Aβ immunoreactive intensity and increased the loads of Aβ plaques, albeit with low to high enhancing effects (Fig. 1A–C,E–G). However, when we applied heating to the EDTA solution, counterstaining of tissue sections with hematoxylin was remarkably thin compared with counterstaining in the FA only method. Notably, the reversed application of the two AR procedures, that is, application of FA treatment and either autoclave heating in EDTA buffer or digestion with PK in this order, showed limited and almost no enhancement, respectively, compared with the single application of FA treatment (data not shown). These results prompted us to try a triple combination of these three AR procedures. The use of the PAF method produced a remarkably stronger enhancement of Aβ immunoreactivity than the two double combinations above (Fig. 1B–D,F–H). In the triple combinations of the three AR methods in different orders other than that used in the PAF method, varying degrees of tissue damage ensued, especially in human brains. Therefore, we refrained from estimating those triple AR combinations. To confirm that this PAF method is universally applicable to Aβ IHC, we examined other Aβ antibodies including 6E10 (Fig. 2A, B, E, F) and 4G8 (Fig. 2C, D, G, H). All of these antibodies showed enhanced Aβ immunoreactivity following the PAF method compared with the FA method, albeit with varied enhancing effects. Omission of the polyclonal or monoclonal primary antibodies in Aβ IHC with the PAF method totally abolished positive immunostaining (Suppl. Fig. S2),

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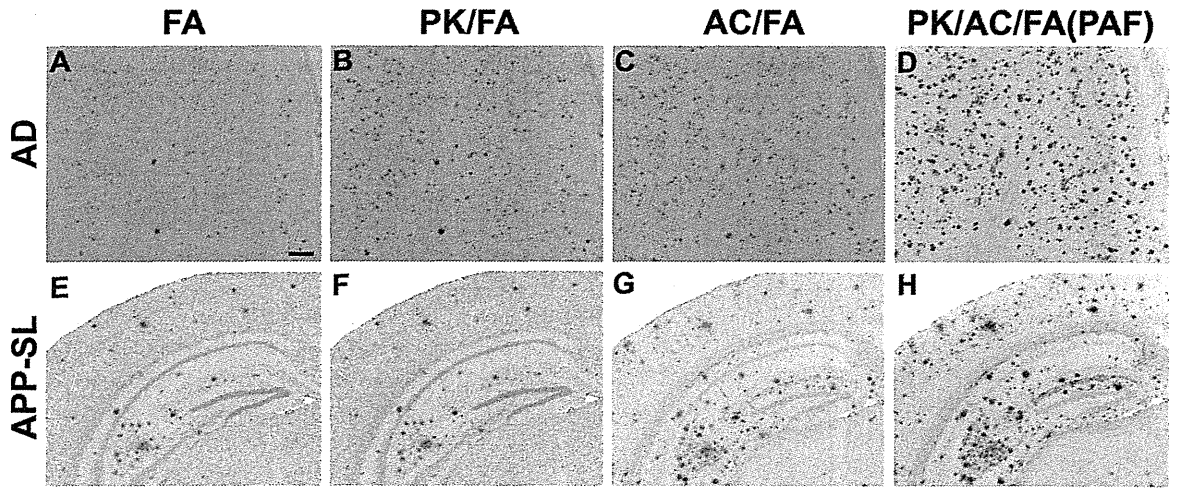

Figure 1. Enhancement of formic acid (FA)–mediated amyloid β peptide (Aβ) antigen retrieval. Serial brain tissue sections from a 74-year-old male patient with Alzheimer disease (A–D) and from a 13-month-old amyloid precursor protein–Swedish/London (APP-SL) mouse of line 7–5 (E–H) were immunostained with anti-Aβ antibody 4702 following pretreatment by FA alone (FA) (A, E); combination of digestion with proteinase K and FA (PK/FA) (B, F); combination of autoclave heating in EDTA buffer and FA (AC/FA) (C, G); and triple combination of digestion with proteinase K, autoclave heating in EDTA buffer, and FA (PK/AC/FA) (D, H). Pictures (A–D) are from the temporal cortex. Scale bar = 200 μm (A–H).

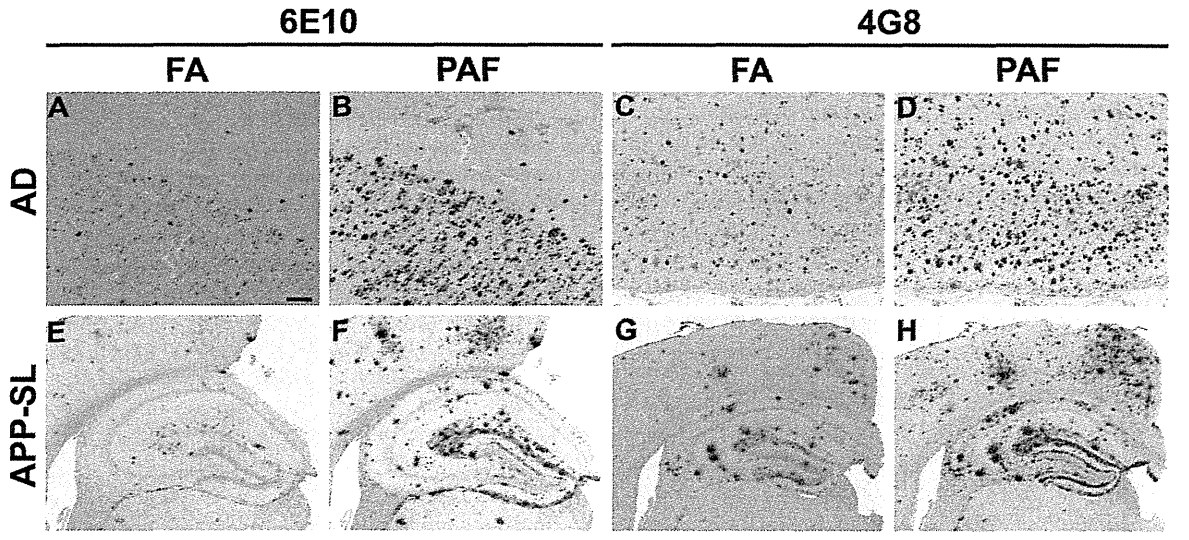

Figure 2. General application of the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as "PAF") method in amyloid β peptide (Aβ) immunohistochemistry. Serial (A–B, C–D, E–F, G–H) brain tissue sections from a 74-year-old male patient with Alzheimer disease (the same patient as shown in Fig. 1) (A–D) and a 16-month-old (E, F) and a 15-month-old (G, H) amyloid precursor protein–Swedish/London (APP-SL) mouse of line 7–5 were immunostained with monoclonal anti-Aβ antibodies 6E10 (A, B, E, F) and 4G8 (C, D, G, H) following pretreatment by the FA (A, C, E, G) and PAF methods (B, D, F, H). Pictures are from the cingulate cortex (A, B) and the frontal cortex (C, D). Scale bar = 200 μm (A–H).

which excludes the possibility that immunoreactivity augmented and disclosed following the PAF method was due to nonspecific staining. In addition, no artifactual immunostaining was observed in the brain sections from the normal younger individuals following Aβ IHC assisted by the PAF method (Suppl. Fig. S3).

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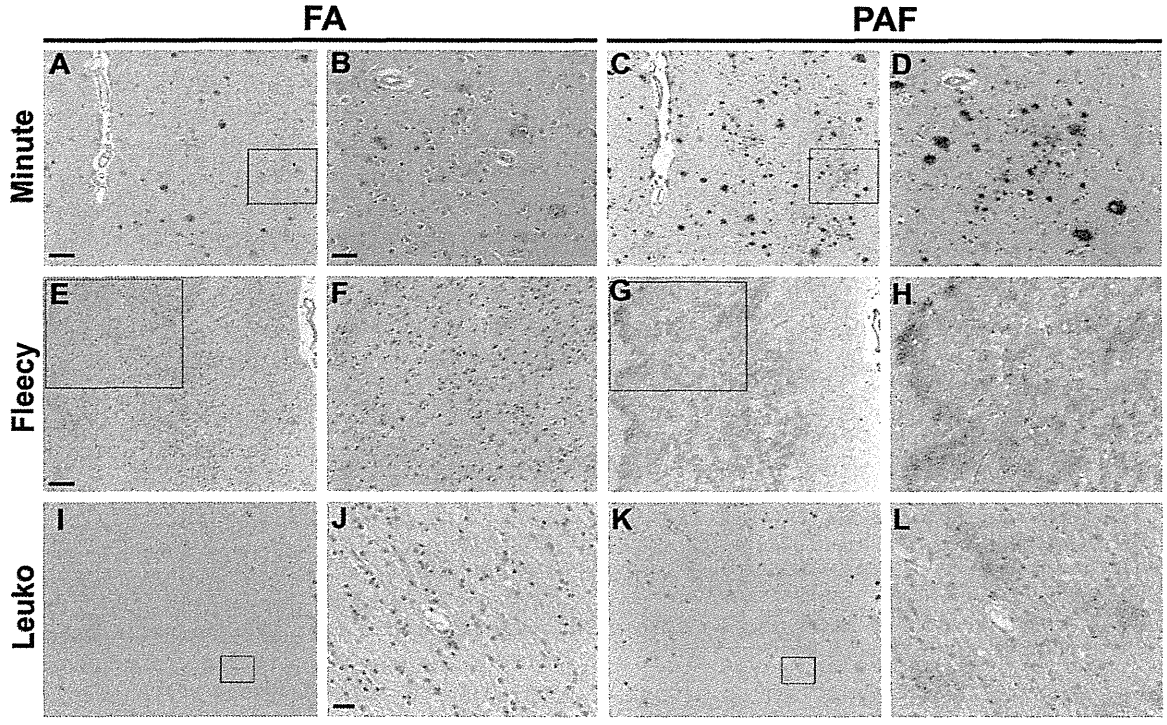

Figure 3. Amyloid β peptide (Aβ) pathology of Alzheimer disease (AD) brains enhanced by the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as "PAF") method. Serial brain tissue sections from a 72-year-old female AD patient (the identical patient as shown in Suppl. Fig. S1A,B) (A–D), a 74-year-old male AD patient (the same patient as shown in Fig. 1) (E–H), and a 74-year-old female AD patient (I–L) were immunostained with the 4702 antibody following pretreatment by the FA (A, B, E, F, I, J) and PAF methods (C, D, G, H, K, L). Pictures are from the frontal cortex (A–D), the entorhinal cortex (E–H), and the frontal white matter (I–L). B, D, F, H, J, and L are higher magnification pictures of areas outlined by squares in A, C, E, G, I, and K, respectively. Scale bars = 200μm (A, C, I, K); 50 μm (B, D, F, H); 100μm (E, G); and 20 μm (J, L).

IHC Analysis of AD Brains by the PAF Method

Serial sections of the AD brains pretreated with either the PAF or FA method were immunostained with the anti-Aβ 4702 antibody, analyzed for pathological Aβ deposits, and compared between these two methods (Fig. 3). In the cerebral cortex and hippocampus of the sections pretreated with the PAF method, larger Aβ plaques (diameter > ~15 μm) showed immunoreactive enhancement with an enlarged robust contour, although there was no apparent increase in the number of these larger Aβ plaques. The prominent effect given by the PAF method was the disclosure of numerous minute-sized (diameter < ~15 μm) fine-granular plaques (hereafter referred to as "minute plaques") in these brain regions, which were not evidently detected by the FA method (Figs. 1A, D and 3A–D). Remarkably, the number of these minute plaques increased with elevation of the total Aβ load (data not shown). Thus, all of the AD brains examined by the PAF method contained a much higher load of Aβ plaques in the cerebral cortex and hippocampus than those by the FA method. In the entorhinal cortex adjacent to the subiculum, large and irregular contours of Aβ staining appeared, which were composed of fine- to coarse-granular or diffuse Aβ deposits following the PAF method. These were distributed from near the subpial layer into the deep cortex (Fig. 3G, H), which were reported as fleecy Aβ deposits (Thal et al. 1999). These Aβ deposits were only faintly or not appreciably stained following FA treatment (Fig. 3E, F). In the cerebral white matter, small but significant amounts of Aβ deposits in the diffuse or granular form were previously shown to occur (Wisniewski et al. 1989; Behrouz et al. 1991; Uchihara et al. 1995). The PAF method gave immunoreactive enhancement and revealed larger amounts of granular Aβ deposits in the cerebral white matter (Fig. 3I–L). The PAF method also enhanced Aβ immunoreactivity of ribbon-like infiltration in the subpial layer of the cerebral cortex and that of cerebral amyloid angiopathy in the vessels of the brain (data not shown). Thus, the PAF method dramatically enhanced the detection level of a spectrum of all morphological forms of Aβ deposits.

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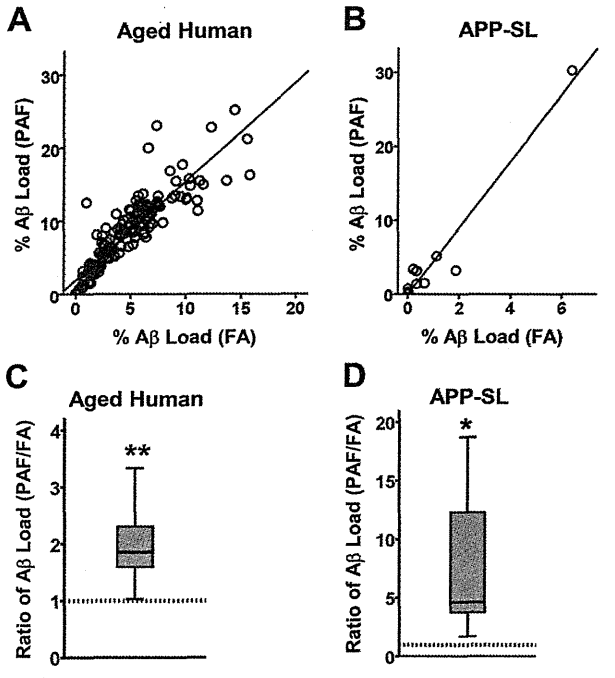

Figure 4. Effect of amyloid β peptide (Aβ) antigen retrieval (AR) by the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as "PAF") method over that by the FA method. (A, B) In immunohistochemistry (IHC) with the 4702 antibody, Aβ loads (%) measured in the fusiform cortex of each case from the aged human brains (A) or in the hippocampus of each from the amyloid precursor protein–Swedish/London (APP-SL) mice (B) following the PAF method were plotted against those following the FA method. Significant correlations between the two AR methods were verified both in the aged human brains (Spearman rank correlation coefficient, r_s=0.92; p=9 × 10^{-69}) and in the mouse brains (r_s=0.80; p=0.003). (C, D) The effect of Aβ AR by the PAF method was significantly higher than that by the FA method both in the aged human and in the mouse brains. In IHC with the 4702 antibody, Aβ-loaded areas measured following the PAF method compared with those following the FA method (set at 1.00; the dotted lines) were 1.59-fold at the 25th percentile, 1.86-fold at the 50th percentile, and 2.31-fold at the 75th percentile in the aged human brains (C) and 3.78-fold at the 25th percentile, 4.64-fold at the 50th percentile, and 12.32-fold at the 75th percentile in the mouse brains (D). (C) Ten (≥3.39) and (D) one (92.79) outliers are not shown. **p=2 × 10^{-28}, *p=0.01; analyzed by Wilcoxon signed-rank test. n=162 from 54 individuals where three regions per case examined (A, C), and n=11 (B, D).

IHC Analysis of the AD Mouse Model by the PAF Method

We examined the brains of the AD APP-SL mice in the same way as for the AD brains. In our previous (Shin et al. 2007) and present studies using conventional Aβ IHC coupled with the FA method, younger APP-SL mice (< ~9 months) showed no occurrence of Aβ deposition, and older mice (≥ ~9 months) exhibited deposition of Aβ plaques that increased its burden with age. Application of Aβ IHC assisted by the PAF method to those younger mice also failed to reveal Aβ deposition. Therefore, in mice showing no evidence of Aβ deposition as evaluated by the FA method, the PAF method did not create any occurrence of Aβ deposition. Thus, these two methods show no difference in their ability to demonstrate the absence of Aβ deposition. In the older mice showing evidence of Aβ deposition, the PAF method was more efficient than the FA method for AR. The enhanced immunoreactive profile was shown to enlarge the sizes and to increase the numbers and immunointensities of Aβ plaques (Figs. 1E,H and 2E–H). Notably, minute Aβ plaques appeared to have a similar morphology as those seen in the AD brains. These minute plaques occur in brain samples containing significant amounts of Aβ burden and prevail dominantly with severity of Aβ burden, as was shown in the AD brain. In 9-month-old APP-SL mice that show an initial appearance of Aβ deposition, Aβ deposits were indiscernible when evaluated by the FA method. These deposits were prominently visualized as distinct Aβ deposits by the PAF method (data not shown). Thus, the PAF method could have an advantage for the retrieval of antigens in Aβ IHC in comparison with the FA method in the AD mouse model as well as humans.

Efficiency of Aβ AR by the PAF Method

We measured the areas of Aβ deposit loads in the serial sections immunostained with the 4702 antibody following the PAF or FA method, and the total sums of the Aβ-loaded areas per the whole area analyzed were compared between the two methods. In the aged human brains, Aβ deposit loads measured in the fusiform cortex by the PAF method were significantly correlated with those by the FA method (p=9 × 10^{-69}) (Fig. 4A). We compared the ratio of the Aβ deposit area from the PAF method with the ratio from the FA method. The AR effect of the PAF method (Fig. 4C) was significantly higher than that of the FA method (1.86-fold at the median) (p=2 × 10^{-28}). In the APP-SL line 7–9 mice, Aβ deposit loads measured in the whole hippocampus by the PAF method were also significantly correlated with those by the FA method (p=0.003) (Fig. 4B). The enhancing effect of the PAF method compared with that of the FA method (the ratio as described above in aged human brains) was significantly higher and 4.64-fold at the median (p=0.01) (Fig. 4D). Thus, the PAF method produced Aβ deposit loads that were consistently and significantly larger than those produced by the FA method.

Evaluation of PK versus Trypsin in Enzymatic Digestion and Some Other Solutions in Autoclave Heating

To obtain a more effective enzymatic digestion than PK digestion, we additionally tested trypsin in the double combination of enzymatic digestion and the FA method. Trypsin

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digestion, when applied prior to the FA method, produced Aβ immunostaining slightly higher in its intensity than the FA method only. However, its efficacy was comparable to that of PK digestion (Suppl. Fig. S4). To obtain a more effective autoclave heating than that in the solution of 10 mM EDTA (pH 6.0) at 121C, we additionally tested solutions of EDTA (pH 3.0 and pH 10.0), DW (pH 3.0, pH 7.1, and pH 10.0), citraconic anhydride (pH 3.0, pH 7.4, and pH 10.0), and sodium citrate (pH 3.0, pH 7.2, and pH 10.0) as well as temperatures of 90C, 105C, and 121C. Among these different conditions, DW (pH 10.0 and 105C) and sodium citrate (pH 7.2 and 105C) produced high Aβ AR effects. A similar effect was observed for EDTA (pH 6.0 and 121C), although sodium citrate slightly damaged the tissue sections (Suppl. Fig. S5). The triple combinations using additional PK or trypsin digestion as the initial step produced higher Aβ AR effects than each of those double combinations, although heating in sodium citrate solution or basic water damaged the tissue sections. Further nonspecific staining was observed in the brain sections of the APP-SL mice applied by the triple combination of trypsin digestion, EDTA autoclaving, and the FA method (Suppl. Figs. S6 and S7). Thus, the triple combination of 1) PK digestion, 2) autoclave heating in 10 mM EDTA (pH 6.0 and 121C), and 3) FA treatment produced the highest Aβ AR effects without damaging tissue sections or producing nonspecific staining.

Discussion

The masking of antigens by aldehyde fixatives or by paraffin-embedding procedures is a problem for IHC studies. To overcome this problem, enzymatic digestion, FA treatment, and high-temperature heating have been developed. Among these, FA treatment is the standard method mainly used for Aβ IHC of FFPE brain tissue sections, although it was originally developed for the immunoreactive enhancement of cerebral amyloids (prion protein and Aβ) and systemic amyloids (amyloid A and prealbumin) (Kitamoto et al. 1987). The pretreatment of protein digestion with an enzyme such as trypsin had been used for IHC but only in a limited application (Battifora and Kopinski 1986; Huang et al. 1976; Mepham et al. 1979). In 1991, the advent of the heating AR method was a breakthrough in the field of IHC. Shin et al. (1991) reported that the procedure of hydrated autoclaving uncovers the masked epitopes of the microtubule-associated protein tau, showing that high-temperature heating serves as an efficient AR method. Shi et al. (1991) reported that microwave heating also shows an AR effect by testing a variety of antigens and antibodies, establishing the milestone of AR for FFPE tissue sections. Moreover, Shi et al. (1996) devised the test battery approach, which can efficiently determine the optimum protocols of AR for each antigen by comparing the immunostaining results between different kinds of solutions, temperatures, and pH (O'Leary 2001). As one good example using the test battery approach, it was demonstrated that AR procedures can also be applied to immunoelectron microscopy for amyloid deposits composed of the κ light chain or transthyretin (Rocken and Roessner 1999).

Aβ AR by FA is proposed because of the unfolding of the conformational amyloid polymers and thereby the exposing of Aβ antigens through acidic hydrolysis (Kitamoto et al. 1987). Further, FA is suggested to esterify serine residues in Aβ peptides and to alter the conformation of the amyloid polymers, as nuclear magnetic resonance imaging has revealed (Klunk et al. 1994). On the other hand, the possible mechanisms underlying AR by high-temperature heating are summarized as follows: 1) breaking of aldehyde-induced cross-linkage involving antigenic proteins, 2) extraction of diffusible blocking proteins, 3) precipitation of antigenic proteins, and 4) increased penetration of antibodies with better access to epitopes due to rehydration of the tissue sections (Suurmeijer and Boon 1993). In addition, the application of divalent/trivalent chelators in high-temperature heating removes metal ions that mask antigenic proteins (Murayama et al. 1999; Yamamoto et al. 2002; Shin et al. 2003). If autoclave high-temperature heating with EDTA chelators (Murayama et al. 1999) is then used prior to FA treatment, with the aim of affecting its activity of Aβ AR, it is likely that FA gains permeability through the tissue sections up to the unmasked Aβ fibrils. Similarly, if digestion with PK is applied prior to FA treatment, then proteolytic digestion of blocking proteins that surround Aβ fibrils might occur and unmask and thereby expose them to FA. In short, the procedures of EDTA autoclaving and PK digestion might assist the access of FA to Aβ fibrils, resulting in reinforcement of Aβ AR of FA. Indeed, we demonstrated that the combination of preceding EDTA autoclaving or PK digestion with subsequent FA treatment enhanced the AR effects of FA, and the aforementioned hypotheses might indeed be true. In support of this hypothesis, FA treatment prior to PK digestion or EDTA autoclaving gave no or only a minimally discernible enhancement in Aβ immunoreactivity compared with single FA treatment (data not shown). Our present results, together with other previous studies, show that PK digestion and EDTA autoclaving apparently differ in the mechanisms of the reinforcement of Aβ AR. If both PK digestion and EDTA autoclaving are combined with the FA method, their effects on Aβ AR by FA might be complementary rather than equivalent. Indeed, the triple combination of PK digestion and EDTA autoclaving with FA treatment provided a further stronger enhancement of Aβ AR.

Our results based on the PAF method suggest that previous IHC studies performed by the conventional FA method might have underestimated the quantitative burden of Aβ pathology and that brains with AD and its mouse models produce much more Aβ accumulation than previously assumed. Further, the presence of minute plaques revealed

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by the PAF method remains to be clarified for its implication for Aβ pathology of AD and its mouse models. Notably, the molecular layer of the hippocampal dentate gyrus is the brain region that produces larger amounts of the minute plaques in the APP-SL mice than in the aged humans. This observation might explain partly, albeit not totally, why the retrieving effects differ between the human and mouse brains.

This powerful PAF method could reveal numerous SPs and various Aβ deposits that have not been detected so far. Therefore, the PAF method could serve as a sensitive IHC tool to give new insights into Aβ pathology of AD and its mouse models. We speculate that the PAF method may be the Aβ AR method with the highest efficiency so far and could be used in place of the conventional FA method.

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Declaration of Conflicting Interests

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References

Ballatore C, Lee VM, Trojanowski JQ. 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci. 8:663–672.

Bataille F, Troppmann S, Klebl F, Rogler G, Stoelcker B, Hofstadter F, Bosserhoff AK, Rummele P. 2006. Multiparameter immunofluorescence on paraffin-embedded tissue sections. Appl Immunohistochem Mol Morphol. 14:225–228.

Battifora H, Kopinski M. 1986. The influence of protease digestion and duration of fixation on the immunostaining of keratins: a comparison of formalin and ethanol fixation. J Histochem Cytochem. 34:1095–1100.

Behrouz N, Defossez A, Delacourte A, Mazzuca M. 1991. The immunohistochemical evidence of amyloid diffuse deposits as a pathological hallmark in Alzheimer's disease. J Gerontol. 46:B209–B212.

Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 297:353–356.

Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science. 256:184–185.

Huang SN, Minassian H, More JD. 1976. Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. Lab Invest. 35:383–390.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. 1994. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron. 13:45–53.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature. 325:733–736.

Kitamoto T, Ogomori K, Tateishi J, Prusiner SB. 1987. Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab Invest. 57:230–236.

Klunk WE, Xu CJ, Pettegrew JW. 1994. NMR identification of the formic acid-modified residue in Alzheimer's amyloid protein. J Neurochem. 62:349–354.

Lee VM, Balin BJ, Otvos L Jr., Trojanowski JQ. 1991. A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. Science. 251:675–678.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A. 82:4245–4249.

Mepham BL, Frater W, Mitchell BS. 1979. The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. Histochem J. 11:345–357.

Murayama H, Shin RW, Higuchi J, Shibuya S, Muramoto T, Kitamoto T. 1999. Interaction of aluminum with PHFtau in Alzheimer's disease neurofibrillary degeneration evidenced by desferrioxamine-assisted chelating autoclave method. Am J Pathol. 155:877–885.

Namimatsu S, Ghazizadeh M, Sugisaki Y. 2005. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. J Histochem Cytochem. 53:3–11.

O'Leary TJ. 2001. Standardization in immunohistochemistry. Appl Immunohistochem Mol Morphol. 9:3–8.

Rocken C, Roessner A. 1999. An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. J Histochem Cytochem. 47:1385–1394.

Selkoe DJ. 1991. The molecular pathology of Alzheimer's disease. Neuron. 6:487–498.

Shi SR, Cote RJ, Yang C, Chen C, Xu HJ, Benedict WF, Taylor CR. 1996. Development of an optimal protocol for antigen retrieval: a 'test battery' approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. J Pathol. 179:347–352.

Shi SR, Key ME, Kalra KL. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 39:741–748.

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Shin RW, Iwaki T, Kitamoto T, Tateishi J. 1991. Hydrated autoclave pretreatment enhances tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. Lab Invest. 64:693–702.

Shin RW, Kruck TP, Murayama H, Kitamoto T. 2003. A novel trivalent cation chelator Feralex dissociates binding of aluminum and iron associated with hyperphosphorylated tau of Alzheimer's disease. Brain Res. 961:139–146.

Shin RW, Ogino K, Shimabuku A, Taki T, Nakashima H, Ishihara T, Kitamoto T. 2007. Amyloid precursor protein cytoplasmic domain with phospho-Thr668 accumulates in Alzheimer's disease and its transgenic models: a role to mediate interaction of Abeta and tau. Acta Neuropathol. 113:627–636.

Suurmeijer AJ, Boon ME. 1993. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. Eur J Morphol. 31:144–150.

Thal DR, Sassin I, Schultz C, Haass C, Braak E, Braak H. 1999. Fleecy amyloid deposits in the internal layers of the human entorhinal cortex are comprised of N-terminal truncated fragments of Abeta. J Neuropathol Exp Neurol. 58:210–216.

Uchihara T, Kondo H, Akiyama H, Ikeda K. 1995. White matter amyloid in Alzheimer's disease brain. Acta Neuropathol. 90:51–56.

Wisniewski HM, Bancher C, Barcikowska M, Wen GY, Currie J. 1989. Spectrum of morphological appearance of amyloid deposits in Alzheimer's disease. Acta Neuropathol. 78:337–347.

Yamamoto A, Shin RW, Hasegawa K, Naiki H, Sato H, Yoshimasu F, Kitamoto T. 2002. Iron (III) induces aggregation of hyperphosphorylated tau and its reduction to iron (II) reverses the aggregation: implications in the formation of neurofibrillary tangles of Alzheimer's disease. J Neurochem. 82:1137–1147.

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REPORT

The TRK-Fused Gene Is Mutated in Hereditary Motor and Sensory Neuropathy with Proximal Dominant Involvement

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Hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P) is an autosomal-dominant neurodegenerative disorder characterized by widespread fasciculations, proximal-predominant muscle weakness, and atrophy followed by distal sensory involvement. To date, large families affected by HMSN-P have been reported from two different regions in Japan. Linkage and haplotype analyses of two previously reported families and two new families with the use of high-density SNP arrays further defined the minimum candidate region of 3.3 Mb in chromosomal region 3q12. Exome sequencing showed an identical c.854C>T (p.Pro285-Leu) mutation in the TRK-fused gene (*TFG*) in the four families. Detailed haplotype analysis suggested two independent origins of the mutation. Pathological studies of an autopsied patient revealed TFG- and ubiquitin-immunopositive cytoplasmic inclusions in the spinal and cortical motor neurons. Fragmentation of the Golgi apparatus, a frequent finding in amyotrophic lateral sclerosis, was also observed in the motor neurons with inclusion bodies. Moreover, TAR DNA-binding protein 43 kDa (TDP-43)-positive cytoplasmic inclusions were also demonstrated. In cultured cells expressing mutant TFG, cytoplasmic aggregation of TDP-43 was demonstrated. These findings indicate that formation of TFG-containing cytoplasmic inclusions and concomitant mislocalization of TDP-43 underlie motor neuron degeneration in HMSN-P. Pathological overlap of proteinopathies involving TFG and TDP-43 highlights a new pathway leading to motor neuron degeneration.

Hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P [MIM 604484]) is an autosomal-dominant disease characterized by predominantly proximal muscle weakness and atrophy followed by distal sensory disturbances.[1] HMSN-P was first described in patients from the Okinawa Islands of Japan, where more than 100 people are estimated to be affected.[2] Two Brazilian HMSN-P-affected families of Okinawan ancestry have also been reported.[3,4]

The disease onset is usually in the 40s and is followed by a slowly progressive course. Painful muscle cramps and abundant fasciculations are observed, particularly in the early stage of the disease. In contrast to the clinical presentations of other hereditary motor and sensory neuropathies (HMSNs) presenting with predominantly distal motor weakness reflecting axonal-length dependence, the clinical presentation of HMSN-P is unique in that it involves proximal predominant weakness with widespread fasciculations resembling those of amyotrophic lateral sclerosis (ALS).[5] Distal sensory loss is accompanied later in the disease course, but the degree of the sensory involvement varies among patients. Neuropathological findings revealed severe neuronal loss and gliosis in the spinal anterior horns and mild neuronal loss and gliosis in the hypoglossal and facial nuclei of the brainstem, which indicates that the primary pathological feature of HMSN-P is a motor neuronopathy involving motor neurons, but not a motor neuropathy involving axons.[1,5] The posterior column, corticospinal tract, and spinocerebellar tract showed loss of myelinated fibers and gliosis. Neuronal loss and gliosis were found in Clarke's nucleus. Dorsal root ganglia showed mild to marked neuronal loss.[1,5] These observations suggest that HMSN-P shares neuropathological findings in part with those observed in familial ALS.[6]

Previous studies on Okinawan kindreds mapped the disease locus to chromosome 3q.[1] Subsequently, we identified two large families (families 1 and 2 in Figure 1A) affected by quite a similar phenotype in the Kansai area of Japan, located in the middle of the main island of Japan and far distant from the Okinawa Islands. We mapped the

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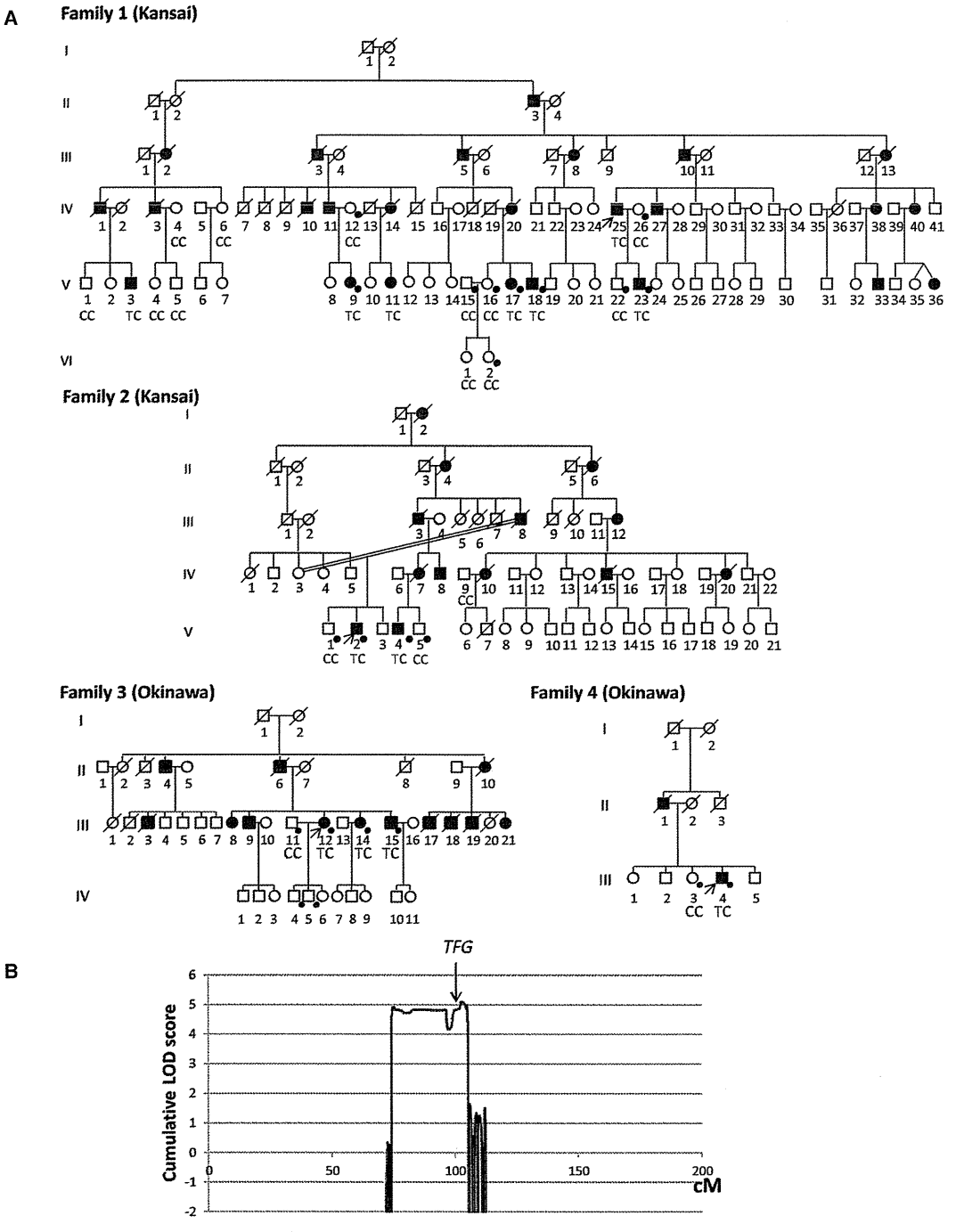

Figure 1. Pedigree Charts and Linkage Analysis
(A) Pedigree charts of families 1 and 2 (Kansai kindreds) and families 3 and 4 (Okinawan kindreds) are shown. Squares and circles indicate males and females, respectively. Affected persons are designated with filled symbols. A diagonal line through a symbol represents a deceased person. A person with an arrow is an index patient. Genotypes of *TFG* c.854 are shown in individuals in whom genomic DNA was analyzed. Individuals genotyped with SNP arrays for linkage analysis and haplotype reconstruction are indicated by dots. (B) Cumulative parametric multipoint LOD scores on chromosome 3 of all the families are shown.

disease locus to chromosome 3q,[7] overlapping with the previously defined locus, which strongly indicates that these diseases are indeed identical.

In addition to the large Kansai HMSN-P-affected families, we found two new Okinawan HMSN-P-affected families (families 3 and 4 in Figure 1A) in our study. In total, 9 affected and 15 unaffected individuals from the Kansai area and four affected and four unaffected individuals from the Okinawa Islands were enrolled in the study. Written informed consent was obtained from

Table 1. Clinical Characteristics of Patients with HMSN-P from Families 1 and 2 from Kansai and Families 3 and 4 from Okinawa

		Family 3			Family 4
	Families 1 and 2	**III-12**	**III-14**	**III-15**	**III-4**
Age at examination (years)	40s–50s	54	52	50	54
Age at onset (years)	37.5 ± 8	44	40	early 20s	41
Initial symptoms	shoulder dislocation and difficulty walking	proximal leg weakness	painful cramps	painful cramps and fasciculation	painful cramps and calf atrophy
Motor					
Proximal muscle weakness and atrophy	+	+	mild	+	+
Painful cramps	+	+	+	+	+
Fasciculations	+	+	+	+	+
Motor ability	bedridden after 10–20 years from disease onset	unable to walk; wheelchair	only mild difficulty climbing stairs	walk with effort	unable to walk; wheelchair
Bulbar symptoms	− ~ +	−	−	−	−
Sensory					
Dysesthesia	+	+	mild	+	+
Decreased tactile sensation	+	+	−	mild	+
Decreased vibratory sensation	+	mild	mild	mild	+
Reflexes					
Tendon reflexes	diminished	diminished	diminished	diminished	diminished
Pathological reflexes	−	−	−	−	−
Laboratory Tests and Electrophysiological Findings					
Serum creatine kinase level	270 ± 101 IU/l	761 IU/l	not measured	625 IU/l	399 IU/l
Hyperglycemia	4/13 patients	−	−	−	+
Hyperlipidemia	3/13 patients	+	−	+	+
Nerve conduction study	motor and sensory axonal degeneration	motor and sensory axonal degeneration	not examined	not examined	motor and sensory axonal degeneration
Needle electromyography	neurogenic changes with fibrillation potentials and positive sharp waves	neurogenic changes with fibrillation potentials and positive sharp waves	not examined	not examined	not examined

The clinical characteristics of the patients from families 1 and 2 were summarized in accordance with the previous studies.[5,6]

all participants. This study was approved by the institutional review boards at the University of Tokyo and the Tokushima University Hospital. Genomic DNA was extracted from peripheral-blood leukocytes or an autopsied brain according to standard procedures.

The clinical presentations of the patients from the four families are summarized in Table 1 and Table S1, available online. Characteristic painful cramps and fasciculations were noted at the initial stage of the disease in all the patients from the four families. Whereas some of the patients showed painful cramps in their 20s, the ages of onset of motor weakness (41.6 ± 2.9 years old) were quite uniform. These patients presented slowly progressive, predominantly proximal weakness and atrophy with diminished tendon reflexes in the lower extremities. Sensory impairment was generally mild. Indeed, one patient (III-4 in family 4) has been diagnosed with very slowly progressive ALS. Although frontotemporal dementia (FTD) is an occasionally observed clinical presentation in patients with ALS, dementia was not observed in these patients. Laboratory tests showed mildly elevated serum creatine kinase levels. Electrophysiological studies showed similar results in all the patients investigated and revealed a decreased number of motor units with abundant positive sharp waves, fibrillation, and fasciculation potentials. Sensory-nerve action potentials of the sural nerve were lost in the later stage of the disease. All these clinical findings were similar to those described in previous reports.[1,3,4]

To further narrow the candidate region, we conducted detailed genotyping by employing the Genome-Wide Human SNP array 6.0 (Affymetrix). Multipoint parametric linkage analysis and haplotype reconstruction were performed with the pipeline software SNP-HiTLink[8] and Allegro v.2[9] (Figure 1A). In addition to the SNP genotyping, we also used newly discovered polymorphic dinucleotide repeats for haplotype comparison (microsatellite marker 1 [MS1], chr3: 101,901,207–101,901,249; and MS2, chr3: 102,157,749–102,157,795 in hg18) around *TFG* (see Table S2 for primer sequences). The genome-wide linkage study revealed only one chromosome 3 region showing a cumulative LOD score exceeding 3.0 (Figure 1B), confirming the result of our previous study.[7] An obligate recombination event was observed between rs4894942 and rs1104964, thus further refining the telomeric boundary of the candidate region in Kansai families (Figure 2A). The Okinawan families (families 3 and 4) shared an extended disease haplotype spanning 3.3 Mb, consistent with a founder effect reported in the Okinawan HMSN-P-affected families,[1] thus defining the 3.3 Mb region as the minimum candidate region.

We then performed exon capture (Sequence Capture Human Exome 2.1 M Array [NimbleGen]) of the index patient from family 3 and subsequent passively parallel sequencing by using two lanes of GAIIx (100 bp single end [Illumina]) and a one-fifth slide of SOLiD 4 (50 bp single end [Life Technologies]). GAIIx and SOLiD4 yielded 2.60 and 2.76 Gb of uniquely mapped reads,[10] respectively. The average coverages were 29.0× and 26.8× in GAIIx and SOLiD4, respectively (Table S3 and Figure S1). In summary, 175,236 single nucleotide variants (SNVs) and 25,987 small insertions/deletions were called.[11] The numbers of exonic and splice-site variants were 14,189 and 127, respectively. In the minimum candidate region of 3.3 Mb, only 11 exonic SNVs were found, and only one was novel (i.e., not found in dbSNP) and nonsynonymous. Direct nucleotide-sequence analysis confirmed the presence of heterozygous SNV c.854C>T (p.Pro285Leu) in TRK-fused gene (*TFG* [NM_006070.5]) in all the patients from families 3 and 4 (Figure 3A and Figure S2[12]). Intriguingly, direct nucleotide-sequence analysis of all *TFG* exons (see Table S4 for primer sequences) of one patient from each of families 1 and 2 from the Kansai area revealed an identical c.854C>T (p.Pro285Leu) *TFG* mutation cosegregating with the disease (Figure 1A and Figure 3A). The base substitution was not observed in 482 Japanese controls (964 chromosomes), dbSNP, the 1000 Genomes Project Database, or the Exome Sequencing Project Database. Pro285 is located in the P/Q-rich domain in the C-terminal region of TFG (Figure 3B) and is evolutionally conserved (Figure 3C). PolyPhen predicts it to be "probably damaging." Because some of the exonic sequences were not sufficiently covered by exome sequencing (i.e., their read depths were no more than 10×) (Figure S1), direct nucleotide-sequence analysis was further conducted for these exonic sequences (Table S5). However, it did not reveal any other novel nonsynonymous variants, confirming that c.854C>T (p.Pro285Leu) is the only mutation exclusively present in the candidate region of 3.3 Mb. All together, we concluded that it was the disease-causing mutation.

Because we found an identical mutation in both Kansai (families 1 and 2) and Okinawan (families 3 and 4) families, we then compared the haplotypes with the c.854C>T (p.Pro285Leu) mutation in the Kansai and Okinawan families in detail. To obtain high-resolution haplotypes, we included custom-made markers, including MS1 and MS2, and new SNVs identified by our exome analysis, in addition to the high-density SNPs used in the linkage analysis. The two Kansai families shared as long as 24.0 Mb of haplotype, and the two Okinawan families shared 3.3 Mb, strongly supporting a common ancestry in each region. When the haplotypes of the Kansai and Okinawan families were compared, it turned out that these families do not share the same haplotype because the markers nearest to *TFG* are discordant at markers 48.5 kb centromeric and 677 bp telomeric to the mutation within a haploblock (Figure 2B). Although the possibility of rare recombination events just distal to the mutation cannot be completely excluded, as suggested by the population-based recombination map (Figure 2B), these findings strongly support the interpretation that the mutations have independent origins and provide further evidence that *TFG* contains the causative mutation for this disease.

Mutational analyses of *TFG* were further conducted in patients with other diseases affecting lower motor neurons (including familial ALS [n = 18], axonal HMSN [n = 26], and hereditary motor neuropathy [n = 3]) and revealed no mutations in *TFG*, indicating that c.854C>T (p.Pro285Leu) in *TFG* is highly specific to HMSN-P.

In this study, we identified in all four families a single variant that appears to have developed on two different haplotypes. The mutation disrupts the PXXP motif, also known as the Src homology 3 (SH3) domain, which might affect protein-protein interactions. In addition, substitution of leucine for proline is expected to markedly alter the protein's secondary structure, which might substantially compromise the physiological functions of TFG.

By employing the primers shown in Table S6, we obtained full-length cDNAs by PCR amplification of the cDNAs prepared from a cDNA library of the human fetal brain (Clontech). During this process, four species of cDNA were identified (Figure S3A). To determine the relative abundance of these cDNA species, we used the primers shown in Table S7 to conduct fragment analysis of the RT-PCR products obtained from RNAs extracted from various tissues; these primers were designed to discriminate four cDNA species on the basis of the size of the PCR products. The analysis revealed that TFG is ubiquitously expressed, including in the spinal cord and dorsal root ganglia, which are the affected sites of HMSN-P (Figure S3B).

Neuropathological studies were performed in a *TFG*-mutation-positive patient (IV-25 in family 1) who died of

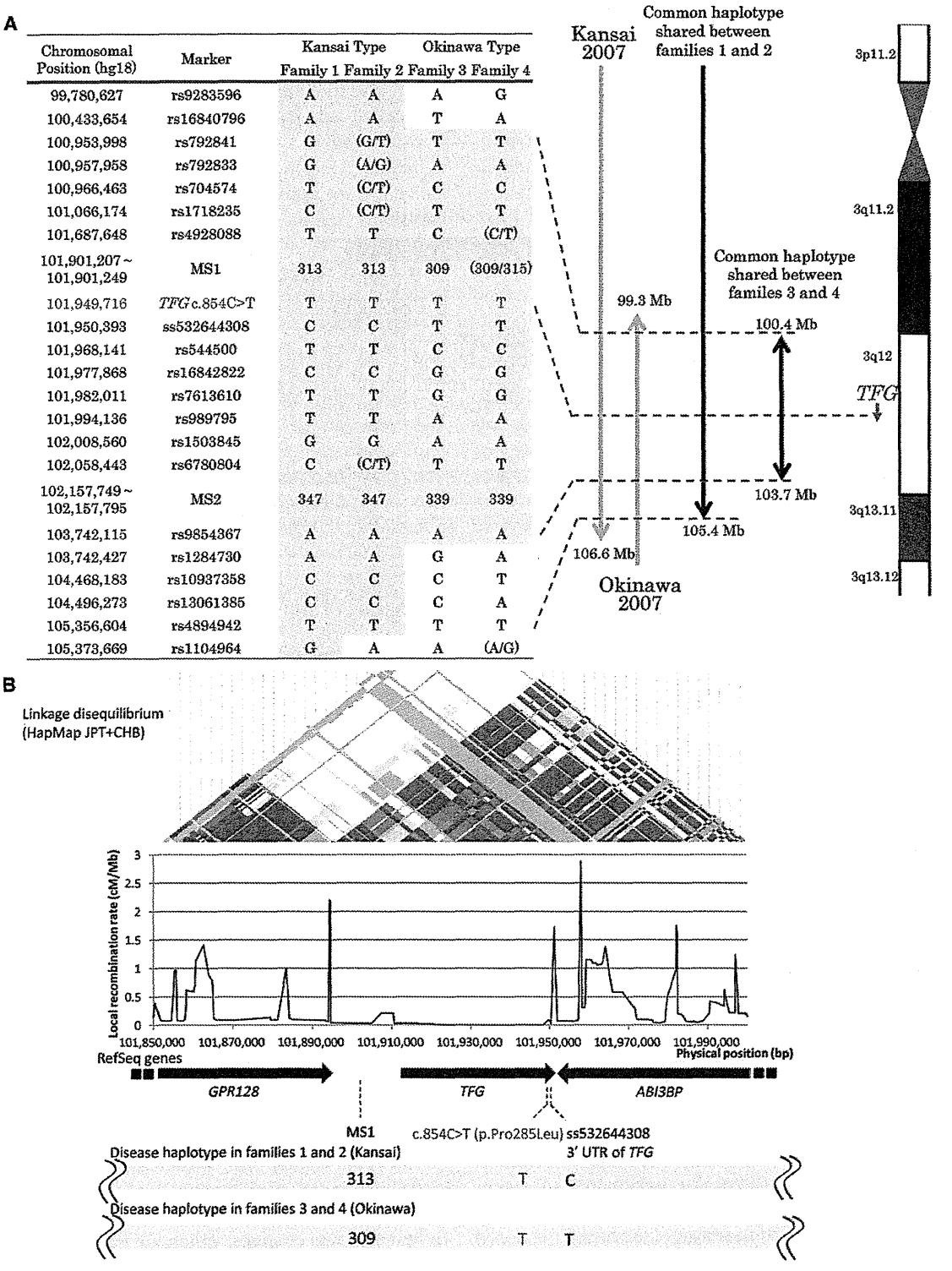

Figure 2. Haplotype Analysis and Minimum Candidate Region of HMSN-P

(A) Haplotypes were reconstructed for all the families with the use of SNP array data and microsatellite markers. Previously reported candidate regions are shown as "Kansai 2007" and "Okinawa 2007."[1,6] Because families 1 and 2 are distantly related, an extended shared common haplotype was observed on chromosome 3, as indicated by a previous study.[6] A reassessment of linkage analysis with high-density SNP markers revealed a recombination between rs4894942 and rs1104964 in family 2, thus refining the telomeric boundary of the candidate region in Kansai families (designated as "Common haplotype shared between families 1 and 2). Furthermore, a shared common haplotype (3.3 Mb with boundaries at rs16840796 and rs1284730) between families 3 and 4 was found, defining the minimum candidate region.

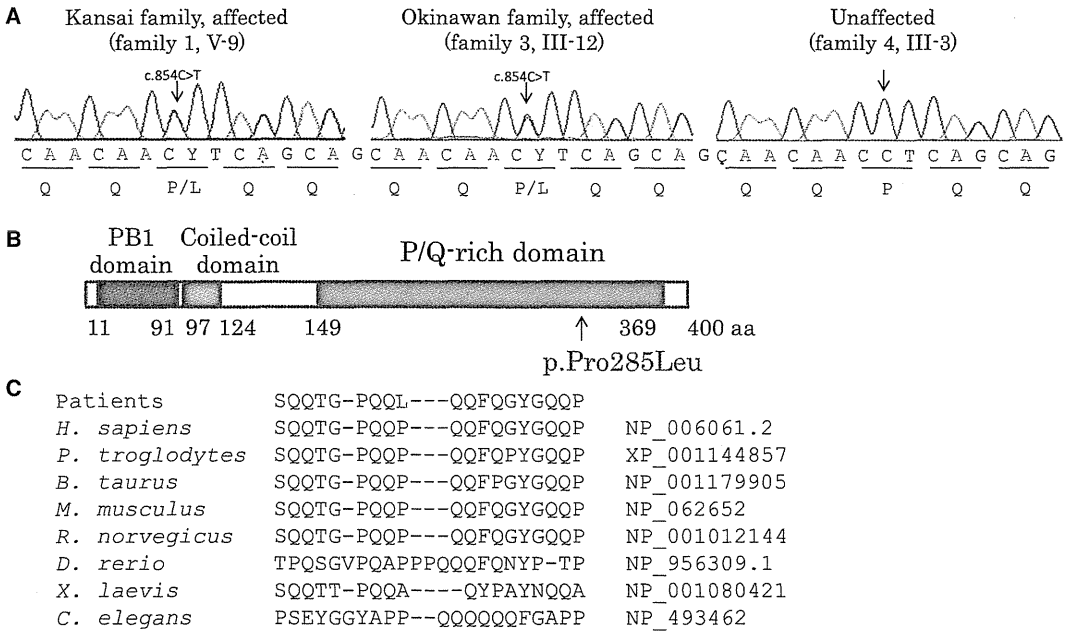

Figure 3. Identification of Causative Mutation
(A) Exome sequencing revealed that only one novel nonsynonymous variant is located within the minimum candidate region. Direct nucleotide-sequence analysis confirmed the mutation, c.854C>T (p.Pro285Leu), in *TFG* in both Kansai and Okinawan families. The mutation cosegregated with the disease (Figure 1A).
(B) Schematic representation of TFG isoform 1. The alteration (p.Pro285Leu) detected in this study is shown below.
(C) Cross-species homology search of the partial TFG amino acid sequence containing the p.Pro285Leu alteration revealed that Pro285 is evolutionally conserved among species.

pneumonia at 67 years of age.[5] Immunohistochemical observations employing a TFG antibody (Table S8) revealed fine granular immunostaining of TFG in the cytoplasm of motor neurons in the spinal cord of neurologically normal controls (n = 3; age at death = 58.7 ± 19.6 years old) (Figure 4A). In the HMSN-P patient, in contrast, TFG-immunopositive inclusion bodies were detected in the motor neurons of the facial, hypoglossal, and abducens nuclei and the spinal cord, as well as in the sensory neurons of the dorsal root ganglia, but were not detected in glial cells (Figures 4B–4D). A small number of cortical neurons in the precentral gyrus also showed TFG-immunopositive inclusion bodies (Figure 4E). Serial sections stained with antibodies against ubiquitin or TFG (Figure 4F) and double immunofluorescence staining (Figure 4G) demonstrated that TFG-immunopositive inclusions colocalized with ubiquitin deposition. Inclusion bodies were immunopositive for optineurin in motor neurons of the brainstem nuclei and the anterior horn of the spinal cord,[5] as well as in sensory neurons of the dorsal root ganglia (data not shown). These data strongly indicate that HMSN-P is a proteinopathy involving TFG.

Because HMSN-P and ALS share some clinical characteristics, we then examined whether neuropathological findings of HMSN-P shared cardinal features with those of sporadic ALS.[13–16] Immunohistochemistry with a TDP-43 antibody revealed skein-like inclusions in the remaining motor neurons of the abducens nucleus and the anterior horn of the lumbar cord (Figures 4H–4I). Phosphorylated TDP-43-positive inclusions were also identified in neurons of the anterior horn of the cervical cord and Clarke's nucleus (Figures 4J–4K). In contrast, TFG immunostaining of spinal-cord specimens from four patients with sporadic ALS (their age at death was 72.3 ± 7.4 years old) revealed no pathological staining in the motor neurons (data not shown). Double immunofluorescence staining revealed that many of the TFG-immunopositive round inclusions in the HSMN-P patient were negative for TDP-43 (Figure 4L), whereas a small number of inclusions were positive for both TFG and TDP-43 (Figure 4M). In addition, to investigate morphological Golgi-apparatus changes, which have recently been found in motor neurons of autopsied tissues of ALS patients,[17] we conducted immunohistochemical analysis by using

(B) Disease haplotypes in the Kansai and Okinawan kindreds are indicated below. Local recombination rates, RefSeq genes, and the linkage disequilibrium map from HapMap JPT (Japanese in Tokyo, Japan) and CHB (Han Chinese in Beijing, China) samples are shown above the disease haplotypes. When disease haplotypes of the Kansai and Okinawan kindreds are compared, the markers nearest to *TFG* are discordant at markers 48.5 kb centromeric and 677 bp telomeric to the mutation within a haploblock, strongly supporting the interpretation that the mutations have independent origins.

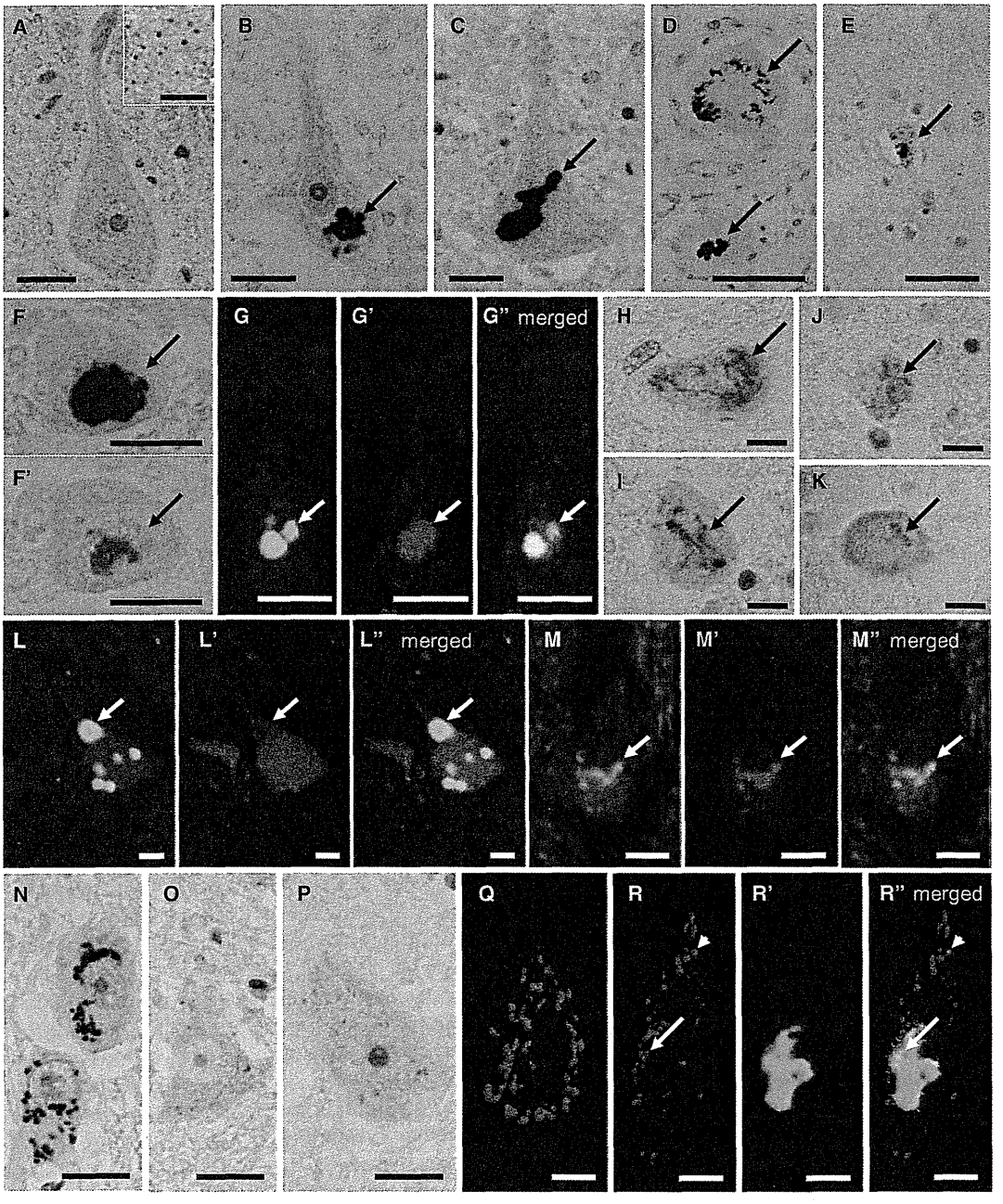

Figure 4. TFG-Related Neuropathological Findings
(A) TFG immunostaining (with hematoxylin counterstaining) of a motor neuron in the spinal cord of a neurologically normal control. A high-power magnified photomicrograph (inset) shows fine granular staining of TFG in the cytoplasm. The scale bars represent 20 μm (main panel) and 10 μm (inset).
(B–E) TFG-immunopositive inclusions of the neurons (with hematoxylin counterstaining) in the hypoglossal nucleus (B), anterior horn of the spinal cord (C), dorsal root ganglion (D, arrows), and motor cortex (E, arrow) of the patient with the *TFG* mutation. The scale bars represent 20 μm (B–D) and 50 μm (E).
(F and F′) Serial section analysis of the facial nucleus motor neuron showing an inclusion body colabeled for TFG (F) and ubiquitin (F′). The scale bars represent 20 μm.
(G–G″) Double immunofluorescence microscopy confirming colocalization of TFG (green) and ubiquitin (red) in an inclusion body of a motor neuron in the hypoglossal nucleus. The scale bars represent 20 μm.
(H and I) TDP-43-positive skein-like inclusions in the motor neurons of the abducens nucleus (H) and anterior horn of the lumbar cord (I). The scale bars represent 20 μm.
(J and K) Phosporylated TDP-43-positive inclusion bodies in the cervical anterior horn (J) and Clarke's nucleus (K). The scale bars represent 20 μm.
(L–L″) Round inclusions (arrows) positive for TFG (green) but negative for TDP-43 (red). The scale bars represent 20 μm.

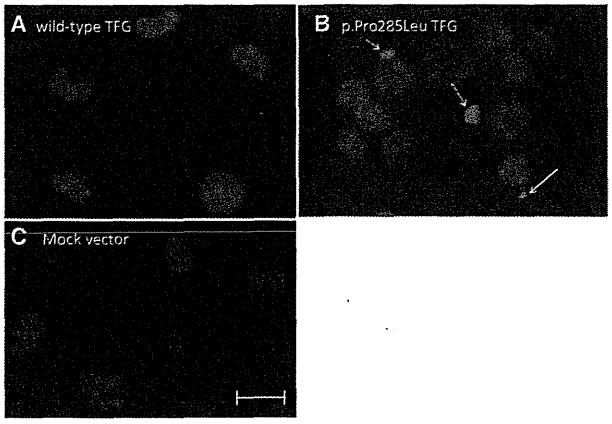

Figure 5. Formation of Cytoplasmic TDP-43 Aggregation Bodies in Cells Stably Expressing Mutant p.Pro285Leu TFG
The coding sequence of *TFG* cDNA was subcloned into pBluescript (Stratagene). After site-directed mutagenesis with a primer pair shown in Table S9, the mutant cDNAs were cloned into the BamHI and XhoI sites of pcDNA3 (Life Technologies). Stable cell lines were established by Lipofectamine (Life Technologies) transfection according to the manufacturer's instructions. Established cell lines were cultured under the ordinary cell-culture conditions (37°C and 5% CO_2) for 5–6 days and were subjected to immunocytochemical analyses. Neuro-2a cells stably expressing wild-type TFG (A), mutant TFG (p.Pro285Leu) (B), and a mock vector (C) are shown. TDP-43-immunopositive cytoplasmic inclusions are absent in the cells stably expressing wild-type TFG or the mock vector (A and C); however, TDP-43-immunopositive cytoplasmic inclusions were exclusively demonstrated in cells stably expressing mutant TFG (p.Pro285Leu), as indicated by arrows (B). Similar results were obtained with HEK 293 cells (not shown). Scale bars represent 10 μm.

a TGN46 antibody. It revealed that the Golgi apparatus was fragmented in approximately 70% of the remaining motor neurons in the lumbar anterior horn. The fragmentation of the Golgi apparatus was prominent near TFG-positive inclusion bodies (Figures 4N–4R). In summary, we found abnormal TDP-43-immunopositive inclusions in the cytoplasm of motor neurons, as well as fragmentation of the Golgi apparatus in HMSN-P, confirming the overlapping neuropathological features between HMSN-P and sporadic ALS.

To further investigate the effect of mutant TFG in cultured cells, stable cell lines expressing wild-type and mutant TFG (p.Pro285Leu) were established from neuro-2a and human embryonic kidney (HEK) 293 cells as previously described.[18] Established cell lines were cultured under the ordinary cell-culture conditions (37°C and 5% CO_2) for 5–6 days and were subjected to immunocytochemical analyses. The neuro-2a cells stably expressing wild-type or mutant TFG demonstrated no distinct difference in the distribution of endogenous TFG, FUS, or OPTN (data not shown). In contrast, cytoplasmic inclusions containing endogenous TDP-43 were exclusively observed in the neuro-2a cells stably expressing untagged mutant TFG, but not in those expressing wild-type TFG (Figure 5). Similar data were obtained from HEK 293 cells (data not shown). Thus, the expression of mutant TFG leads to mislocalization and inclusion-body formation of TDP-43 in cultured cells.

TFG was originally identified as a part of fusion oncoproteins (NTRK1-T3 in papillary thyroid carcinoma,[19] TFG-ALK in anaplastic large cell lymphoma,[20] and TFG/NOR1 in extraskeletal myxoid chondrosarcoma[21]), where the N-terminal portions of TFG are fused to the C terminus of tyrosine kinases or a superfamily of steroid-thyroid hormone-retinoid receptors acting as a transcriptional activator leading to the formation of oncogenic products. Very recently, TFG-1, a homolog of TFG in *Caenorhabditis elegans*, and TFG have been discovered to localize in endoplasmic-reticulum exit sites. TFG-1 acts in a hexameric form that binds the scaffolding protein Sec16 complex assembly and plays an important role in protein secretion with COPII-coated vesicles.[22] It is noteworthy that mutations in genes involved in vesicle trafficking[23,24] (such genes include *VAPB*, *CHMP2B*, *alsin*, *FIG4*, *VPS33B*, *PIP5K1C*, and *ERBB3*) cause motor neuron diseases, emphasizing the role of vesicle trafficking in motor neuron diseases. Thus, altered vesicle trafficking due to the *TFG* mutation might be involved in the motor neuron degeneration in HMSN-P. The presence of TFG-immunopositive inclusions in motor neurons raises the possibility that mutant TFG results in the misfolding and formation of cytoplasmic aggregate bodies, as well as altered vesicle trafficking.

An intriguing neuropathological finding is TDP-43-positive cytoplasmic inclusions in the motor neurons; these inclusions have recently been established as the fundamental neuropathological findings in ALS.[13,14] Of note, expression of mutant, but not wild-type, TFG in cultured cells led to the formation of TDP-43-containing cytoplasmic aggregation. These observations are similar

(M–M″) An inclusion immunopositive for both TFG (green) and TDP-43 (red) is observed in a small number of neurons. The scale bars represent 20 μm.
(N) Normal Golgi apparatus in the neurons of the intact thoracic intermediolateral nucleus. The scale bar represents 20 μm.
(O and P) Fragmentation of the Golgi apparatus with small, round, and disconnected profiles in the affected motor neurons of the lumbar anterior horn. The scale bars represent 20 μm.
(Q–R″) Immunohistochemical observations of the Golgi apparatus and TFG-immunopositive inclusions employing antibodies against TGN46 (red) and TFG (green), respectively. The scale bars represent 10 μm.
(Q) Normal size and distribution (red) in a motor neuron without inclusions.
(R–R″) The Golgi apparatus was fragmented into various sizes and reduced in number in the lumbar anterior horn motor neuron with TFG-positive inclusions (green). The fragmentation predominates near the inclusion (arrow), whereas the Golgi apparatuses distant from the inclusion showed nearly normal patterns (arrow head).

to what has been described for ALS, where TDP-43 is mislocalized from the normally localized nucleus to the cytoplasm with concomitant cytoplasmic inclusions. Cytoplasmic TDP-43 accumulation and inclusion formation have also been observed in motor neurons in familial ALS with mutations in *VAPB* (MIM 608627) or *CHMP2B* (MIM 600795).[25,26] Furthermore, TDP-43 pathology has been demonstrated in transgenic mice expressing mutant VAPB.[27] Although the mechanisms of mislocalization of TDP-43 remain to be elucidated, these observations suggest connections between alteration of vesicle trafficking and mislocalization of TDP-43. Thus, common pathophysiologic mechanisms might underlie motor neuron degenerations involving vesicle trafficking including TFG, as well as VAPB and CHMP2B. Because TDP-43 is an RNA-binding protein, RNA dysregulation has been suggested to play important roles in the TDP43-mediated neurodegeneration.[28] Furthermore, recent discovery of hexanucleotide repeat expansions in *C9ORF72* in familial and sporadic ALS/FTD (MIM 105550)[29,30] emphasizes the RNA-mediated toxicities as the causal mechanisms of neurodegeneration. Observations of TDP-43-positive cytoplasmic inclusions in the motor neurons of the patient with HMSN-P raise the possibility that RNA-mediated mechanisms might also be involved in motor neuron degeneration in HMSN-P.

In summary, we have found that *TFG* mutations cause HMSN-P. The presence of TFG/ubiquitin- and/or TDP-43-immunopositive cytoplasmic inclusions in motor neurons and cytosolic aggregation composed of TDP-43 in cultured cells expressing mutant TFG indicate a novel pathway of motor neuron death.

Supplemental Data

Supplemental Data include three figures and nine tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows.

1000 Genomes Project Database, http://www.1000genomes.org/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
HapMap, http://hapmap.ncbi.nlm.nih.gov/
NHLBI GO Exome Sequencing Project, https://esp.gs.washington.edu/drupal/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
PolyPhen, http://genetics.bwh.harvard.edu/pph/
RefSeq, http://www.ncbi.nlm.nih.gov/projects/RefSeq/
UCSC Human Genome Browser, http://genome.ucsc.edu/

References

1. Takashima, H., Nakagawa, M., Nakahara, K., Suehara, M., Matsuzaki, T., Higuchi, I., Higa, H., Arimura, K., Iwamasa, T., Izumo, S., and Osame, M. (1997). A new type of hereditary motor and sensory neuropathy linked to chromosome 3. Ann. Neurol. *41*, 771–780.
2. Nakagawa, M. (2009). [Wide spectrum of hereditary motor sensory neuropathy (HMSN)]. Rinsho Shinkeigaku *49*, 950–952.
3. Maeda, K., Sugiura, M., Kato, H., Sanada, M., Kawai, H., and Yasuda, H. (2007). Hereditary motor and sensory neuropathy (proximal dominant form, HMSN-P) among Brazilians of Japanese ancestry. Clin. Neurol. Neurosurg. *109*, 830–832.
4. Patroclo, C.B., Lino, A.M., Marchiori, P.E., Brotto, M.W., and Hirata, M.T. (2009). Autosomal dominant HMSN with proximal involvement: new Brazilian cases. Arq. Neuropsiquiatr. *67* (3B), 892–896.
5. Fujita, K., Yoshida, M., Sako, W., Maeda, K., Hashizume, Y., Goto, S., Sobue, G., Izumi, Y., and Kaji, R. (2011). Brainstem and spinal cord motor neuron involvement with optineurin inclusions in proximal-dominant hereditary motor and sensory neuropathy. J. Neurol. Neurosurg. Psychiatry *82*, 1402–1403.
6. Takahashi, H., Makifuchi, T., Nakano, R., Sato, S., Inuzuka, T., Sakimura, K., Mishina, M., Honma, Y., Tsuji, S., and Ikuta, F. (1994). Familial amyotrophic lateral sclerosis with a mutation in the Cu/Zn superoxide dismutase gene. Acta Neuropathol. *88*, 185–188.
7. Maeda, K., Kaji, R., Yasuno, K., Jambaldorj, J., Nodera, H., Takashima, H., Nakagawa, M., Makino, S., and Tamiya, G. (2007). Refinement of a locus for autosomal dominant hereditary motor and sensory neuropathy with proximal dominancy (HMSN-P) and genetic heterogeneity. J. Hum. Genet. *52*, 907–914.
8. Fukuda, Y., Nakahara, Y., Date, H., Takahashi, Y., Goto, J., Miyashita, A., Kuwano, R., Adachi, H., Nakamura, E., and Tsuji, S. (2009). SNP HiTLink: A high-throughput linkage analysis system employing dense SNP data. BMC Bioinformatics *10*, 121.
9. Gudbjartsson, D.F., Thorvaldsson, T., Kong, A., Gunnarsson, G., and Ingolfsdottir, A. (2005). Allegro version 2. Nat. Genet. *37*, 1015–1016.

10. Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754–1760.
11. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079.
12. Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26.
13. Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., et al. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science *314*, 130–133.
14. Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., and Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem. Biophys. Res. Commun. *351*, 602–611.
15. Hasegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., Beach, T.G., Buratti, E., Baralle, F., Morita, M., et al. (2008). Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Ann. Neurol. *64*, 60–70.
16. Inukai, Y., Nonaka, T., Arai, T., Yoshida, M., Hashizume, Y., Beach, T.G., Buratti, E., Baralle, F.E., Akiyama, H., Hisanaga, S., and Hasegawa, M. (2008). Abnormal phosphorylation of Ser409/410 of TDP-43 in FTLD-U and ALS. FEBS Lett. *582*, 2899–2904.
17. Stieber, A., Chen, Y., Wei, S., Mourelatos, Z., Gonatas, J., Okamoto, K., and Gonatas, N.K. (1998). The fragmented neuronal Golgi apparatus in amyotrophic lateral sclerosis includes the trans-Golgi-network: Functional implications. Acta Neuropathol. *95*, 245–253.
18. Kuroda, Y., Sako, W., Goto, S., Sawada, T., Uchida, D., Izumi, Y., Takahashi, T., Kagawa, N., Matsumoto, M., Matsumoto, M., et al. (2012). Parkin interacts with Klokin1 for mitochondrial import and maintenance of membrane potential. Hum. Mol. Genet. *21*, 991–1003.
19. Greco, A., Mariani, C., Miranda, C., Lupas, A., Pagliardini, S., Pomati, M., and Pierotti, M.A. (1995). The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. Mol. Cell. Biol. *15*, 6118–6127.
20. Hernández, L., Pinyol, M., Hernández, S., Beà, S., Pulford, K., Rosenwald, A., Lamant, L., Falini, B., Ott, G., Mason, D.Y., et al. (1999). TRK-fused gene (TFG) is a new partner of ALK in anaplastic large cell lymphoma producing two structurally different TFG-ALK translocations. Blood *94*, 3265–3268.
21. Hisaoka, M., Ishida, T., Imamura, T., and Hashimoto, H. (2004). TFG is a novel fusion partner of NOR1 in extraskeletal myxoid chondrosarcoma. Genes Chromosomes Cancer *40*, 325–328.
22. Witte, K., Schuh, A.L., Hegermann, J., Sarkeshik, A., Mayers, J.R., Schwarze, K., Yates, J.R., 3rd, Eimer, S., and Audhya, A. (2011). TFG-1 function in protein secretion and oncogenesis. Nat. Cell Biol. *13*, 550–558.
23. Dion, P.A., Daoud, H., and Rouleau, G.A. (2009). Genetics of motor neuron disorders: New insights into pathogenic mechanisms. Nat. Rev. Genet. *10*, 769–782.
24. Andersen, P.M., and Al-Chalabi, A. (2011). Clinical genetics of amyotrophic lateral sclerosis: What do we really know? Nat Rev Neurol *7*, 603–615.
25. Ince, P.G., Highley, J.R., Kirby, J., Wharton, S.B., Takahashi, H., Strong, M.J., and Shaw, P.J. (2011). Molecular pathology and genetic advances in amyotrophic lateral sclerosis: an emerging molecular pathway and the significance of glial pathology. Acta Neuropathol. *122*, 657–671.
26. Cox, L.E., Ferraiuolo, L., Goodall, E.F., Heath, P.R., Higginbottom, A., Mortiboys, H., Hollinger, H.C., Hartley, J.A., Brockington, A., Burness, C.E., et al. (2010). Mutations in CHMP2B in lower motor neuron predominant amyotrophic lateral sclerosis (ALS). PLoS ONE *5*, e9872.
27. Tudor, E.L., Galtrey, C.M., Perkinton, M.S., Lau, K.-F., De Vos, K.J., Mitchell, J.C., Ackerley, S., Hortobágyi, T., Vámos, E., Leigh, P.N., et al. (2010). Amyotrophic lateral sclerosis mutant vesicle-associated membrane protein-associated protein-B transgenic mice develop TAR-DNA-binding protein-43 pathology. Neuroscience *167*, 774–785.
28. Lee, E.B., Lee, V.M., and Trojanowski, J.Q. (2012). Gains or losses: Molecular mechanisms of TDP43-mediated neurodegeneration. Nat. Rev. Neurosci. *13*, 38–50.
29. DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J., et al. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron *72*, 245–256.
30. Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L., et al; ITALSGEN Consortium. (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron *72*, 257–268.