with the commercial phosphorylation-independent antibody, suggesting that all of the inclusion types previously described contain phosphorylated TDP-43. Similar staining patterns were obtained using pS379 (Figs 2A–C), pS403/404 (see Figs 2D–F), pS409 (see Figs 2G–I), and pS410 (see Figs 2J–L). Preabsorption of the antibodies with phosphopeptide immunogens abolished the labeling of these structures (data not shown).

Immunoelectron microscopic examination of the

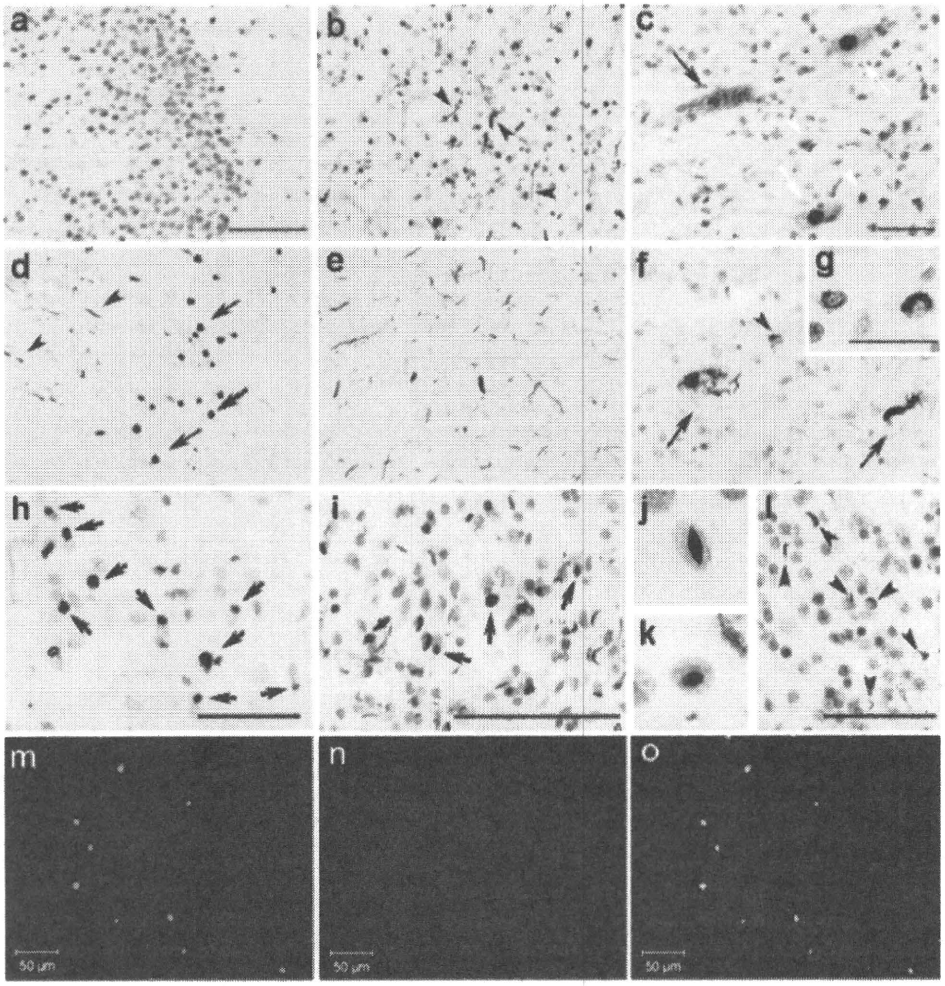

Fig 1. Immunohistochemical comparison of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) brains using the phosphorylation-independent anti–TAR DNA-binding protein of 43kDa (TDP-43) antibody (ProteinTech) (A–C) and the phosphorylation-dependent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

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

Immunoblot Analysis of Phosphorylated TDP-43

Immunoblot analyses of sarkosyl-insoluble fractions extracted from the brains of control, AD, FTLD-U, and ALS cases with the phosphorylation-independent TDP-43 antibody (ProteinTech) always showed a band of 43kDa and also showed an additional 45kDa band that was present only in FTLD-U and ALS cases, as described previously[12,13] (Fig 4A). The phosphorylation-dependent antibodies specific for pS409/410 (see Fig 4B), pS409 (see Fig 4C), pS410 (see Fig 4D), pS403/404 (see Fig 4E), and pS379 (see Fig 4F) did not recognize the normal 43kDa band, showing a single band at approximately 45kDa, several smaller fragments at approximately 25kDa, and indistinct smears in FTLD-U and ALS cases but not in control and AD cases (see Figs 4B–F). The intensity of the approximately 25kDa fragments tended to be greater than that of the 45kDa band in FTLD-U (see Figs 4B–E) and in ALS (see Figs 4B, D). As for the immunohistochemical findings, the antibody to pS409/410 showed the most intense labeling (see Fig 4B). All of the immunoreactive bands were completely abolished by dephosphorylation, which was performed with lambda protein phosphatase (8,000U/ml; New England Biolabs) at 30°C for 2 hours.

Immunoblot Distinction between Clinicopathological Subtypes of TDP-43

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

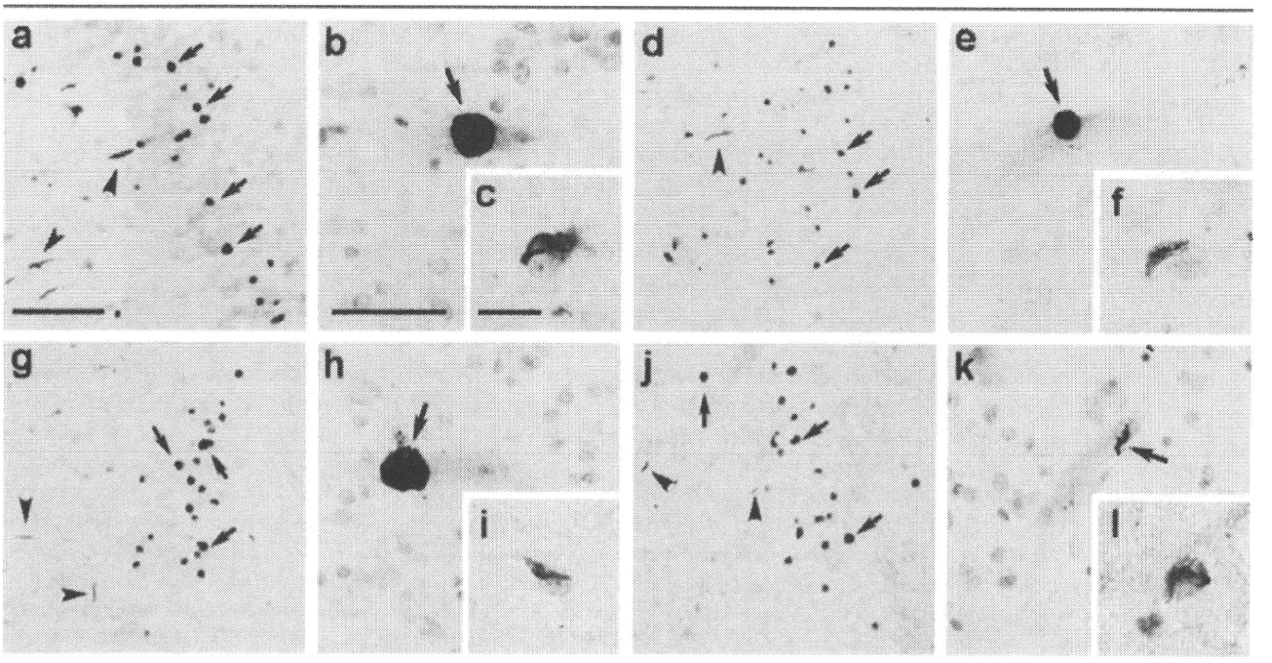

Fig 2. Immunohistochemistry of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) brains and amyotrophic lateral sclerosis (ALS) spinal cords using the phosphorylation-dependent anti–TAR DNA-binding protein of 43kDa (TDP-43) antibodies specific for pS379 (A–C), pS403/404 (D–F),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

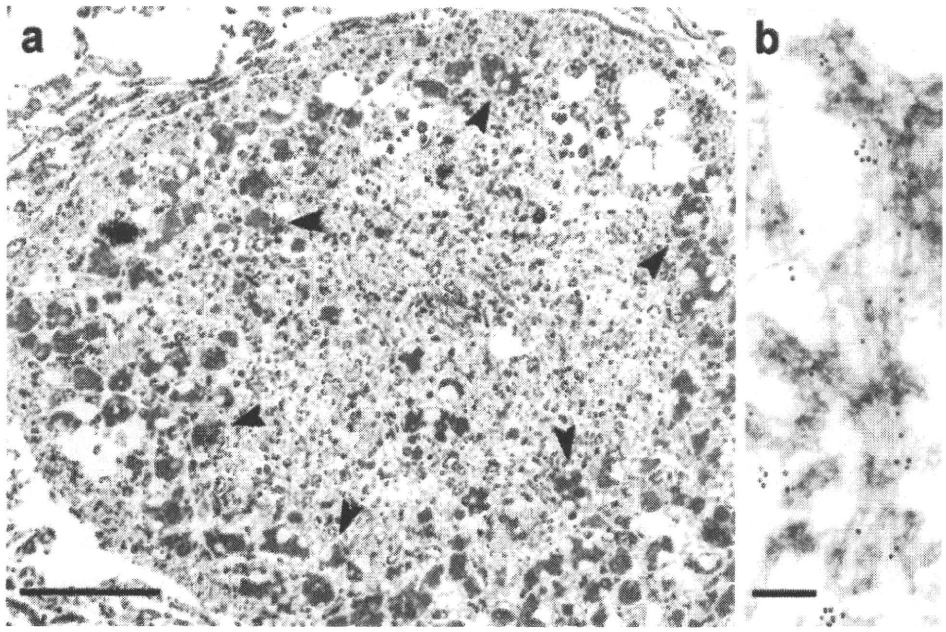

Fig 3. (A) A low-power immunoelectron micrograph of a phosphorylated TAR DNA-binding protein of 43kDa (TDP-43)–positive motoneuronal inclusion in the spinal cord of an amyotrophic lateral sclerosis (ALS) patient. The irregularly shaped structure surrounded by lipofuscins (arrowheads) *is the inclusion.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

Phosphorylation Epitopes Are Generated by Casein Kinase-1

To investigate the kinase responsible for the abnormal phosphorylation of TDP-43, we treated recombinant TDP-43 in vitro with CK1, CK2, and GSK3β. Immunoblot analyses of the recombinant TDP-43 showed that phosphorylation by CK1 caused a reduction in gel mobility of TDP-43 to approximately 45kDa and strong immunoreactivity to the phosphorylation-specific antibodies (Fig 6A). TDP-43 phosphorylated by CK2 was only weakly immunoreactive for these antibodies (see Fig 6A), and that phosphorylated by GSK3β was negative (data not shown). Kinase activity capable of generating the approximately 45kDa TDP-43 with pS409/410 epitopes was also detected in crude rat brain extracted with a high concentration (10–20mM) of $MgCl_2$ (data not shown). This kinase activity was not inhibited by the CK2 inhibitor heparin, suggesting that CK1 may be the major kinase in brain extract. Interestingly, increased levels of sodium dodecyl sulfate–stable TDP-43 oligomers were observed after phosphorylation by CK1 (see Fig 6B). Furthermore, based on immunoelectron microscopic analysis, recombinant TDP-43 phosphorylated by CK1 formed abundant filaments when applied on a carbon-coated copper grid (see Fig 6C), whereas nonphosphorylated recombinant TDP-43 formed few filaments (data not shown).

Discussion

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

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

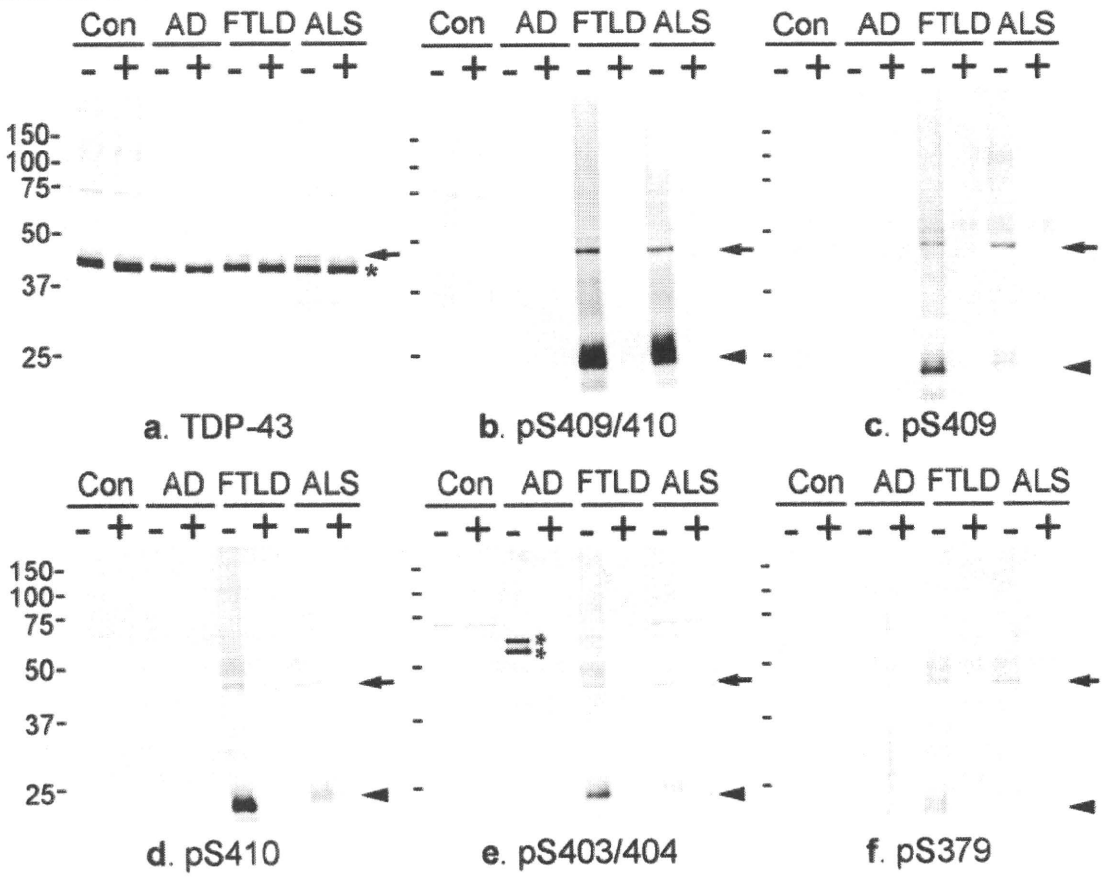

Fig 4. (A) Immunoblot analyses of sarkosyl-insoluble, urea-soluble fractions from control, Alzheimer's disease (AD), frontotemporal lobar degeneration (FTD; frontotemporal lobar degeneration with ubiquitinated inclusions [FTLD-U]), and amyotrophic lateral sclerosis (ALS) brains with phosphorylation-independent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

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

There is a close relation between the pathological subtypes of TDP-43 proteinopathy and the immunoblot pattern of C-terminal fragments of phosphorylated TDP-43. These findings confirm and extend Sampathu and colleagues'[31] and Neumann and coworkers'[12] previous reports that showed C-terminal fragment composition varied between cases with type 1 and 2 pathology. Furthermore, we have shown that cases with type 3 pathology have a band pattern that is mixed or intermediate. These results parallel our earlier findings of differing C-terminal tau fragments in progressive supranuclear palsy and corticobasal degeneration, despite identical composition of tau isoforms.[32] Taken together, these results suggest that elucidating the mechanism of C-terminal fragment origination may shed light on the pathogenesis of several neurodegenerative disorders involving TDP-43 proteinopathy and tauopathy.

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

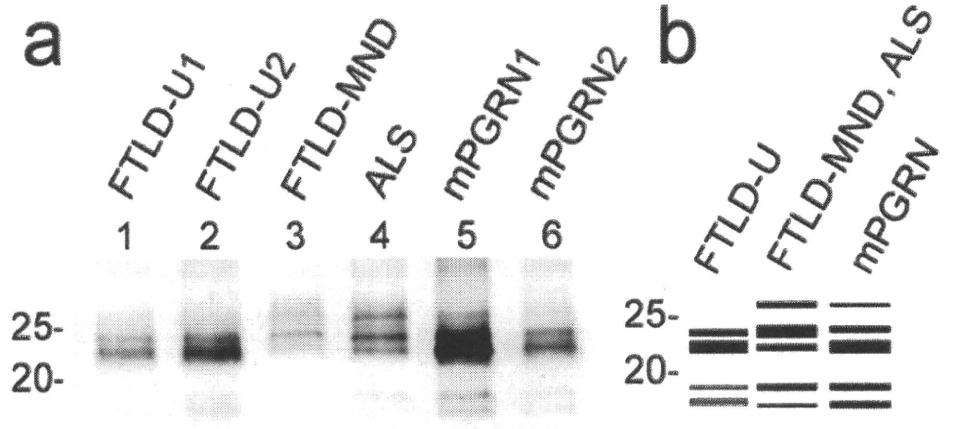

Fig 5. A relation between the clinicopathological subtypes of TAR DNA-binding protein of 43kDa (TDP-43) proteinopathies and the band pattern of the C-terminal fragments of phosphorylated TDP-43. (A) Immunoblots of the sarkosyl-insoluble, urea-soluble fractions from sporadic frontotemporal lobar degeneration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

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

The results suggest that CK1 is involved in the abnormal phosphorylation and accumulation of TDP-43. In this study, the treatment of recombinant TDP-43 by CK1 generates the same phosphorylation epitopes that are recognized by phosphorylation-dependent antibodies. In addition, phosphorylation at these epitopes facilitates filament formation. In comparison, several protein kinases have been reported to be responsible for phosphorylating tau and α-synuclein. They include, for tau phosphorylation,[34–37] GSK3β, cyclin-dependent kinase 5, mitogen-activated protein kinase, and mitogen-activated protein/microtubule affinity-regulating kinase, and for α-synuclein phosphorylation,[38–40] CK1, CK2, and G-protein–coupled receptor kinase 5.

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

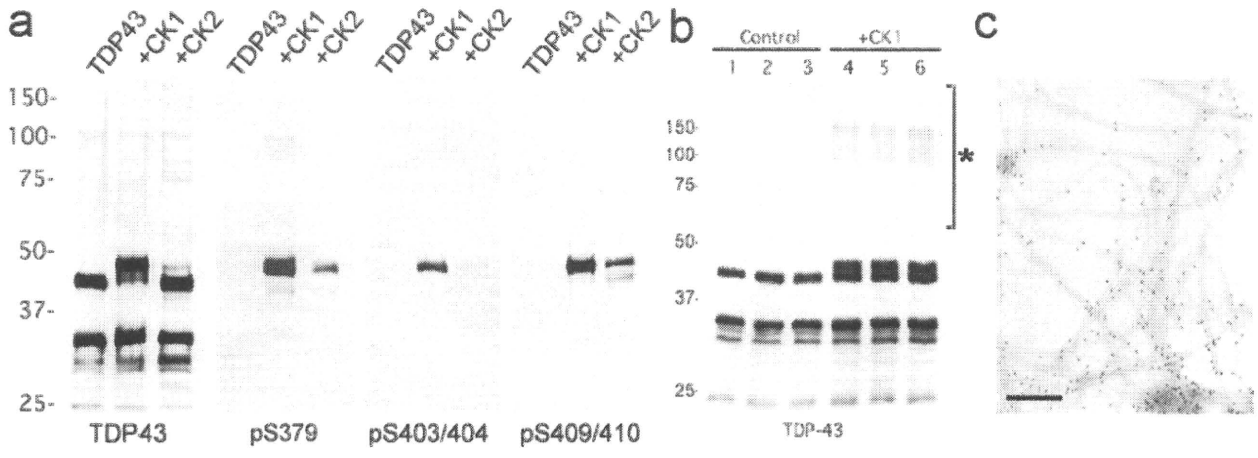

Fig 6. (A) Immunoblot analyses of recombinant TAR DNA-binding protein of 43kDa (TDP-43) phosphorylated in vitro. The crude extract from E. coli *that expressed human TDP-43 is treated with casein kinase-1 (CK1) and CK2 at 30°C for 14 hours, and probed with a phosphorylation-independent antibody against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

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

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

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Age at onset influences on wide-ranged clinical features of sporadic amyotrophic lateral sclerosis

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ABSTRACT

Purpose: To profile the detailed clinical features of sporadic amyotrophic lateral sclerosis (ALS) on large-scale samples in Japan.
Methods: We assessed the clinical features of sporadic ALS patients in Japan, based on the nationwide registration system of the Ministry of Health, Labor and Welfare of Japan. We described 3428 new cases registered cases between 2003 and 2006 to analyze initial symptoms and related clinical features, 4202 cases registered in the single year of 2005 to describe the cross-sectional overview of the ALS patients, and a total of 2128 cases with tracheostomy positive pressure ventilation (TPPV) from all of the registration data from 2003 to 2006 to describe the features of ALS patients with TPPV.
Results: The patients with an older age at onset progressed more rapidly to the TPPV stage than those with a younger age at onset. The subpopulation of patients with long-standing TPPV showed ophthalmoplegia, while its appearance rate was less in the patients with an older age at onset than in those with a younger age at onset. Furthermore, age at onset strongly influenced the frequency of initial symptoms: dysarthria, dysphagia, neck weakness and respiratory disturbance were more frequent in patients with an older age at onset, while upper or lower limb weakness was observed more frequently in patients with a younger age at onset. In addition, those initial symptoms were still the most prominent at the follow-up stage, suggesting that the initial symptoms determine the major clinical features even in advanced illness.
Conclusions: Our present study demonstrated that symptomatic features of ALS are strongly influenced by the age at onset by the large scale of samples.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is one of the most devastating neurodegenerative diseases affecting upper and lower motor neurons preferentially, and shows progressive muscle wasting of the limb, bulbar and respiratory musculatures. Almost half of ALS patients expire within three years of onset, primarily due to respiratory failure [1–6]. Approximately 5–10% of ALS patients show a familial trait, while more than 90% of the patients are sporadic, and the causal mechanism of the motor neuron degeneration is largely unknown. Although many clinical trials of potential therapeutic agents for the treatment of sporadic ALS have been performed [7], effective therapeutics against motor neuron degeneration in ALS except for riluzole [8,9] have not been developed. The clinical features of ALS have been established for the most part. However, many aspects of symptomatic manifestations such as the influence of age at onset on clinical features, the frequency of rare symptoms and many other symptomatic details have not been well characterized, particularly

Abbreviations: ALS, amyotrophic lateral sclerosis; TPPV, tracheostomy positive pressure ventilation.

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based on a nationwide scale sample. In Japan, the proportion of the ALS patients with TPPV is relatively higher than in other countries [10,11]. Rare symptoms such as ophthalmoplegia are more frequently seen in those who receive TPPV to prolong survival [12,13], so the clinical profile of ALS patients in Japan might have unique features. Data concerning the clinical features are important to establish an early diagnosis, treatment plan, and prognostic estimation, as well as to design clinical trials.

The aim of this study was to profile the detailed clinical features of sporadic ALS on large-scale samples in Japan.

2. Research design and methods

A nationwide registration of patients with intractable diseases including ALS has been conducted by the Ministry of Health, Labor and Welfare of Japan since 1974. When a patient is diagnosed as having ALS, the patient can apply for registration in this system, and receive financial support from the state for medical expenses incurred for the treatment of ALS, independent of the disease severity. In 2003, a data collection system was developed for research use of this registration system. Concurrently with that, the registration form for ALS was revised substantially. Since 2003, the annual renewal of registration of each patient has been conducted. The data from registration forms were input to the database in each prefectural office and consolidated in the Ministry of Welfare, Health and Labor of Japan. In the revised registration form, the overview of the clinical state is to be indicated, including the severity, neurological symptoms, activities of daily living and conditions of tube feeding or non-invasive positive pressure ventilation (NIPPV) and TPPV of ALS patients in Japan on a nationwide scale. Using the data accumulated from 2003 to 2006, we analyzed the clinical features of sporadic ALS patients in Japan. Clinical profiles of sporadic ataxias in Japan were previously described using this registration system [14].

The inclusion criteria of the registration system for ALS are: 1) adult onset, steady progressive course; 2) the presence of clinical or electrophysiological evidence of lower motor neuron (LMN) degeneration in at least two topographical anatomic regions (brainstem, cervical, thoracic or lumbosacral region), together with clinical evidence of upper motor neuron (UMN) degeneration in at least one region; and 3) the absence of electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs. Therefore the patients registered in this system satisfy definite, probable or possible ALS based on the revised El Escorial Criteria [15] for the diagnosis of ALS.

The data collection system was developed in 32 of 47 prefectures in Japan. In proportion to the total population, 63% of total registered patients in Japan were integrated into the computerized database. The data were comprised of initial registration form and renewal registration form. When a patient was diagnosed as ALS, the initial registration form was used to apply for the system, and the renewal registration form was used in the following year. However, the information on the patients initially registered before 2003 was comprised of data from only renewal registration.

After 2003, 3694 ALS patients were newly registered in the system. Records were eliminated from the analysis if information was missing for age at onset and age at registration. Ninety-four patients were also excluded who had a family history of motor neuron disease or an abnormality of genes related to neurodegenerative disease such as the SOD1 mutation. The inclusion age range was above 20 years at onset. After these data clearing, the data from a total of 3428 patients were available. In order to analyze the age at onset, initial symptoms and related clinical features, we used this data set.

In a single year, 2005, 4546 ALS patients were registered using the initial registration form or renewal registration form. The number included those initially registered before 2003. To describe the cross-sectional overview of the medical and social conditions of ALS patients in Japan, we used this data set. After the data described above were excluded, the data from 4202 patients were used.

From 2003 to 2006, 2440 ALS patients with TPPV were registered at least once, mostly using the renewal registration form. The number included those initially registered before 2003. To describe the conditions of ALS patients with TPPV, we analyzed this data set. After the data cleaning, the data from 2128 patients with TPPV were used.

All of the patients provided written informed consent for the research use of the data, and the anonymity of the data was strictly secured. We implemented the guidelines for research use of the data from the nationwide registration system of intractable diseases and the ethics guidelines for clinical studies endorsed by the Japanese government. The research project was approved by the Ministry of Health, Labor and Welfare, Japan, and by the ethics committee of Nagoya University Graduate School of Medicine.

2.1. Assessment of clinical features

Age at onset was considered as the time of the patient's initial awareness of weakness. As for the initial symptoms, six symptoms including dysarthria, dysphasia, respiratory disturbance, weakness of neck, weakness of upper extremities and weakness of lower extremities were noted. In most cases, one symptom was assessed as an initial symptom, however, two or more symptoms may be recorded. The activities of daily living and clinical symptoms were assessed by 6 items from the 12 items of ALSFRS-R (Speech, Swallowing, Handwriting, Dressing and Hygiene, Walking and Dyspnea). The Japanese version of ALSFRS-R was validated previously for ALS, showing that the assessment values are highly equivalent among well-trained neurologists, general physicians and nurses, and that intra-rater assessment values are also highly equivalent [16]. Intra-rater and inter-rater reliability of each item of the Japanese version of ALSFRS-R were also validated. The presence of oculomotor disturbance was assessed through a bedside neurological examination.

2.2. Data analysis

All variables were summarized using descriptive statistics, including mean, standard deviation (S.D.), and percentages. Correlations

Table 1
Clinical features of patients newly registered from 2003 to 2006 (n=3428)

Age at onset (years, mean±S.D.)	65.4±10.7
Male/female (%)	57.8/42.2
Duration from disease onset to registration (years, mean±S.D.)	1.7±2.2
Symptoms at registration (%)	
Dysarthria	64.2
Dysphagia	57.8
Weakness of neck	70.0
Respiratory distress	34.2
Weakness of upper extremities	86.6
Weakness of lower extremities	76.2
Initial symptoms (%)	
Dysarthria	36.3
Dysphagia	21.1
Weakness of neck	7.1
Respiratory disturbance	6.3
Weakness of upper extremities	48.1
Proximal dominant	26.1
Distal dominant	50.8
Diffuse	23.0
Weakness of lower extremities	34.1
Proximal dominant	19.7
Distal dominant	42.6
Diffuse	37.8

Table 2
Cross-sectional living conditions of patients registered in 2005 (n=4202)

Living condition	Frequency (%)
At work or school	6.7
Household work	6.5
Under home care	58.2[a]
In hospital	27.5[a]
In nursing-care facility	2.4

[a] 1.2% of patients overlap.

between age at onset and duration from disease onset to invasive procedures were analyzed using Pearson's correlation coefficient, and the cumulative incident curves of two age groups were assessed by the log-rank test. Difference of frequencies of symptoms between two age groups was assessed by the chi-square test. p-values <0.05 were considered to be statistically significant. Calculations were performed using the statistical software package SPSS 15.0J for Windows (SPSS Japan Inc., Tokyo Japan).

3. Results

3.1. Clinical features of sporadic ALS patients

The mean age at onset was 65.4±10.7 years, the male to female ratio was 1.37:1, and the mean duration from disease onset to registration was 1.5±1.4 years. The initial symptom was dysarthria in 36.3%, dysphagia in 21.1%, weakness of neck in 7.1%, respiratory disturbance in 6.3%, weakness of the upper extremities in 48.1%, weakness of lower extremities in 34.1%, when allowing overlapping descriptions (Table 1). When we analyzed these demographic clinical features between male and female patient groups, age at onset was slightly higher in the female patients. The proportion of the patients with bulbar symptom onset was higher in the female patients, whereas, the proportion of the patients with weakness of upper extremities was higher in the male patients (Supplemental Table 1).

The cross-sectional state of living conditions of ALS patients in Japan in 2005 is shown in Table 2. The proportion of the patients at work or school was 6.7%, 6.5% engaged in household work, 58.2% under home care, 27.5% in hospital and 2.4% in a nursing-care facility. The state of nutrition and respiratory support is shown in Table 3. The frequency of patients with a gastrostomy tube was 28.7%, and 7.8% were using a nasogastric tube. NIPPV was used by 7.2% of the patients, and 29.3% were under TPPV. The clinical profiles of the patients with TPPV were shown in Table 4. Mean duration from introduction of TPPV was 3.7 years, and 42.2% of the patients with TPPV were living under home care.

3.2. Age at onset influences progression of disease assessed by duration from onset to introduction of TPPV

The mean interval between the onset of disease and the introduction of TPPV was 3.0 years. Intervals from the disease onset to the introduction of TPPV became shorter as the age at onset advanced (Fig. 1A). There was a significant correlation between the

Table 3
Nutritional and respiratory support of patients registered in 2005 (n=4202)

Nutritional and respiratory support	Frequency (%)
Tube feeding	
Gastrostomy tube	28.7
Nasogastric tube	7.8
NIPPV [a]	
Intermittent use	2.0
All-night use	2.6
All-day use	2.6
TPPV [b]	29.3

[a] Non-invasive positive pressure ventilation.
[b] Tracheostomy positive pressure ventilation.

Table 4
Clinical profiles of patients with TPPV (n=2128)

Male/female (%)	59.9/40.1
Age at onset (years, mean±SD)	59.8±11.7
Duration of disease (years, mean±SD)	6.7±5.0
Duration from disease onset to introduction of TPPV	3.0±3.2
Duration from TPPV introduction	3.7±3.5
Living conditions	
Under home care (%)	42.2[a]
In hospital (%)	57.4[a]
In nursing-care facility (%)	2.1

[a] 1.8% of patients overlap.

age at onset and the interval from disease onset to introduction of tube feeding or TPPV, when analyzed using Pearson's correlation coefficient (r=−0.39 p<0.001). Since 65 years was the mean age of onset, we assessed the cumulative frequency of TPPV in subgroups of patients with an age at onset of 65 years or more and less than 65 years, showing that the duration from onset to introduction of TPPV was significantly shorter in patients with an onset age of 65 years or older (p<0.001) (Fig. 1B). The age at onset influences the progression from onset to the advanced stage assessed by the introduction of TPPV.

3.3. Appearance of ophthalmoplegia under TPPV influenced by age at onset

In the patients with long-standing TPPV, rare symptoms such as ophthalmoplegia were frequently observed. Ophthalmoplegia, which is particularly well assessed by bedside examination, was seen in only

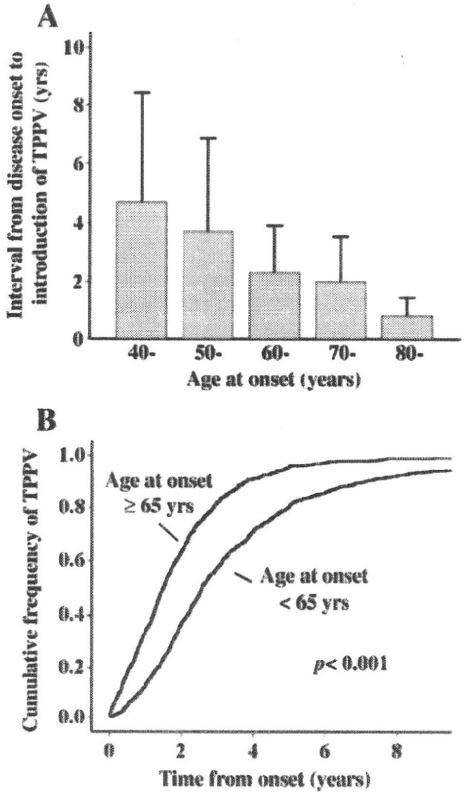

Fig. 1. Relationship between age at onset and introduction of tube feeding and TPPV. Interval from disease onset to introduction of TPPV (A) is shown. An older age at onset strongly correlates to shorter intervals from onset to TPPV. Cumulative frequencies of patients with TPPV in the patient population with an onset age older or younger than 65 years are shown (B). Cumulative curves for patients with an onset age of 65 years or more show significantly shorter intervals between disease onset and introduction of TPPV than those with an onset age of under 65 years of age, suggesting that age at onset markedly influences the time from onset to introduction of TPPV. n=2128.

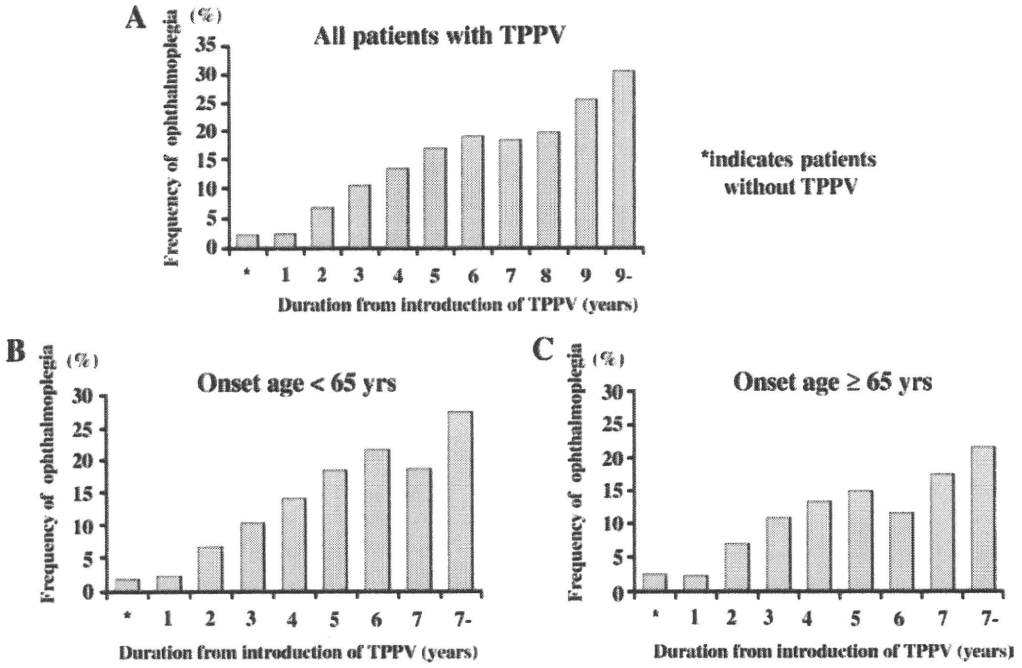

Fig. 2. Frequency of ophthalmoplegia in patients under TPPV, in terms of duration of TPPV and the influence of onset age on its appearance. Ophthalmoplegia rarely occurs in patients without TPPV (*), while its occurrence gradually increases with advanced duration of TPPV (A). Following 9 years of TPPV, almost 30% of patients show ophthalmoplegia. Frequencies of ophthalmoplegia in the patient population with onset age older or younger than 65 years are shown in B and C. Ophthalmoplegia is less frequent in patients with an age at onset of 65 years or older (C). The total frequency of ophthalmoplegia in the patients with onset age older than 65 years or younger than 65 years is 8.3% and 15.1%, respectively. A significant difference exists between them by the chi-square test ($p<0.001$). $n=2128$.

2.0% of the patients without TPPV. The frequency of ophthalmoplegia was increased with the advanced duration of TPPV (Fig. 2A). However, ophthalmoplegia was observed in 30% of patients under TPPV for more than 9 years.

The appearance of ophthalmoplegia under long-standing TPPV is also influenced by the age at onset (Fig. 2B,C). The patients with an age at onset under 65 years showed a higher frequency of appearance of oculomotor symptoms than those with an age at onset over 65 years (Fig. 2B,C). The total frequency of ophthalmoplegia in the patients under TPPV with an onset age of older than 65 years or younger than 65 years was 8.3% and 15.1%, respectively. A significant difference was found between them by the chi-square test ($p<0.001$). These observations suggest that a younger age at onset advances the appearance of ophthalmoplegia compared to patients with an older age at onset. The average time from onset to introduction of TPPV was, however, 1.86± 1.70 years in the patients with an onset age over 65 years, and 3.60± 3.72 years in those with an onset age of younger than 65. This difference influenced the appearance rate of ophthalmoplegia.

3.4. Age at onset influences the frequency of initial symptoms

We analyzed the relationships between the age at onset and the initial symptoms. Dysarthria and dysphagia as the initial symptoms were markedly increased in patients with an advanced age at onset

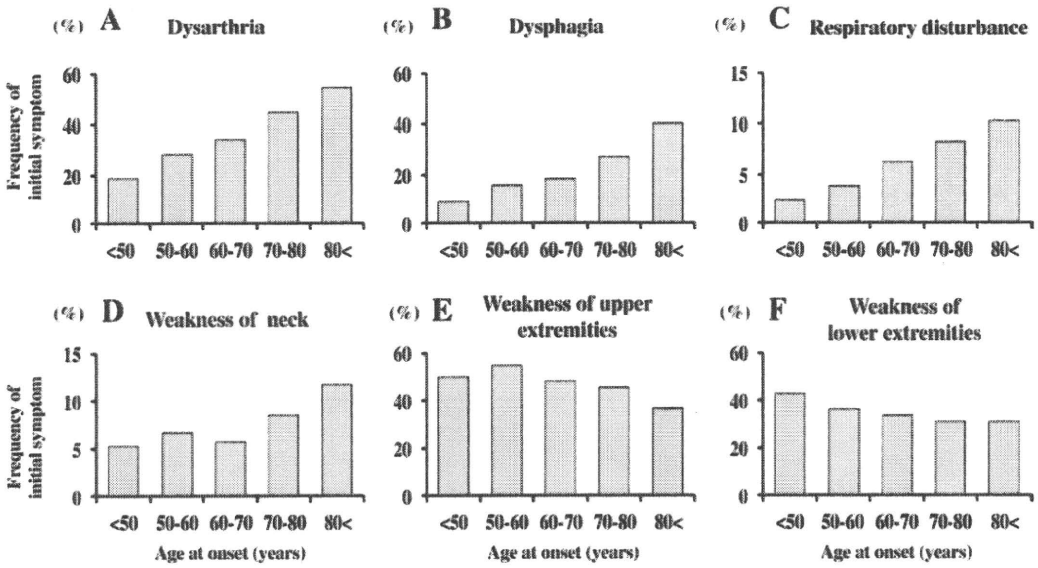

Fig. 3. Age at onset and frequency of initial symptoms. Dysarthria (A), dysphagia (B), respiratory disturbance (C) and weakness of neck (D) are increased in frequency as an initial symptom as the age at onset increases. In contrast, weakness of the upper extremities (E) or lower extremities (F) decreased as the onset age increases. $n=3428$.

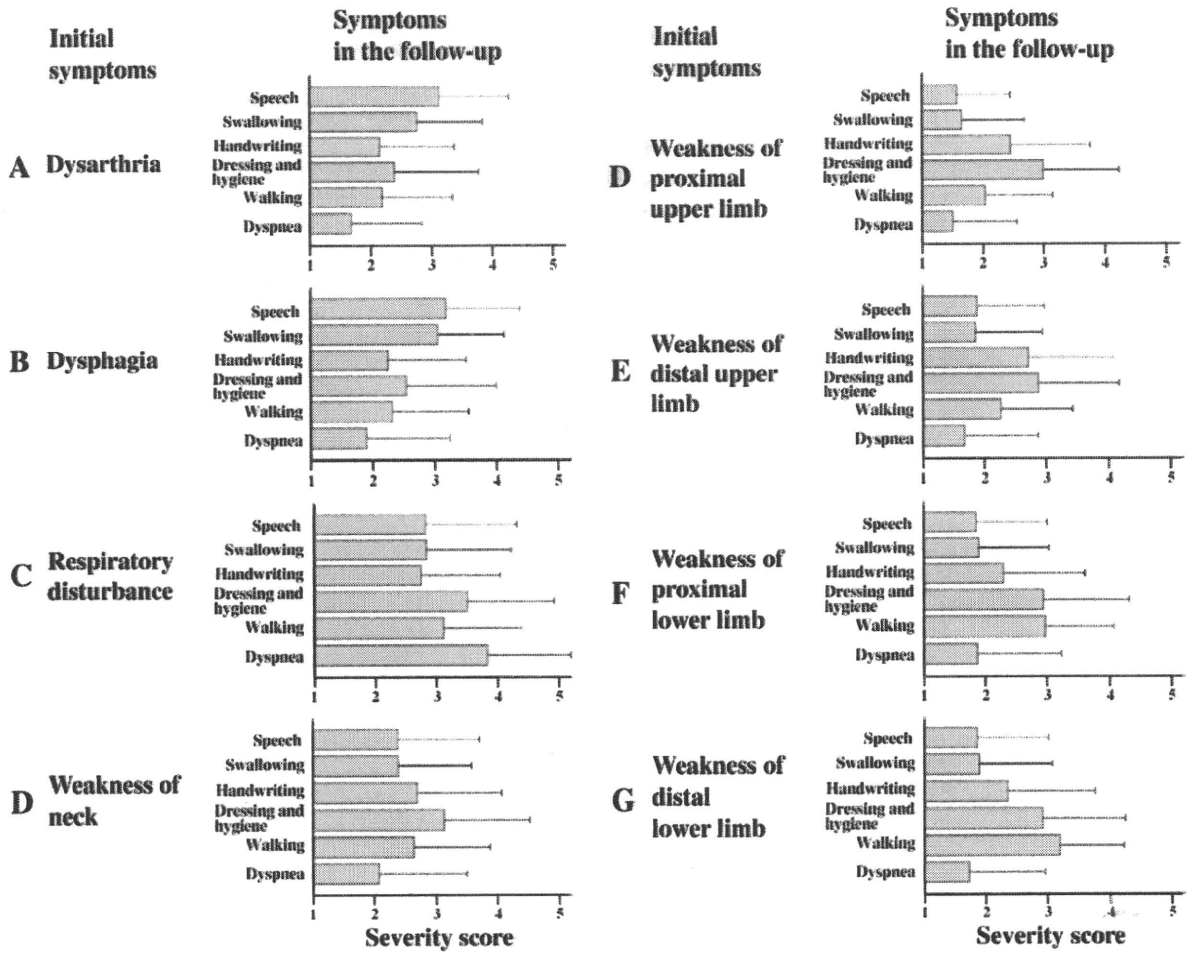

Fig. 4. Relationship between initial symptoms and symptoms at the follow-up stage. Severity scores of Speech, Swallowing, Handwriting, Dressing and Hygiene, Walking and Dyspnea are shown as subscales of ALSFRS-R. The score of "5" represents the most severe state, and "1" represents the absence of the symptom. Initial symptoms remain the most prominent or related symptoms even in the follow-up stage for 1.7±2.2 years from onset, suggesting that initial symptoms significantly determine the prominent features of symptoms throughout the disease course. $n=3428$.

(Fig. 3A,B). On the other hand, weakness in the upper or lower limbs as an initial symptom was seen more frequently in patients with a younger age at onset, and these frequencies gradually decreased with increasing age at onset. As for the respiratory disturbance and dropping head due to weakness of the neck muscles, the frequencies increased gradually with increasing age at onset. When we divided the patients between those with an onset age of older than 65 years and those younger than 65 years and analyzed the data with the chi-square test, the differences in frequencies of dysarthria, dysphagia, respiratory disturbance, weakness of upper extremities and weakness of lower extremities as initial symptoms were also significant between those groups ($p<0.001$, $p<0.001$, $p<0.001$, $p=0.001$, $p=0.019$, respectively). The difference in the frequency of neck weakness was not significant ($p=0.07$), although the tendency was apparent, and may be due to the small number of patients with neck weakness as an initial symptom. These observations suggest that age at onset is a determining factor of the features of the initial symptoms. Correlations between age at onset and the frequency of initial symptoms were similarly observed in the male and female patient groups (Supple. Fig. 1).

3.5. Initial symptoms determine major clinical features in follow-up stage

We examined the relationship between the initial symptoms and the symptoms assessed by 6 items of ALSFRS-R at examination at 1.7± 2.2 years after the onset (Fig. 4). At the follow-up stage, the patients who showed a bulbar symptom as an initial symptom showed speech or swallowing disturbance as a major symptom in the follow-up stage. Patients that showed respiratory disturbance as an initial symptom also showed dyspnea as the most prominent disturbance; patients with weakness of distal upper limb muscles showed the most prominent disturbance in handwriting and dressing; patients with weakness of proximal upper limbs showed prominent disturbance in dressing and hygiene; and patients with weakness of lower limbs, either proximal or distal, all showed a prominent disturbance in walking. These observations strongly suggested that the initial symptoms remained the most prominent or related symptoms even in the follow-up stage, and support the view that the initial symptoms determine the clinical features of the individual patient even in the follow-up stage. A similar tendency was observed in the male and female patient groups (Supple. Fig. 2).

4. Discussion

The results of the present study demonstrate the characteristic clinical profiles of Japanese sporadic ALS patients. A very high rate of Japanese ALS patients (29.3%) were under TPPV compared to patients in North America or Europe [10,11,17,18] which are 2.1–5.4%, respectively. The frequency of patients showing rare symptoms such as ophthalmoplegia increased with disease progression, particularly under long-standing TPPV.

A striking observation in the present study is that the age at onset greatly influences the wide-ranging clinical features, including the initial symptoms, progression to the endstage assessed by introduction of TPPV, and the frequency of rare symptom in the long-standing course. A higher incidence of bulbar involvement in patients with an older age at onset has been reported in some previous studies [19–23]. We extended these observations in that almost all of the initial symptoms, such as dysphagia, dysarthria, upper or lower limb weakness, respiratory failure and head dropping are strongly influenced by the age at onset. This observation was also confirmed in the subpopulation of male and female patients. In addition, since the initial symptoms also determine the prominent clinical phenotypes in the follow-up stage as demonstrated in this study, age at onset may influence not only the initial symptoms, but also the entire clinical phenotypes of sporadic ALS. The underlying mechanism for the onset age influence on the initial manifestation of the symptoms is unknown. Furthermore, we do not know the mechanism by which patients with a younger age at onset tend to show a higher frequency of rare symptoms. Further study is needed to resolve these issues, although one may speculate that subpopulations of the motor neurons may be differentially vulnerable to the aging process. In several sporadic neurodegenerative diseases, age at onset has been suggested to be an influencing factor for the spatial development of neural involvement, and, thus, for the features of clinical manifestations [24]. In Parkinson's disease, for instance, patients with an older age at onset have been suggested to have a tendency to show a higher cognitive dysfunction and autonomic dysfunction [25–27], whereas, those with a younger age at onset have an increased tendency toward dystonia and a diurnal fluctuation of symptoms [28,29]. Taking these observations together with our findings on ALS, age at onset may be a more important factor modifying clinical manifestations in sporadic neurodegenerative diseases than previously thought.

Age at onset also influenced the interval from the onset to the time of introduction of TPPV. Reserved respiratory function is known to decrease with advancing age [19]. Therefore, the short interval between the onset and the introduction of TPPV may be explained by the smaller reserved respiratory capacity in elderly patients. Indeed, serial examinations of the respiratory function in elderly patients start at a lower vital capacity and reach a critical point more quickly than younger patients [19,30]. It is congruent with the fact shown in the previous reports [1,3,5,6,22], that younger ALS patients survive longer than older patients.

Therefore, in taking into account the age at onset, initial symptoms, occurrence of rare symptoms and progression, the age at onset greatly affects the clinical profiles of sporadic ALS patients. In addition, the onset age-related initial symptoms are important to estimate the patient's prognosis as well as the design of clinical trials [31].

A high proportion of ALS patients in Japan are under TPPV compared to patients in other countries, possibly for social, cultural and economic reasons [13,17,18]. The presence of a subgroup of patients extending involvement to other systems beyond motor neurons, such as oculomotor, autonomic, sensory and higher functional systems, has been described in Japanese ALS patients under long-term TPPV treatment [32–36]. Pathologically, these patients show an extensive involvement of the tegmentum of the brainstem, substantia nigra, Clarke's dorsal nuclei and spinocerebellar tract, and frequent involvement of the thalamus and globus pallidus. Our present observations have confirmed these reports on sporadic Japanese ALS patients, particularly those with long-standing TPPV, and demonstrated that these subpopulations with a rare extension of involvements include almost 30% of the patients with 9 years or more under TPPV, particularly those assessed for oculomotor system involvement. However, further studies are needed to determine whether all the patients would eventually show an extended involvement beyond the motor system or whether these patients with an extended form are restricted to a given subpopulation. This is an important issue to determine the natural history of sporadic ALS. Since European and American ALS patients are not generally maintained on TPPV treatment for a longer period as Japanese patients, extended involvement is very rarely observed in Europe or North America.

In summary, we have presented the clinical profiles of sporadic Japanese ALS patients based on a large-scale sample. As demonstrated, age at onset may be a remarkable factor influencing wide-ranging clinical profiles including the progression and prognosis. We should take account of this observation in cohort studies or clinical trials.

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Appendix A

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jns.2008.09.024.

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Temporal trends and geographic clusters of mortality from amyotrophic lateral sclerosis in Japan, 1995–2004

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ABSTRACT

The present study examined temporal trends and geographic clustering of amyotrophic lateral sclerosis (ALS) mortality in Japan, during 1995–2004, using vital statistics based on death certificates. ALS was usually diagnosed by neurologists according to clinical guidelines that complied with the El Escorial Criteria. The underlying cause of death for ALS was coded as G12.2A. Regression analysis was used to examine temporal trends. Spatial scan statistic was used to detect any area of elevated risk as a cluster. A total of 12,173 (6864 male and 5309 female) ALS deaths were reported. Annual crude mortality rate per 100,000 population was 1.07 (1.26 for males and 0.89 for females) in 2004. Although the overall temporal trend was stable, the trend increased in the 70+ years age group (p for trend, <0.001 in males and <0.05 in females), while it declined in the under 70 years age group (p for trend, <0.01 for both sexes). Male preponderance and M/F ratio remained nearly constant over time. Three clusters were detected: two ($p<0.005$ in males and $p<0.05$ in females) in northeast and one ($p<0.05$ in males) in west-central Japan. Further research is needed to clarify contributing factors for the observed trends and clusters in ALS mortality.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is still a fatal neurodegenerative disease characterized by the selective loss of upper and lower motor neurons. Five to 10% of cases of ALS are familial; the others are believed to be sporadic [1]. While advances have been made in identifying disease-causing genes for familial ALS, very little is known about susceptibility genes or other risk factors for sporadic ALS. Many putative environmental risk factors (i.e., heavy metals, solvents, electrical and electromagnetic fields, poliovirus, mechanical trauma, heavy physical activity, cigarette smoking, and diet) have been previously reported; however, age and a family history are the only established risk factors for ALS [1–4].

Variation in mortality over time and by geographic location, sex and ethnicity can often be a source of etiological clues [5]. The mortality rate from motor neuron disease (MND), of which ALS accounts for 85% or more [1,5], was reported to have steadily increased from the 1950s to the 1990s in western countries [3,5–10]. In some European countries and the United States, a greater increase in ALS mortality was observed in females than in males in the past 30 years, causing a decrease in the male to female (M/F) ratio [5,8–10].

Contrary to the trends noted in many other countries, Japan has shown an unusual pattern of mortality from MND for decades. The age-adjusted MND mortality rate rose from the mid 1950s, peaking in the early 1960s, and declined in the early 1970s [11,12]. Thereafter, the rate slowly increased to that in the early 1950s for a period of 20 years between 1970 and 1990 [11]. A recent study reported that it decreased from 1995 through 2001, and the M/F ratio slightly increased [13].

The Western Pacific form of ALS, referred to as ALS and parkinsonism-dementia complex (ALS/PDC), was identified in the 1950s in three distinct geographic isolates: Guam, western New Guinea and the Kii Peninsula of Japan [14,15]. Over the past four decades, the incidence of ALS/PDC has markedly declined in Guam [14]. On the other hand, a continuing high prevalence and incidence in Hohara in Mie prefecture [16] and Kozagawa in Wakayama prefecture [17] in the Kii peninsula are reported, although they temporarily declined in the 1980s.

In this study we examined the national ALS mortality data of Japan to reveal the recent temporal trends in ALS mortality and investigated whether or not any geographic clusters of ALS deaths exist in particular regions.

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2. Methods

The World Federation of Neurology Research Group on Neuromuscular Diseases published the El Escorial Criteria (EEC) in 1994 [18]. Based on these criteria, the research committee on ALS of Japan has updated the guidelines for diagnosis and treatments of ALS, and recommended it for clinicians and researchers [19]. ALS was usually diagnosed by neurologists using guidelines that complied with the El Escorial Criteria.

In 1995, the Statistics and Information Department of the Ministry of Health, Labour and Welfare, Japan, changed the coding for disease classification from that in the ninth version of International Classification of Diseases (ICD-9) to that in the tenth version (ICD-10). ICD-9 and ICD-10 designated MND with the respective four-digit codes of 3352 and G12.2. For the vital statistics of Japan after the year 1995, they added the capital letters A and B to G12.2 to differentiate ALS from other MND: G12.2A for ALS and G12.2B for primary lateral sclerosis, progressive bulbar palsy, spinal muscular atrophy, and other or unspecified MND. They followed the international rules for mortality statistics based on the underlying cause of death. The underlying cause of death was defined by the World Health Organization as the disease or injury that initiated the train of morbid events directly leading to death or the circumstances of the accident or violence that produced the fatal injury.

The national ALS mortality data from 1995 through 2004 were used for our present study. The data used were taken from the national mortality database of vital statistics based on death certificates, after obtaining permission for use from the Statistics and Information Department. The variables in the data file included the codes of the underlying cause of death for ALS along with age at death, sex, and place of residence where the deceased had lived. It did not contain any personal identifiable information (e.g., individuals' names or residential addresses).

The total number of deaths due to ALS from 1995 through 2004 was counted, and age-specific mortality rates were calculated according to 5-year age intervals. Annual crude mortality rates in 1995–2004 were calculated as the number of ALS deaths per million persons per year on the basis of the Japanese population for the respective year.

To examine temporal trends in deaths from ALS, annual age-adjusted mortality rates were calculated by the direct method using the total population of the 2005 census as a standard population. Linear regression analysis was used to examine temporal trends in mortality as well as M/F ratios in individual years as a continuous variable. Joinpoint regression analysis was used to provide annual percentage changes (APC) with 95% confidence intervals and p values for trends [20,21]. It was also performed in the groups of age at death, <70 and 70+ years. Statistical significance was determined as a p value for trend less than 0.05.

A cluster is defined as a geographically bounded group of occurrences of sufficient size and concentration to be unlikely to have occurred by chance without any assumptions about the shape or form of the cluster [22]. To detect clusters, the flexible spatial scan statistic [23–26] was used in our present study. It can detect clusters of any size and form located anywhere in the study region, whether or not they cross administrative borders. The most likely cluster can be detected as that with the maximum likelihood. P values were obtained using Monte Carlo hypothesis testing, comparing the test statistic from the observed data set with the test statistics from 999 random data sets generated under the null hypothesis of no clustering. Statistical significance was determined as a p value less than 0.05.

The flexible spatial scan statistic can be applied to geographically aggregated data. So, to eliminate the effects of age, we employed this statistic using the number of observed and expected deaths from ALS based on the vital statistics for each of the secondary medical care zones (SMCZ). At the time of this study, there were 359 SMCZs for medical care planning, each of which consisted of neighboring municipalities in all 47 prefectures of Japan, according to the Medical Service Law.

3. Results

A total of 12,173 (6864 male and 5309 female) ALS deaths were reported in Japan in the period between 1995 and 2004. Fig. 1 shows mortality rates due to ALS by 5-year age groups. The age-specific mortality rates rose steadily with age group up to the age of 75–79 years and then sharply declined for those aged 80 years and older.

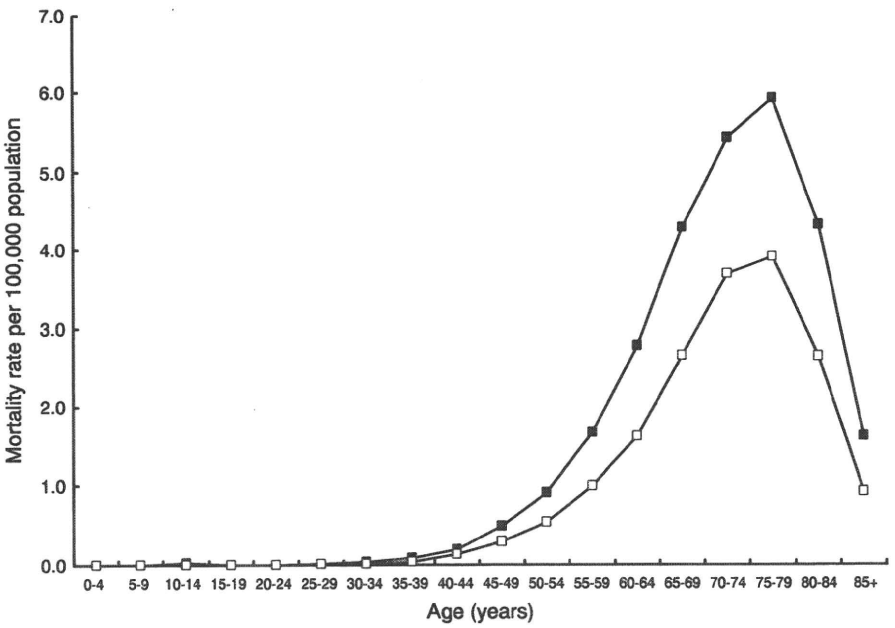

Fig. 1. Age-specific mortality rates from amyotrophic lateral sclerosis (ALS) by sex, Japan, 1995–2004 (■ = males and □ = females).

Table 1
Annual mortality rate per 100,000 population from amyotrophic lateral sclerosis (ALS), Japan, 1995–2004.

Year	Number of deaths			Crude mortality rate			Age-adjusted mortality rate[a]		
	Total	Male	Female	Total	Male	Female	Male	Female	M/F ratio
1995	1031	579	452	0.82	0.94	0.71	1.27	0.81	1.56
1996	1166	658	508	0.93	1.07	0.79	1.43	0.90	1.59
1997	1144	634	510	0.91	1.03	0.79	1.34	0.88	1.52
1998	1179	666	513	0.93	1.08	0.79	1.38	0.86	1.60
1999	1181	673	508	0.93	1.09	0.78	1.36	0.83	1.64
2000	1202	666	536	0.95	1.07	0.83	1.33	0.86	1.55
2001	1225	692	533	0.96	1.11	0.82	1.34	0.85	1.57
2002	1337	761	576	1.05	1.22	0.88	1.43	0.88	1.64
2003	1338	749	589	1.05	1.20	0.90	1.38	0.87	1.58
2004	1370	786	584	1.07	1.26	0.89	1.41	0.85	1.67

[a] Direct age-adjusted to the year 2005 census population. *P* values for trends are 0.200 for males, 0.899 for females and 0.122 for M/F ratios.

Annual crude mortality rates per 100,000 population increased from 0.94 to 1.26 for males and from 0.71 to 0.89 for females. After adjustment by age, ALS mortality rates have been stable during this period in both males and females. Mortality rates were higher for males than for females over the entire period. The M/F ratios remained almost constant (Table 1).

After stratification by age, age-adjusted mortality rates showed completely different temporal trends in the age groups of <70 years and 70+ years (Figs. 2 and 3). Age-adjusted mortality rates have significantly increased in the older age group (p for trend, <0.001 in males and <0.05 in females), while they have significantly decreased in the younger age group (p for trend, <0.01 for both sexes).

The most likely, second, and third clusters are shown in Table 2, and each cluster is located on maps in Figs. 4 and 5. For males, two statistically significant clusters were identified, but both clusters were detected in different regions from the Kii Peninsula. In a part of the Kii Peninsula including Hohara and Kozagawa villages, which have been well known hyperendemic ALS foci for many years, a third cluster containing 236 observed deaths was detected, but it was not statistically significant. For females, one statistically significant cluster was identified. It was adjacent to the most likely cluster found in males, and some areas of these two clusters overlapped.

4. Discussion

Previous studies have reported a rise and fall in mortality from MND or ALS in Japan over the past five decades [11–13]. Our present study demonstrated that the recent temporal trend in age-adjusted mortality rate due to ALS was stable during the 10-year period between 1995 and 2004, since the introduction of ECC and ICD-10. When stratified by age into <70 and 70+ years at death, we found opposite directions in age-adjusted mortality rates for the two groups: a clear upward trend in the older age group and a downward trend in the younger age group. This pattern is unique compared to those reported from previous studies [2,3,5,6,8–13]. In the United States, an increasing trend is seen for all ages except age 45 to 54 years [5]. In the United Kingdom and Norway, the trend is upward in the 60+ or 65+ years age group but it is static in the under 60 years age group [8,10].

The increased ALS mortality in the older age group is common to all studies. It is noteworthy that in our present study there was a decreasing trend in the under 70 years age group. One of the hypotheses explaining this phenomenon is that age at onset may be chronologically delayed with time. According to a 10-year prospective population-based study conducted in Italy, mean onset age (SD) has become slightly higher: 64.2 (11.2) years in 1995–1999 and 65.4 (11.1) years in 2000–2004 [27]. Although the data are cross-sectional in Japan, there is a substantial difference in age at onset between 61.8 (12.2) years in 1989–1999 [17] and 65.4 (10.7) years in 2003–2006 [28]. As another hypothetical explanation, it may be that survival duration has been prolonged by the improvement of treatment such as pharmacotherapy (e.g., rilusole) combined with nutritional and respiratory support [1]. Remarkable progress in home-visit nursing care has been made in Japan, with the universal availability of

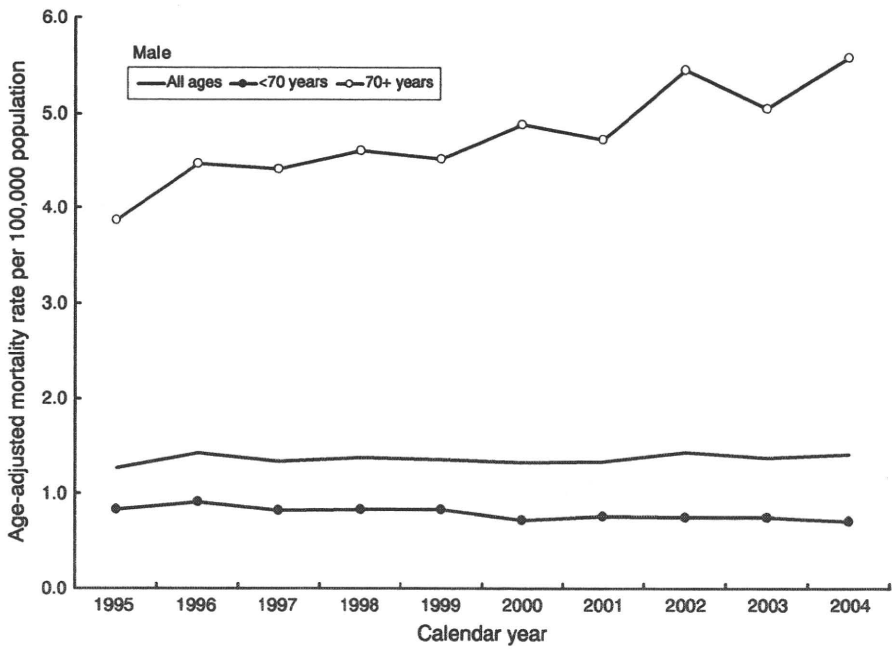

Fig. 2. Age-adjusted mortality rates from amyotrophic lateral sclerosis (ALS) in males, Japan, 1995–2004. The annual percentage changes (95% confidence intervals) were 0.5(−0.3, 1.4), −2.1(−3.2, −1.0) and +3.3(2.1, 4.5) for all age groups, age group of <70 years and age group of 70+ years, respectively.

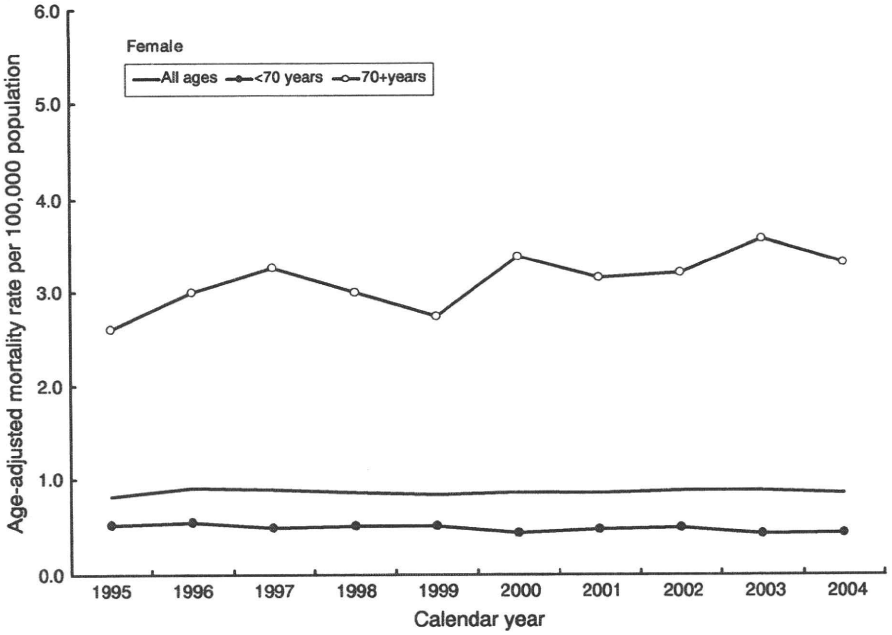

Fig. 3. Age-adjusted mortality rates from amyotrophic lateral sclerosis (ALS) in females, Japan, 1995–2004. The annual percentage changes (95% confidence intervals) were 0.0 (−0.7, 0.8), −2.3(−3.6, −0.9) and +2.3(0.3, 4.1) for all age groups, age group of <70 years and age group of 70+ years, respectively.

mechanical ventilatory support for ALS patients. A very high proportion of Japanese ALS patients (29.3%) received tracheotomy positive pressure ventilation (TPPV) compared to those in North America and Europe (2.1–5.4%) [28–33]. Based on the large-scale registered data on patients with ALS in Japan, about 40% of those under TPPV received care at home and almost 30% survived 9 years or more after the introduction of TPPV [28]. In our clinical experience, younger patients and their families are more likely to choose TPPV than older ones (unpublished). As a result, younger patients could survive longer. This may lead to prolongation of the age at death, particularly for younger patients, which may cause mortality to decrease in younger patients and consequently increase in older patients. To determine the duration of survival, both age at symptom-onset and age at death are required, but our present study lacked the former. Further research is needed to justify the aforementioned hypotheses.

Regarding the M/F ratio of ALS mortality over time, we found male preponderance that remained stable in our present study. This finding is inconsistent with the reported narrowing of the male to female gap in mortality [4,8–10] and incidence [2–4,34]. As some authors pointed out, the reduced M/F ratio may be partly caused by the changing lifestyle (e.g., smoking) of women, which has become more similar to that of men, in western countries [3,4,34]. According to OECD health data, for example, the prevalence of smoking has become similar for both sexes in western countries, but it is still markedly different between sexes in Japan (58.8% for males and 15.2% for females in 1995; 46.9% for males and 13.2% for females in 2004) [35]. In addition, we have to take into account gender difference in socioeconomic status that might affect patients' choice of treatment and long-term care. But we could not examine this factor because of the limited data in our current study. Further investigation is required to verify potential factors explaining the M/F ratio of ALS mortality over time.

The present study identified three statistically significant clusters of ALS mortality. Two (A1 in Fig. 4 and B1 in Fig. 5), which partially overlapped, were located in the northeastern part of the mainland of Japan. As shown in Fig. 4, one more cluster (A2) was geographically separated from the other (A1), at a short distance from the Kii Peninsula. In the Kii Peninsula, a cluster (A3) was observed but it did not reach statistical significance at the SMCZ level. Recent epidemiological surveys reported a continuing high incidence and prevalence in the subfoci of Hohara and Kozagawa villages of the Kii Peninsula [16,17,36]. Our cluster analysis may not be able to detect clustering in the two villages, which are much smaller units of area than SMCZ. These four clusters (A1–A3 and B1) are promising candidate areas for further analytical studies on ALS.

The limitations of our present study should be taken into account. One is case ascertainment for ALS based on the underlying cause of

Table 2
Geographic clusters of ALS mortality detected using flexible spatial statistics, Japan, 1995–2004.

Cluster	Approximate cluster location[a]	Number of SMCZs[b]	Expected cases	Observed cases	Relative risk	*p* value
Males (N = 6864)						
Most likely cluster	A1: a part of Niigata–Gunma–Nagano–Fukushima	12	115.70	181	1.56	0
Second cluster	A2: a part of Hyogo–Kyoto–Osaka	7	182.16	254	1.39	0.03
Third cluster	A3: a part of Kii Peninsula (Wakayama–Mie–Nara–Osaka)	11	178.34	236	1.32	0.36
Females (N = 5309)						
Most likely cluster	B1: a part of Gunma–Tochigi–Saitama	12	118.67	178	1.50	0.02
Second cluster	B2: a part of Hokkaido Island	11	172.54	231	1.34	0.26
Third cluster	B3: a part of Aichi–Gifu–Shizuoka–Shiga	12	276.71	384	1.26	0.3

[a] The clusters of A1–A3 and B1–B3 correspond to those on maps in Figs. 4 and 5, respectively.
[b] Abbreviated secondary medical care zones.

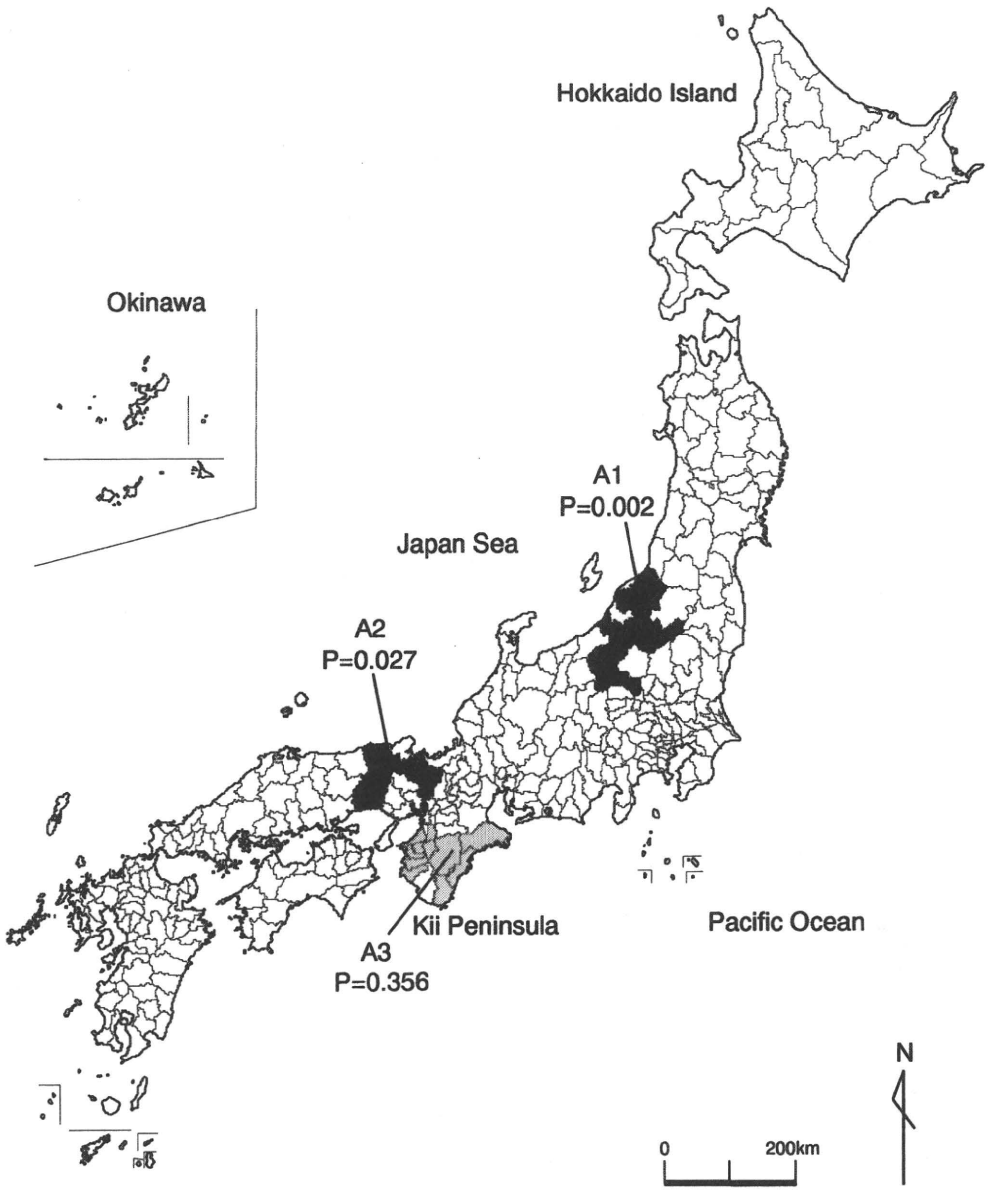

Fig. 4. Geographic clusters of age-adjusted deaths from amyotrophic lateral sclerosis (ALS) for males, Japan, 1995–2004. A1, A2 and A3 are the most likely, second and third geographic clusters, respectively (black (A1 and A2) and gray (A3) clusters with and without statistical significance, respectively).

death recorded on death certificates. This is not so serious, however, considering the following points: (1) ALS was usually diagnosed by neurologists, (2) the number of neurologists was large (e.g., 8555 as of March 31, 2009), and (3) neurologists have generally followed the clinical guidelines based on ECC since the recommendation of its use by the research committee on ALS of Japan [18,19]. The other is regional disparity affecting geographic clustering (e.g., number of neurologists and access to specialized medical care). Taking the following points together, it seems that the degree of regional disparity is small. The average number of neurology clinics/departments was 4.75 per 100,000 population [37]. The areas with higher or lower concentrations of neurology clinics/departments were not consistent with the locations in which the clusters of ALS mortality were detected in our present study. Basically, all ALS patients have been guaranteed free medical access by the provision of financial aid from universal medical insurance since 1961, countermeasures against intractable diseases including ALS since 1972, and nursing-care insurance since 2000.

In conclusion, we have provided new evidence of ALS mortality in Japan during the 10-year period between 1995 and 2004: 1) The overall temporal trend in age-adjusted ALS mortality is stable. 2) The trend is going up in the 70+ years age group while it is going down in the under 70 years age group. 3) Male preponderance and M/F ratio remain nearly constant. 4) Some geographic clusters are detected. Current thinking on complex diseases like ALS is that multiple genetic and environmental factors contribute to disease liability [38]. Further research is needed to clarify contributing factors for the observed trends and clusters in ALS mortality.

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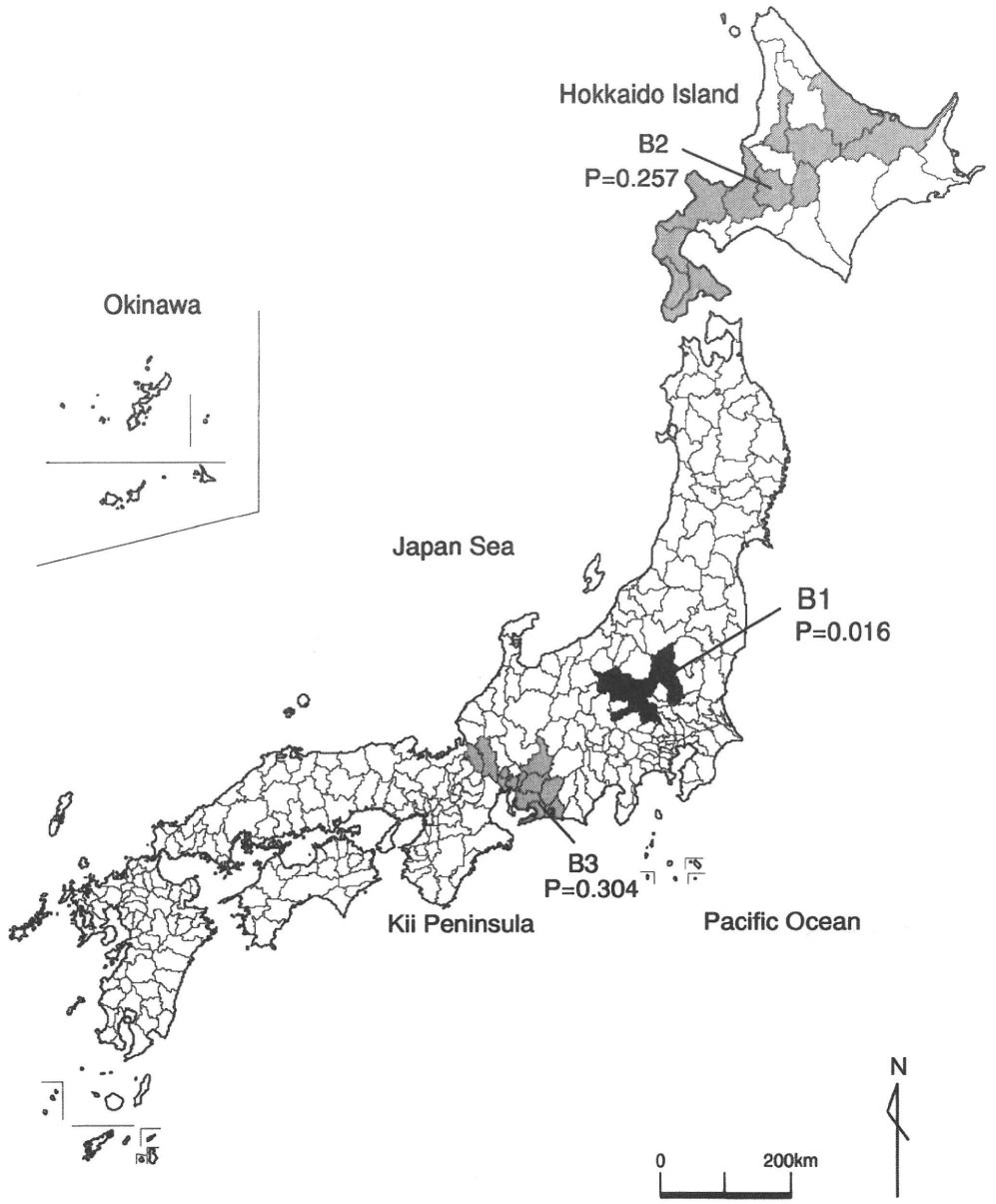

Fig. 5. Geographic clusters of age-adjusted deaths from amyotrophic lateral sclerosis (ALS) for females, Japan, 1995–2004. B1, B2 and B3 are the most likely, second and third geographic clusters, respectively (black (B1) and gray (B2 and B3) clusters with and without statistical significance, respectively).

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