symptoms were confirmed by the family or other observers, whenever possible. All patients were screened for mutations in SOD1, TARDBP, and ANG, and none of these mutations was detected in any of the patients. Thus all patients were ones with sALS. Written informed consent was obtained from each of the patients prior to his/her participation in this study. The study was conducted with the approval of the ethics committees at the participating institutions.

2.2. SNP analysis

All of the subjects were unrelated Japanese individuals. Using standard protocols, genomic DNA was extracted from the peripheral blood leukocytes. We used a primer pair containing the new SNP (rs2275294). Each PCR was performed with 5 ng of genomic DNA derived from ALS subjects, and the amplification was performed in the GeneAmp PCR system 9700 (PE Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 60–65 °C for 30 s, extension at 72 °C for 2 min, and post-extension at 72 °C for 7 min. The PCR products served as templates for direct sequencing by the fluorescent dye-terminator cycle sequencing method [1]. Then, we examined the risk (C) allele of the new SNP (rs2275294). The genotypes with the risk allele are CC homozygous and CT heterozygous, and one without the risk allele is TT homozygous.

2.3. Retrospective observation

We conducted a retrospective review and evaluated both the outcomes and clinical manifestations of these patients. Survival time, which was an endpoint in this study, was defined as the number of months from symptom onset until death from any cause or of tracheostomy for institution of permanent mechanical ventilation, and incidence of non-invasive ventilation. The patient characteristics entered for the univariate analyses were the age at onset, sex, site (bulbar/spinal) of symptom onset, time from onset to diagnosis and riluzole use. In regard to riluzole use, we defined riluzole use as continuous treatment with 100 mg drug daily. The longest retrospective observation period of time for ALS patients was 72 months.

2.4. Statistical analysis

For general statistical analyses, we used the SPSS v.11.0.1 program. Chi-square test for independent testing was applied to a two-by-two contingency table with sex, site (bulbar/spinal) of symptom onset, and history/no history of riluzole use. Student's t-test was applied to the mean age at onset and the mean survival time. Mann–Whitney's U-test was applied to the mean time from onset to diagnosis. The effects of prognostic factors on the survival were assessed using the Kaplan–Meier life-table method for all 176 patients according to the rs2275294 C allele carrier status. The log-rank test was used to assess the equality of the outcome functions. Multivariable analysis was performed with the Cox proportional hazard model. The following variables were included in the model: the risk allele (C allele; with vs. without), sex (male vs. female), the age at onset (included as continuous variable), the site of onset (bulbar vs. spinal), riluzole (use vs. no), and the time from onset to diagnosis (included as continuous variable). All tests were two-tailed and significance was set at $P<0.05$.

3. Results

The risk allele (C allele; CC and CT genotypes) of the new SNP (rs2275294) in the ZNF512B gene was detected in 128 (72.7%) of the 176 patients with ALS. The mean age at onset of ALS was 62.7 (± 11.1) years in patients without the risk allele and 63.8 (± 10.7) years in those with the risk allele, the difference not being significant (t-test, $P=0.56$, Table 1). The site (bulbar/spinal) of onset was 19/29

in patients without the risk allele and 43/85 in those with the risk allele, the difference not being significant (chi-square test, $P=0.46$, Table 1). The mean time from onset to diagnosis was also not significantly different between the two groups (Mann–Whitney's U-test, $P=0.32$, Table 1). Furthermore, no influence of the use of riluzole was found in this study (chi-square test, $P=0.98$, Table 1).

We could confirm the endpoints of 72 patients among the analyzed 176 patients without censure. The mean survival time was 36.6 (± 19.7) months in patients without the risk allele and 24.3 (± 13.8) months in those with the risk allele, so the mean survival time with the risk allele was shorter than the one without the risk allele, the difference being significant (t-test, $P<0.01$, Fig. 1). Moreover in regard to the prognosis after onset, the Kaplan–Meier survival curves for patients with versus without the risk allele according to the rs2275294 C allele carrier status revealed a significantly lower survival probability in those with the risk allele (log-rank test, $P<0.01$, Fig. 2).

In the multivariate analysis accounting for all investigated variables, significant effects for the risk allele ($P=0.043$), the age of onset ($P=0.002$), the site of onset ($P=0.049$) and the time from onset to diagnosis ($P<0.001$) were found. The genotype with the risk allele (C allele; CC and CT) was an independent prognostic factor (hazard ratio, 1.807; 95% confidence interval, 1.018–3.209). The values for sex and riluzole were not significant (Table 2).

4. Discussion

The results suggest that ALS patients with the risk (C) allele of the new SNP (rs2275294) might have shorter survival compared with ALS patients without the risk allele. In the survival time and the Kaplan–Meier analysis, the patients with the risk allele had a markedly shorter survival by 72 months. The original study of Iida et al. [1] included 1305 ALS patients while there were only 176 ALS patients in our study. This might have influenced the results. Moreover in the study of Iida et al. [1] the risk (C) allele frequency is approximately 50%, while it is almost 75% in our study. The greatest reason for this difference of the two studies is the restriction of definite ALS patients in our study, excluding probable ALS patients. Thus we considered that the sample size of our study was smaller than the original study, but this had reliability as well as originality.

Previous studies have reported a number of prognostic factors in ALS patients; the most consistent are the age at onset, site of symptom onset and longer time from the first symptom to diagnosis [10–13]. In addition, some investigators have reported male gender as a favorable prognostic factor, although the incidence between men and women is about the same in familial disease [13,14] and recently Byrne et al. reported that patients with ALS and the C9orf72 repeat expansion seem to present reduced survival [15]. Although in our study the C9orf72 repeat expansion was not analyzed, the frequency of the C9orf72 repeat expansion among Japanese patients is

Table 1
Baseline characteristics of ALS patients.

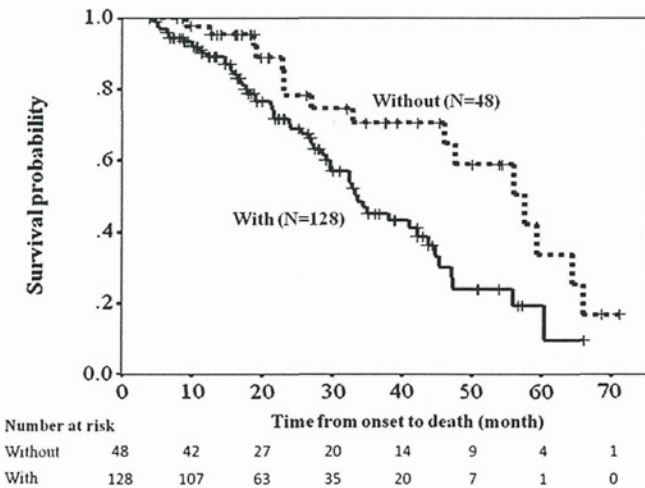| Characteristic	Without the risk allele (N = 48)	With the risk allele (N = 128)	P value
Sex (male/female)	30/18	67/61	0.23 (Chi-square test)
The mean age at onset (SD) (year)	62.7 (± 11.1)	63.8 (± 10.7)	0.56 (Student's t-test)
The site of onset (bulbar/ spinal)	19/29	43/85	0.46 (Chi-square test)
Riluzole ($\pm$)	25/23	67/61	0.98 (Chi-square test)
The mean time from onset to diagnosis (SD) (month)	16.8 (± 14.2)	13.1 (± 9.3)	0.32 (Mann–Whitney's U-test)

Bulbar/spinal = bulbar/spinal-onset ALS, SD = standard deviation.
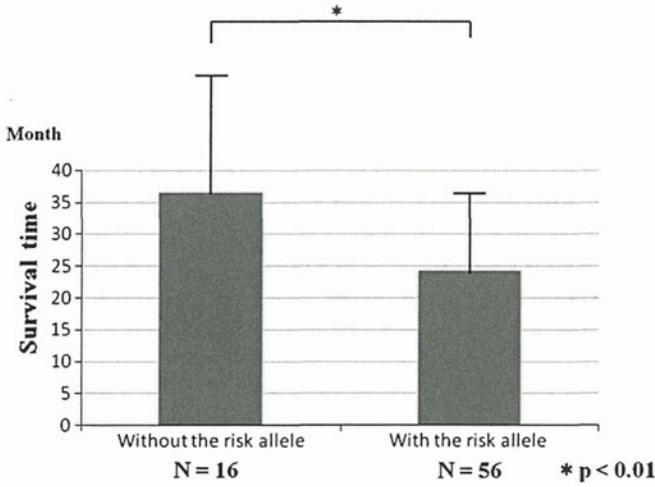
Fig. 1. Survival time for ALS patients: We could confirm the endpoints of 72 patients among the 176 analyzed patients. The mean survival time was 36.6 (±19.7) months in patients (N=16) without the risk allele and 24.3 (±13.8) months in those (N=56) with the risk allele (C allele; CC and CT genotypes). The mean survival time with the risk allele was shorter than the one without the risk allele, the difference being significant (t-test, $P<0.01$).

much lower than in Western populations [16]. Thus we considered that the *C9orf72* repeat expansion had not influenced our study and knowledge of the pattern of disease progression and survival time is of utmost importance for the clinical care of ALS patients and appropriately directed research on ALS. Evaluation of early prognostic variables and a proper understanding of "at first exam" factors related to survival may influence the selection of patient cohorts for clinical trials and identification of practical indicators that clinicians might find useful in the management of ALS patients. In this retrospective study of patients diagnosed as having ALS, we found that the ZNF512B gene was a prognostic factor influencing survival, independent of the sex, age at onset, time from onset to diagnosis, site (bulbar/spinal) of symptom onset and riluzole use [17]. This study revealed that the survival probability of patients with the risk allele (rs2275294, C allele) in the ZNF512B gene was significantly lower than that in those without the risk (C) allele.

The attempts to establish the genetic basis of survival for sALS by identifying susceptibility genes have had little success [18,19]. To date results from candidate gene studies have identified several

Table 2
Cox regression analysis.

Characteristic	Hazard ratio	95% CI	P value
The risk allele (C allele; CC/CT)	1.807	(1.018–3.209)	0.043[a]
Sex (male)	0.673	(0.403–1.125)	0.131
The age at onset	1.004	(1.016–1.072)	0.002[a]
The site of onset (bulbar)	1.716	(1.001–2.942)	0.049[a]
Riluzole (no)	1.333	(0.805–2.209)	0.264
The time from onset to diagnosis	0.960	(0.939–0.982)	<0.001[a]

CI is confidence interval.
[a] Significant covariates.

susceptibility genes [20], although the mechanism by which risk is conferred is not known [13]. But recently Chiò et al. reported that the SNP (rs12608932) located in the intron of the UNC13A gene associated with susceptibility to ALS significantly influenced survival in Italian ALS patients [21]; the mechanism of the effect of the UNC13A gene on ALS survival is still unclear. On the other hand we could indicate the mechanism of the ZNF512B gene on ALS survival as follows.

To date, studies on the ZNF512B gene and ALS have been based mainly on in vitro analysis results of studies conducted by Iida et al. [1]. However, our study indicated that the new SNP influenced the phenotype of ALS patients. Proteomics analysis has suggested that the protein encoded by the ZNF512B gene is a transcription factor that promotes the expression of a downstream gene in the signal transduction pathway of TGF-β [5], which is essential for the protection and survival of neurons [6–8], and several studies have reported elevated serum and plasma levels of TGF-β in ALS patients [22,23]. However, ZNF512B gene expression is reduced in ALS patients with the risk (C) allele of the new SNP, and their serum and plasma levels of TGF-β decrease, resulting in the weakening of their neuronal protection signals. Thus the survival probability of ALS patients with the risk (C) allele decreases (Fig. 3). On the basis of this hypothesis, development of therapies based on the neuroprotective ability of TGF-β has been expected. Day et al. conducted a study in which they administered intraperitoneal injections of TGF-β to a mouse model of ALS that carried a disease-related SOD1 mutation [24]; they demonstrated that the motor performance of the mouse model of ALS improved. Thus, their study revealed that systemic treatment with TGF-β may protect motor neurons from toxic protein insults in the short term, although the long-term effects of TGF-β on ALS have yet to be determined. The disruption of TGF-β signaling is an important molecular event in the pathogenesis of motor neuron diseases [25] and Nakamura et al. reported that activation of the TGF-β

Fig. 2. Kaplan–Meier survival curves for ALS patients: Kaplan–Meier survival curves for ALS patients (N=48) without (broken line) versus those (N=128) with (solid line) the risk allele (C allele; CC and CT genotypes) for all 176 patients according to the rs2275294 C allele carrier status. Vertical bars indicate censored data. A significant difference was found between the two survival curves (log-rank test, $P<0.01$).
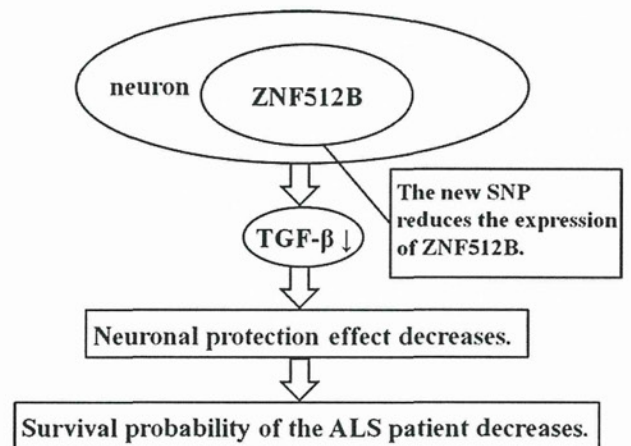

Fig. 3. The mechanism by which the ZNF512B gene might serve as a prognostic factor in ALS patients: In patients with the new SNP (rs2275294), the ZNF512B gene expression is reduced, which results in the weakening of the neuronal protection signals by TGF-β and, therefore, reduction in the survival probability of the ALS patients with the risk allele (C allele; CC and CT genotypes).

signaling system is protective against aggregate formation of cyto-plasmically mislocalized TDP-43 and may be a potential therapeutic approach to delay the progression of ALS [26]. Our study might also indicate the beneficial effect of TGF-β for the protection and survival of neurons in actual ALS patients. Taking these reports together with our data, it may be reasonable to suggest that the ZNF512B gene may serve as a new clinical prognostic factor in ALS patients. Thus, it is very important to examine the ZNF512B gene in ALS patients for prognosis prediction and appropriate treatment selection.

In conclusion, an association between the SNP rs2275294 within the ZNF512B gene and survival in ALS has been confirmed. Our retrospective study revealed that the genotype of the ZNF512B gene is a very useful new prognostic factor in patients with ALS. This study is the first, as per our knowledge, to indicate that the association between the new susceptibility gene for ALS and its pathway could be identified and the findings may open the path to new insights concerning therapy for this devastating disease.

Conflict of interest

The authors have no conflict of interests to declare.

Acknowledgements

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Novel deletion mutations of *OPTN* in amyotrophic lateral sclerosis in Japanese

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by selective motor neuron death in the brain and spinal cord. Many disease genes for ALS have been identified; however, each disease gene is responsible for very small fractions of ALS. Recently, mutations of the gene encoding optineurin (*OPTN*) are reported in familial and sporadic ALS. *OPTN* is also responsible for a small number of ALS, 3.8% of familial and 0.29% of sporadic ALS in Japanese. The low prevalence may be an underestimation due to incomplete screening of the mutation. To examine *OPTN* mutations more extensively, we screened the *OPTN* deletions using a quantitative PCR system. We examined 710 Japanese ALS subjects who had previously been found to have no *OPTN* mutations by a screening using a PCR-direct sequence strategy. We identified 3 kinds of deletions in 5 patients; one was homozygous, and the remaining were heterozygous. All deletions occurred due to the Alu-mediated recombination and are expected to result in null alleles. Our results suggest that the *OPTN* deletion mutation in ALS is not infrequent and the prevalence of the *OPTN* mutation in Japanese sporadic ALS is considerably high.
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Keywords: Amyotrophic lateral sclerosis; Optineurin; Deletion; Alu-mediated recombination

1. Introduction

Optineurin is a 577 amino acids multifunctional protein involved in NF-κB regulation, vesicular trafficking, immune response and transcription regulation (Chalasani et al., 2009). Optineurin is encoded by *OPTN*, whose mutations cause primary open-angle glaucoma (POAG); 3 disease-causing mutations (E50K, 691_692insAG, and R545Q) and one nonsynonymous substitution (M98K) have been described (Rezaie et al., 2002).

Recently, Maruyama et al. (2010) preformed a homozygosity mapping in consanguineous families with amyotrophic lateral sclerosis (ALS), and identified *OPTN* as a causative gene for ALS. They examined a total of 689 Japanese ALS subjects (92 familial ALS (fALS), and 597 sporadic ALS (sALS)), and found 3 types of *OPTN* mutations, a homozygous deletion of an exon; a homozygous nonsense mutation (p.Q398X) and a heterozygous missense mutation. We also examined *OPTN* mutations in Japanese ALS and found 2 kinds of missense mutations in one fALS and 2 sALS patients (Iida et al., 2012). Taken together, the results of the 2 reports show that the *OPTN* mutation is found in 3.8% of fALS and 0.29% of sALS (Iida et al.,
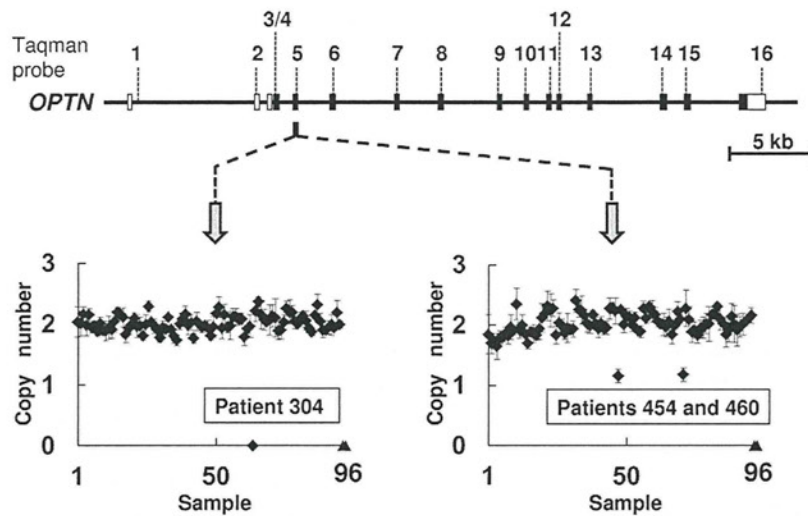
Fig. 1. TaqMan qPCR assay for detection of *OPTN* deletions. Top: Genomic structure of the *OPTN* gene and the position of the TaqMan probes for the assay. Open rectangles indicated the 5′- and 3′-untranslated regions, and filled rectangles the coding regions. Bottom: Scatter-plot analysis views of the assay for the exon 5 probe that indicate homozygous (left) and heterozygous (right) deletions.

2012). The low prevalence is quite similar to other ALS disease genes. In ALS genes other than *OPTN, SOD1* is the most prevalent in the Japanese. Its mutational frequency in sALS is 1.6% (7/439) (Akimoto et al., 2011). *TARDBP* is the second most prevalent ALS gene in the Japanese, and its mutational frequencies in fALS and sALS are 3.0% and 0.29%, respectively (Iida et al., 2010). However, these figures may be an underestimation due to incomplete screening of the mutation, because the mutation screenings in both studies were based on a PCR-direct sequence method, in which it is easy to overlook heterozygous deletions. Notably, Maruyama et al. (2010) reported a deletion mutation.

To examine *OPTN* mutations more extensively, we screened the *OPTN* deletion by quantitative PCR covering *OPTN* exons. We have identified 5 deletions in 710 ALS subjects who had previously been examined for *OPTN* mutations by a PCR-direct sequence method with negative results.

2. Methods

A total of 710 Japanese ALS patients (685 were sporadic and 25 familial) were examined in this study. All DNA samples were obtained from the Biobank Japan project (Nakamura, 2007). Their clinical information was described previously (Iida et al., 2012). They had previously been examined for *OPTN* mutations by a PCR-direct sequence method and were found to have no mutations (Iida et al., 2012). The control subjects consisted of a general population recruited through several medical institutions in Japan as previously described (Nakajima et al., 2010). Written informed consent was obtained from all the subjects. The ethical committees at the participating institutions approved this project.

To examine the copy number of *OPTN*, we employed a quantitative PCR (qPCR) method as previously described (Hosono et al., 2009). A total of 15 TaqMan probes for the qPCR covering all of 16 *OPTN* exons were designed with the aid of Primer Express software v2.0 (Applied Biosystems) (Fig. 1, top). All but 2 probes were designed in the exons. Probe 1 was designed not in exon 1 but in intron 1 because of the high GC content of exon 1. Probe 3/4 that should cover exons 3 and 4 was designed in exon 4, because the 2 exons were very near (only 96 bp apart). The RNase P gene was used as a reference gene (Applied Biosystems). All assays were performed with TaqMan Universal PCR Master Mix (Applied Biosystems) according to the recommended protocol.

The breakpoint sequences of patients were determined by direct sequencing of PCR amplicons using primer sets described in Supplementary Table 1. The 3730xl DNA analyzer (Applied Biosystems) was used according to a standard protocol. The breakpoints were determined by comparing the sequences of the patients with reference sequences. Repeat sequences were examined by the RepeatMasker program (www.repeatmasker.org/).

3. Results

We screened the copy number abnormalities of *OPTN* using a system composed by 15 sets of qPCR assays based on the TaqMan platform. We found 3 kinds of deletions in 5 among 710 ALS patients (Table 1): one was known (Maruyama et al. 2010) and homozygous; 4 were novel and heterozygous. All deletions were detected in sporadic ALSs and were not detected in 470 unrelated Japanese controls. All deletion breakpoints were in Alu repeats, suggesting that they occurred due to the Alu-mediated recombination. The

Table 1
Clinical features and *OPTN* deletions

Patient ID	Gender	Age at onset (year)	Site	Family history	Deletion	
					Exon	Status
9	Female	43	Spinal	No	1, 2, 3, 4	Heterozygous
256	Female	44	Bulbar	No	3, 4, 5	Heterozygous
304	Male	43	Spinal	No	5	Homozygous
454	Female	65	Bulbar	No	3, 4, 5	Heterozygous
460	Female	62	Bulbar	No	3, 4, 5	Heterozygous

Alu repeats involved in the recombination were all different in the 3 types of deletions. The breakpoint sequences were all different.

Patient 304 lost the signal for Probe 5 (Fig. 1), while two-copy signals for Probes 3/4 and 6 were retained, indicating that the patient had deletions of both alleles of the genome containing exon 5. To identify the deletion breakpoints, we designed a set of the PCR primers in exon 4 and intron 5 (Supplementary Table 1). The PCR yield a 3.2-kb amplicon of the expected size in a control, while it yielded only a single 1.4-kb amplicon in the patient (Supplementary Fig. 1a). Direct sequencing of the PCR amplicons showed that the breakpoint of the deletion (Deletion 1) was flanked by Alu repeats in introns 4 and 5 (Fig. 1). The deletion was the same as the one previously reported in fALS, which was also homozygous (Maruyama et al., 2010). Deletion 1 is predicted to cause a frameshift, which would result in a premature stop codon in exon 6.

Patients 256, 454 and 460 lost signals for Probes 3/4 and 5 heterozygously (Fig. 1). A set of PCR primers in introns 2 and 5 (Supplementary Table 1) amplified a ~700-bp fragments in the patients and a 4-kb fragment in a control, respectively (Supplementary Fig. 1b). Direct sequencing of the PCR amplicons showed that the 3 patients had the same deletion (Deletion 2), which had breakpoints in the 5' part of an Alu repeats in intron 2 and the 3' part of another Alu repeats in intron 5 (Fig. 2). Deletion 2 is predicted to result in a null allele because the translation start site of *OPTN* is in exon 3. No mutations in nondeleted alleles of these patients were found.

A novel deletion (Deletion 3) was identified in Patient 9. The TaqMan screening system showed that genomic regions for Probes 1, 2, and 3/4 were deleted heterozygously in this patient, while that for Probe 5 was not deleted (Fig. 3, top). To identify the deletion breakpoint, we designed long PCR forward primers at every ~10 kb from exon 1 and
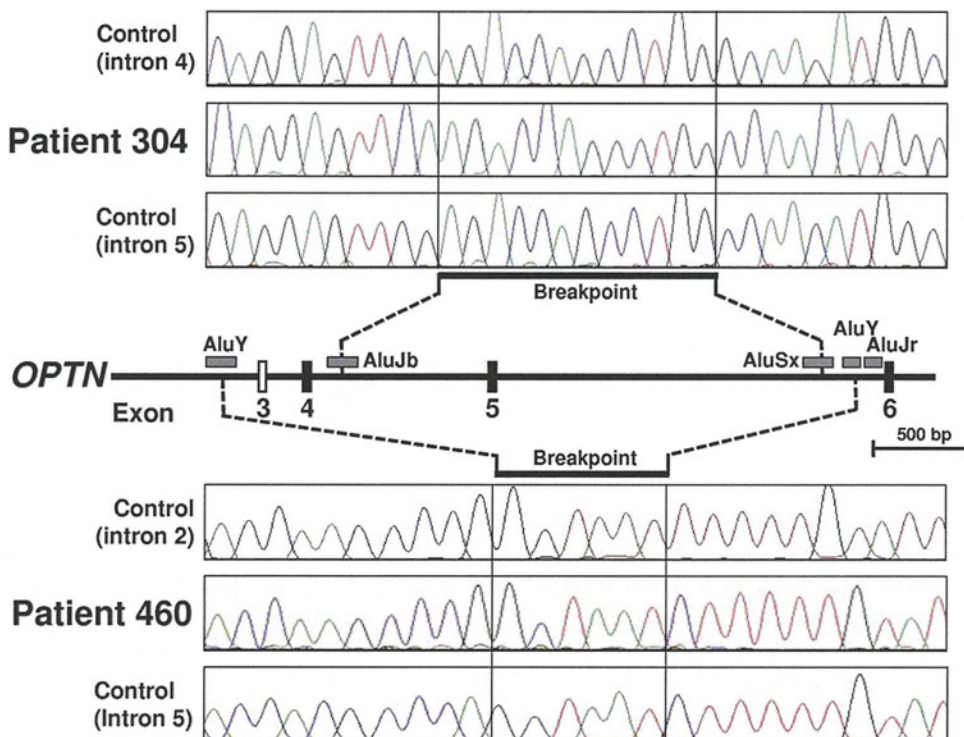

Fig. 2. Analysis of deletion breakpoints for Deletion 1 (Patient 304) and 2 (Patient 460). A map of deletions and Alu repeats in *OPTN* and the DNA sequences of the breakpoints. Open rectangles indicated the 5'-untranslated region, and filled rectangles the coding regions.
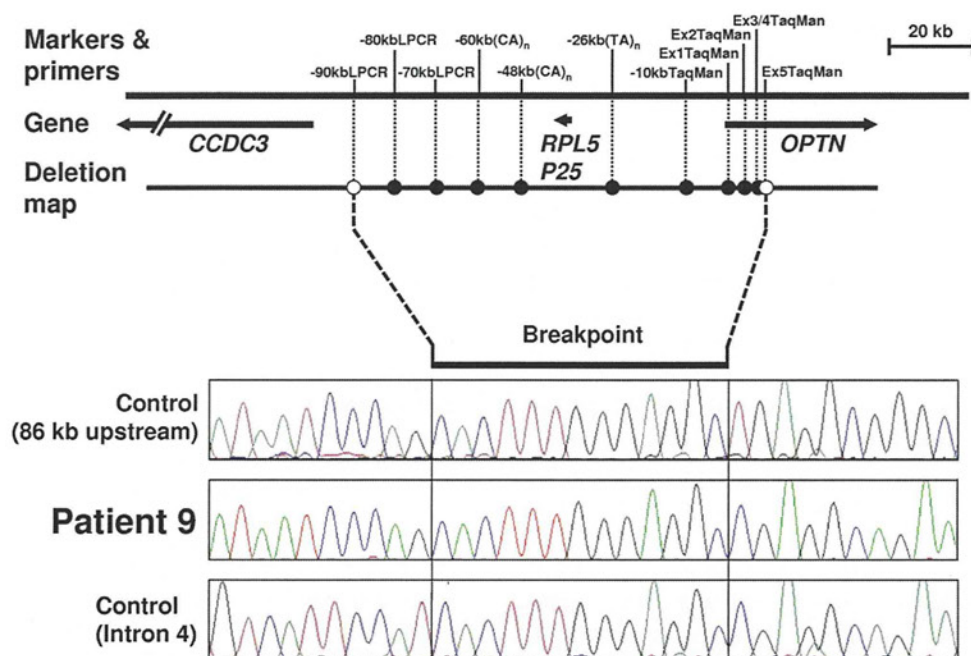
Fig. 3. Identification of Deletion 3 in Patient 9. Top: a map of the genomic region 5' upstream of *OPTN*. Positions of TaqMan probes, dinucleotide polymorphism markers (CA) and PCR primers are indicated. Open circles: retained markers, solid circles: deleted markers. Genes are represented by arrows. RPL5P25: ribosomal protein L5 pseudogene 25. CCDC3: coiled-coil domain containing 3. Bottom: DNA sequences of the breakpoints. The deletion encompasses 86 kb upstream to exon 1 to intron 4.

a reverse primer in exon 5 (ex5-RP), and investigated up to the 92 kb upstream region from the exon 1 (Fig. 3, top). A long PCR of the patient DNA using a forward primer (up-90k-FP) in 92 kb upstream from the *OPTN* transcription start site and the reverse primer detected a 7-kb PCR amplicon (Supplementary Fig. 1c). Direct sequencing of the PCR amplicon showed that the deletion (Deletion 3) occurred between Alu repeats at 85.8 kb upstream of *OPTN* and in intron 4 of *OPTN* (Fig. 3). No mutation in another allele in the patient was found.

4. Discussion

In this study, we investigated copy number abnormalities of *OPTN* in 710 Japanese ALS by using a TaqMan-qPCR assay and found deletions in 5 patients with sALS. Taken together with the previously study (Maruyama et al., 2010), all but one deletion contained exon 5, suggesting that the exon is a hotspot for *OPTN* deletion in ALS. Our clinical information suggests that those patients with *OPTN* deletions did not have POG. In Maruyama's report (2010), *OPTN* deletions were not found in 200 normal Japanese, nor > 6800 POG patients. We did not find any deletions in our controls. Hence, we concluded that *OPTN* deletions are ALS-specific events.

Maruyama et al. (2010) postulated 2 disease-causing mechanisms of *OPTN* mutations: a loss of function based on the identification of a homozygous deletion (Deletion 1) and a homozygous nonsense mutation, and a gain of function based on the identification of the heterozygous missense mutation. In our study, Deletion 1 was also identified in a homozygous state. Because the last exon of *OPTN* is exon 16, the mutant mRNA from Deletion 1 would be subjected to the nonsense mediated mRNA decay (Bhuvanagiri et al., 2010), resulting in a null allele. Deletions 2 and 3 are expected to be null alleles, because exon 4 that contained the translation start site is lost in the deletions. In all 4 patients who had those deletions, the exon-by-exon direct sequence showed no mutation in other alleles. Therefore, haplo-insufficiency is the most likely disease-causing mechanism for these deletions. Del Bo et al. (2011) reported the *OPTN* mutation screening in 274 Italian ALS patients (161 fALS and 113 sALS). Their finding is supportive for haplo-insufficiency. They identified 6 novel mutations including a nonsense mutation (p.G23X), all of which occurred in heterozygous states.

The 5 deletions were all located in Alu repeats. These data showed homologous Alu-mediated recombination as the mechanism for *OPTN* deletion mutations. Alu repeats represent a sequence of approximately 300 bp in length (Deininger and Batzer, 1999), and are dispersed throughout 10.6% of the human genome (Lander et al., 2001). The *OPTN* contains 38 Alu repeats, which occupies 21% of the 38 kb gene region. Alu repeats density is particularly high in introns 2, 4 and 5; 45%, 32% and 43%, respectively. They are 3–4 times higher than the average density in the human genome. The high density of Alu repeat appears to predispose the *OPTN* to a high frequency of Alu-mediated deletions.

Various inherited disorders and cancer have been caused by unequal homologous recombination between Alu repeats (Deininger and Batzer, 1999). Alu repeats are divided into 3 subfamilies; the older (Alu Jo, Jb, etc.), intermediate (Alu Sc, Sx, Sq, etc.), and the younger (Alu Y, Ya, Yb, etc.) (Batzer et al., 1996). The Alu Y is evolutionarily younger among all Alu elements with an age of 25 million years. The Alu Sx and Alu Jb are 30–35 and < 60 million years, respectively (Franke et al., 2009). So far, ~0.3% of all human genetic diseases seems to have resulted from Alu mediated recombination (Deininger and Batzer, 1999). The Alu repeats are known to be hot spots for recombination events (Deininger et al., 2003). A high density of Alu repeats is likely to result in a high level of unequal homologous recombination (Batzer and Deininger, 2002). Also, the recombination rate between Alu repeats belonging to different subfamilies should vary as a function of a pairwise sequence divergence between repeats (Batzer and Deininger, 2002). Namely, the older Alu repeats that have higher pairwise divergence (~15–20%) are much less likely to recombine than the youngest ones (Batzer and Deininger, 2002). In von Hippel–Lindau disease, the frequency of intra-Alu subfamily recombinations correlates with both the pairwise sequence divergence between the recombining Alu elements and the age of the subfamily, whereas inter-Alu subfamily recombinations are less frequent and have, on average, higher pairwise sequence divergences between the recombining Alu elements (Franke et al., 2009). In our study, the recombination in Deletion 2 occurred between elements belonging to the younger Alu Y, the younger subfamilies with pair-wise divergence 8%. The recombination in Deletion 1 and 3 occurred between inter-Alu subfamily: Alu Jb—AluSx with 29% divergence in Deletion 1 and Alu Y—Alu Jb with 26% divergence in Deletion 3.

In the previous study, we had examined the same patient cohort using PCR-direct sequence and found point mutations of *OPTN* in 2 sALS (Iida et al., 2012). Taken together with the result of the present study, the prevalence of *OPTN* mutation in Japanese sALS is 1.0%. It is 3.5 times higher than that in *TARDBP* (Iida et al., 2010). Thus, *OPTN* is the second most prevalent sALS gene in the Japanese after *SOD1*. It remains to be examined whether, in other ALS genes, chromosomal abnormalities including deletion may be left undetected and their prevalence is underestimated.

Disclosure statement

The authors declare that they have no competing financial interests.

Acknowledgments

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.12.037.

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ORIGINAL ARTICLE

Creatinine/cystatin C ratio as a surrogate marker of residual muscle mass in amyotrophic lateral sclerosis

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Abstract

Aim: Identification of sensitive surrogate markers that indicate disease progression in amyotrophic lateral sclerosis might be useful in clinical trials and clinical care. Determination of the creatinine/cystatin C (Cr/CysC) ratio eliminates the effect of renal function on serum creatinine levels; therefore, we considered that the ratio might serve as a surrogate marker of residual muscle mass. We studied the Cr/CysC ratio as a useful surrogate marker of residual muscle mass in patients with amyotrophic lateral sclerosis.

Methods: A total of 103 participants were recruited: 62 patients with amyotrophic lateral sclerosis and 41 healthy controls. Serum levels of Cr and CysC were measured in both groups. We subsequently investigated the correlation between the Cr/CysC ratio and disease severity in patients with amyotrophic lateral sclerosis.

Results: The ratio was significantly lower in the amyotrophic lateral sclerosis group than in the control group. Furthermore, the ratio decreased as the severity of amyotrophic lateral sclerosis increased. The Cr/CysC ratio might be a better and more reliable method than the serum Cr level as a means of monitoring residual muscle mass of the entire body in patients with amyotrophic lateral sclerosis.

Conclusion: The present results show that the Cr/CysC ratio might be a suitable candidate for a useful and quantitative surrogate marker for the assessment of disease severity and progression in patients with amyotrophic lateral sclerosis.

Introduction

Amyotrophic lateral sclerosis (ALS) is a uniformly fatal and debilitating disease; therefore, there is tremendous interest in developing effective therapies to slow or halt the progression of this disease. Current study designs often use a primary end-point of either death from ALS or initiation of long-term mechanical ventilation. This design requires a relatively long observation time to determine whether there is a positive treatment effect. Thus, identification of sensitive surrogate markers that indicate disease progression in ALS could be useful for the rapid identification of beneficial drugs, prompt exclusion of ineffective candidates and to determine clinical care for ALS patients. Surrogate markers of disease progression would provide a means of more rapid monitoring of drug efficacy in clinical trials.[1-5]

Muscle atrophy is a disease-defining feature of ALS, and clinical experience shows that atrophy might correlate with progressive weakness. Muscle atrophy is a qualitative marker of disease progression, but as yet, there is no clear quantitative marker of atrophy. Thus, as daily life activities decrease, the severity grade of ALS approximates to the residual skeletal muscle volume. A parameter that reflects muscle volume

would enable estimation of disease severity.[6-8] The serum level of creatinine (Cr) is currently considered to be the most useful blood parameter that reflects the severity of motor dysfunction in spinal and bulbar muscular atrophy[9]; serum Cr levels were found to be correlated with the ALS Functional Rating Scale-Revised (ALSFRS-R) score in patients with spinal and bulbar muscular atrophy (correlation coefficient = 0.566, $P < 0.001$). However, because serum Cr almost exclusively originates from the skeletal muscle and its levels are dependent of renal function, we considered that the use of serum Cr levels as an accurate marker in ALS patients might be questioned.

Cystatin C (CysC), a known cysteine protease inhibitor, could potentially be used as a surrogate marker of glomerular filtration rates (GFR).[10,11] CysC is a non-glycosylated, 13.3-kDa basic protein that contains two disulfide bridges, and it is produced by all nucleated cells. It is considered to be unaffected by any factors (e.g. muscle mass, lean tissue mass, age, ambulation, circadian rhythm and sex) other than renal function status.[12-14] Furthermore, CysC is independent of the body muscle volume, and it is excreted from the kidneys in the same manner as Cr. Thus, the Cr/CysC ratio, which remains almost constant irrespective of renal function

in individuals with neuromuscular diseases, is theoretically considered to be a good surrogate marker of muscle volume in ALS patients. In the present study, we compared the Cr-based estimated GFR (eGFR) with the CysC-based eGFR in ALS patients and healthy controls. The Cr/CysC ratio was further comparatively analyzed according to disease severity in ALS patients.

Methods

ALS patients and controls. Amyotrophic lateral sclerosis was diagnosed according to the revised El Escorial criteria.[15] A total of 62 patients serially diagnosed with definite ALS at Jichi Medical University Hospital in Shimotsuke, Japan, were enrolled in the present study. These ALS patients had no prior history of renal disease, no known concomitant disease and were not participating in any experimental treatment. A total of 41 subjects free from diseases characterized by muscle atrophy were recruited as controls in the present study. The mean age of the study participants was 62.9 ± 9.6 years for ALS patients and 61.8 ± 12.4 years for healthy controls. Clinical variables of ALS patients were analyzed for age, sex, onset site, symptom duration and grading according to the ALSFRS-R score.[16] In the current study, 10 patients experienced a bulbar onset and 52 patients experienced a limb onset of disease. The mean symptom duration was 4.4 ± 5.1 years (Table 1). Classification of disease severity based on the severity scale established by the modified Rankin scale (mRs; Table 2) revealed five patients with a disease severity of grade 1, nine with grade 2, 13 with grade 3, 13 with grade 4 and 22 with grade 5 (Table 1). Informed consent was obtained from all patients and healthy controls.

Measurements. All study participants were actively engaged in their usual daily life activities. Blood samples

Table 2 Modified Rankin scale

Grade	Description
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent, and requiring constant nursing care and attention
6	Dead

were collected in distinct serum-separator tubes and analyzed for serum Cr and CysC. Serum Cr levels were measured using an enzymatic method at our hospital laboratory. Serum CysC levels were measured using colloidal gold particles coated with anti-CysC antibodies at SRL Laboratory (Tokyo, Japan).[17]

eGFR (mL/min/1.73 m^2) was determined by measuring serum Cr levels using the following equation developed by the Committee on Chronic Kidney Disease of the Japanese Society of Nephrology[18]: men, Cr-based eGFR = 194 × Cr$^{-1.094}$ × age$^{-0.287}$; women, eGFR = 194 × Cr$^{-1.094}$ × age$^{-0.287}$ × 0.739. In addition, eGFR (mL/min/1.73 m^2) was determined by measuring serum CysC levels using the following equation developed by A Rule[19]: men and women, CysC-based eGFR = 66.8 × CysC$^{-1.30}$. The ratio of Cr (mg/dL) to CysC (mg/L) × 10 was defined.

Statistical analyses. For general statistical analyses, we used the SPSS v.11.0.1 program (Tokyo, Japan). Student's *t*-test was applied to the mean eGFR for serum Cr. Mann–Whitney's *U*-test was applied to the mean age, mean eGFR for serum CysC, and mean ratios of serum Cr to serum CysC between ALS patients and healthy controls. The χ^2-test for independent testing was applied to a two-by-two contingency table with sex between both groups. We analyzed differences in the Cr/CysC ratio between severity grades (mRs) by ANOVA followed by Tukey's honestly significant difference post-hoc test.

Correlation coefficients between serum Cr levels, Cr/CysC ratios and ALSFRS-R scores were determined. A scatter diagram between the serum levels and ALSFRS-R score was constructed. The correlation coefficient between Cr/CysC ratios and ALS duration was determined, and a scatter diagram between both sides was made. Correlations were determined using Spearman's rank-correlation coefficient. All tests were two-tailed and significance was set at $P < 0.05$.

Results

In this current study, clinical parameters (sex and age) were not significantly different between the ALS and control groups (Table 1).

Table 1 Clinical background of amyotrophic lateral sclerosis patients and control subjects

	ALS patients ($n = 62$)	Control subjects ($n = 41$)	P-value
Sex (male/female)	39/23	19/22	0.10 (χ^2-test)
Age (years)	62.9 ($\pm$9.6)	61.8 ($\pm$12.4)	0.52 (Mann–Whitney's *U*-test)
Symptom duration (years)	4.4 ($\pm$5.1)		
Bulbar onset/limb onset	10/52		
ALSFRS-R	25.8 ($\pm$17.3)		
Severity scale (mRs)	Grade 1 5		
	Grade 2 9		
	Grade 3 13		
	Grade 4 13		
	Grade 5 22		

Data were expressed by mean (standard deviation).
ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; mRs, modified Rankin scale.

The mean Cr/CysC ratios were 8.2 ± 2.2 for the control group and 5.5 ± 3.3 for the ALS group, the value being significantly lower in the ALS group (Mann–Whitney's U-test, $P = 0.01$; Table 3). The mean eGFR determined by serum Cr levels was 381.6 ± 486.9 mL/min/1.73 m^2 for the ALS group, which was significantly different (Mann–Whitney's U-test, $P < 0.001$; Table 3) from the control group value of 98.5 ± 57.9 mL/min/1.73 m^2. However, the mean eGFR determined by serum CysC was 103.3 ± 24.8 mL/min/1.73 m^2 for the ALS group, which was not significantly different from the control group value of 97.6 ± 21.6 mL/min/1.73 m^2. Thus, Cr-based eGFR in the ALS group was markedly higher than any other values. This is probably explained by reduced serum Cr levels in ALS patients on account of the reduced residual muscle mass. The mean Cr/CysC ratios were not significantly different according to sex between both groups (Table 3).

We compared the mean Cr/CysC ratios with disease severity (Fig. 1a) in ALS patients. The ratio was 10.13 ± 1.27 for grade 1 disease severity, 7.82 ± 1.1 for grade 2, 6.47 ± 1.92 for grade 3, 6.3 ± 2.78 for grade 4 and 2.37 ± 2.25 for grade 5. Significant differences were observed in the Cr/CysC ratio between severity grades 1–3, 1–4, 1–5, 2–5, 3–5 and 4–5 ($P < 0.05$). Thus, the ratio linearly decreased with an increase in disease severity (Fig. 1a). A relatively high Cr/CysC ratio observed in the patient group with grade 4 disease severity could be explained by the fact that this group included a number of patients with the bulbar-palsy type of ALS, in which the muscle mass in the four extremities is relatively well preserved. Therefore, we compared the mean Cr/CysC ratios after excluding patients with this type of ALS. This analysis revealed a ratio of 10.13 ± 1.27 for grade 1 disease severity, 7.82 ± 1.1 for grade 2, 6.47 ± 1.92 for grade 3, 4.6 ± 1.42 for grade 4 and 1.6 ± 1.58 for grade 5 (Fig. 1b). Significant differences were observed in the Cr/CysC ratio between severity grades 1–3, 1–4, 1–5, 2–4, 2–5, 3–5 and 4–5 ($P < 0.05$). A steady decrease in the ratio with an increase in disease severity became more evident when patients with the bulbar-palsy type of ALS were excluded from the analysis.

The scatter plot showed strong simple correlations between the Cr/CysC ratio and disease severity in ALS patients as determined by the ALSFRS-R score (correlation coefficient = 0.84, $P < 0.001$; Fig. 2). Furthermore, the plot showed that ALS severity increased with a decrease in the Cr/CysC ratio. A correlation between serum Cr levels and disease severity in ALS patients as determined by the ALS-FRS-R score was also recognized (correlation coefficient = 0.78, $P < 0.001$; Fig. 2). The correlation coefficient of the Cr/CysC ratios was higher than that of serum Cr levels.

The relationship between the Cr/CysC ratio and ALS duration is shown on the scatter plot; the plot shows that the Cr/CysC ratio decreased with an increase in the duration of ALS (correlation coefficient = 0.75, $P < 0.001$; Fig. 3a). Figure 3b shows the percentage of patients with the Cr/CysC ratios of ≥ 5 and those with ratios of <5 as classified by disease severity. The percentage of patients with ratios of <5 was markedly higher in patients with grade 4 and grade 5 disease severity (71% and 94%, respectively).

Discussion

We believe that CysC levels are not basically affected by ALS itself. By calculating the serum Cr/CysC ratio, we eliminated the effect of renal function on serum Cr, which is dependent on the muscle mass of the entire body. We subsequently used this ratio as a surrogate marker of the residual muscle mass throughout the body. The Cr/CysC ratio was significantly lower in the ALS group, which was characterized by a decreased residual muscle mass, than that in the control group. Furthermore, the ratio decreased with an increase in ALS severity. This finding suggested that changes in the Cr/CysC ratio might reliably reflect the decrease in muscle mass throughout the body with an increase in ALS severity. According to the severity scale used in the present study, patients with the bulbar-palsy type of ALS, in whom the residual muscle mass was relatively conserved, were assigned higher grades of disease severity. Therefore, we also carried out the analysis after excluding patients with this type of ALS. With the exclusion of these patients from the analysis, it became even more evident that the Cr/CysC ratio reliably reflected the residual muscle mass of the entire body. Correlation of the Cr/CysC ratio was higher than that

Table 3 The ratios of creatinine to cystatin C, and the estimated glomerular filtration rate of creatinine and cystatin C in amyotrophic lateral sclerosis patients and controls

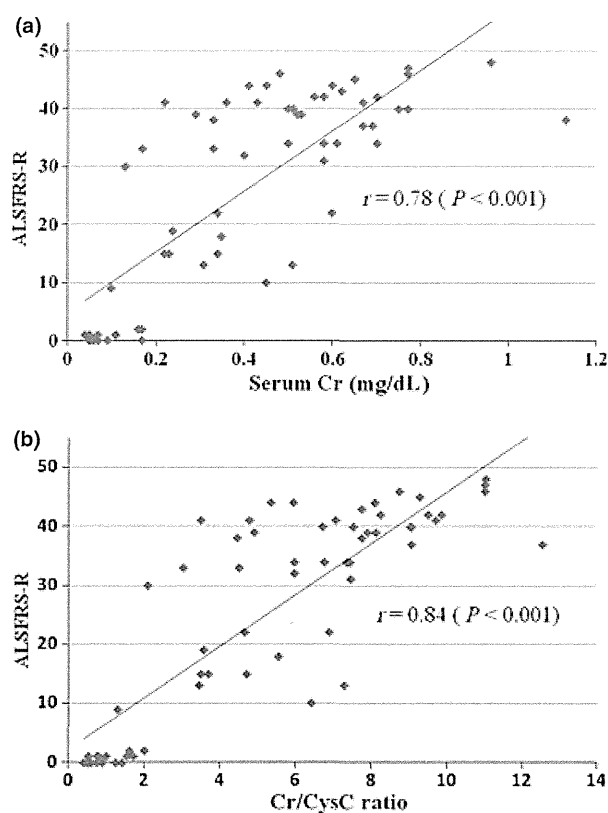|	ALS patients (n = 62)	Control subjects (n = 41)	*P*-value
The ratio of Cr (mg/dL) to CysC (mg/L) ×10 (Cr/CysC)	5.5 (±3.3)	8.2 (±2.2)	0.01* (Mann–Whitney *U*-test)
Cr-based eGFR (mL/min/1.73 m^2)	381.6 (±486.9)	98.5 (±57.9)	<0.001* (Mann–Whitney *U*-test)
CysC-based eGFR (mL/min/1.73 m^2)	103.3 (±28.4)	97.6 (±21.6)	0.28 (Student's *t*-test)
	Male	Female	*P*-value
ALS patients (Cr/CysC ratio)	5.2 (±3.7)	5.9 (±2.5)	0.37 (Student's *t*-test)
Control subjects (Cr/CysC ratio)	8.8 (±2.6)	7.6 (±1.8)	0.09 (Student's *t*-test)

*Data were expressed by mean (standard deviation). Significant difference.
Cr, creatinine; CysC, cystatin C; eGFR, estimated glomerular filtration rate.
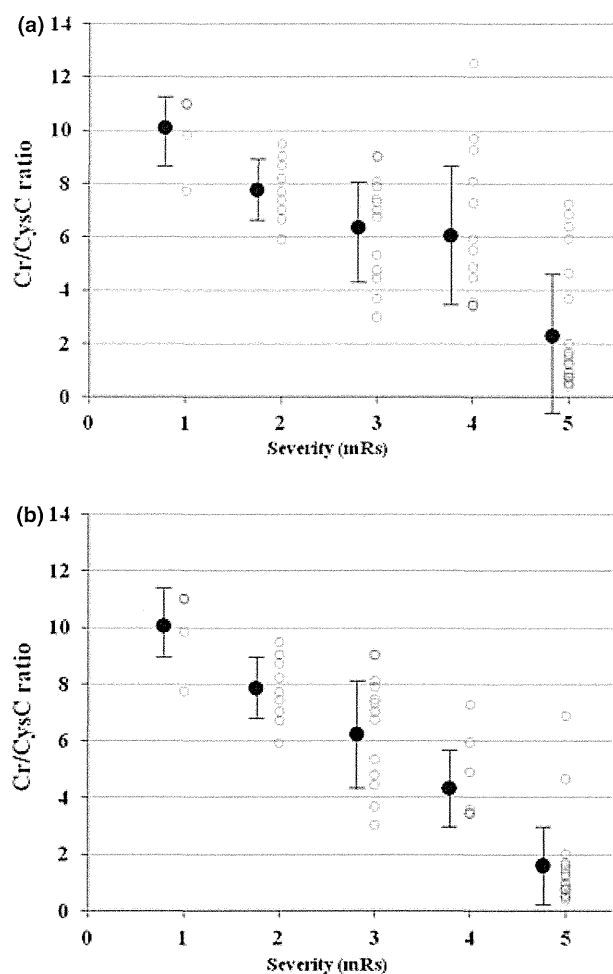
Figure 1 The mean creatinine/cystatin C (Cr/CysC) ratios were compared by disease severity (modified Rankin scale [mRs]) in amyotrophic lateral sclerosis (ALS) patients, and differences were analyzed by ANOVA followed by Tukey's honestly significant difference post-hoc test. (a) The ratio decreased linearly as disease severity increased (severity grades 1–3, 1–4, 1–5, 2–5, 3–5 and 4–5; $P < 0.05$). (b) After excluding patients with the bulbar-palsy type of ALS, the steady decrease of the ratio with increasing disease severity became more apparent (severity grades 1–3, 1–4, 1–5, 2–4, 2–5, 3–5 and 4–5; $P < 0.05$).

Figure 2 The scatter diagram showed strong simple correlations between (a) Cr level, (b) creatinine/cystatin C (Cr/CysC) ratio and the severity by Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) score in amyotrophic lateral sclerosis (ALS) patients. The plot showed that ALS severity increased with a decrease in both. The correlation coefficient of Cr/CysC ratio was higher than that of Cr level.

of serum Cr levels. The results also indicated that the Cr/CysC ratio might be better and more reliable than serum Cr levels for monitoring the residual muscle mass of the entire body in ALS patients. In addition, in ALS patients with serum Cr/CysC ratio of <5, daily life activities were markedly restricted. Therefore, this value might constitute a critical cut-off point during the clinical course of the disease.

To date, insensitive, non-parametric surrogate markers, which presume quantitative assessment of the disease severity in ALS patients, such as forced vital capacity, and the ALSFRS-R score have been used to assess disease progression.[16,20] We suggest that the Cr/CysC ratio might permit quantitative assessment of disease severity in ALS patients, assessment of therapeutic responses and determination of

disease progression. Lee CD *et al.* presented an alternative approach that involved assessment of muscle thickness by muscle ultrasound (MUS) in ALS patients.[7] These authors reported that MUS is sufficiently sensitive as a potential surrogate marker to quantitatively detect changes in muscle thickness over time. Their study proposed biceps brachii as a suitable candidate for study; however, it is likely that assessment of multiple muscles would be required to account for the heterogeneity of ALS. Furthermore, muscle gene expression changes in skeletal muscle that could reliably define the degree of disease severity were reported.[21] However, we think that the current study presents a more convenient method for assessing disease status and progression than these approaches. Additional methods of measuring limb muscle mass include magnetic resonance imaging (MRI) and bioelectric impedance analysis (BIA). Applications for measuring residual muscle mass in ALS patients by MRI and BIA have been previously reported.[8,22] In particular, BIA is reported to be safe, portable, highly reliable and relatively inexpensive.[23] However, both methods can measure only a limited segment of body muscles. As a result of the heterogeneity of ALS that often affects different muscles
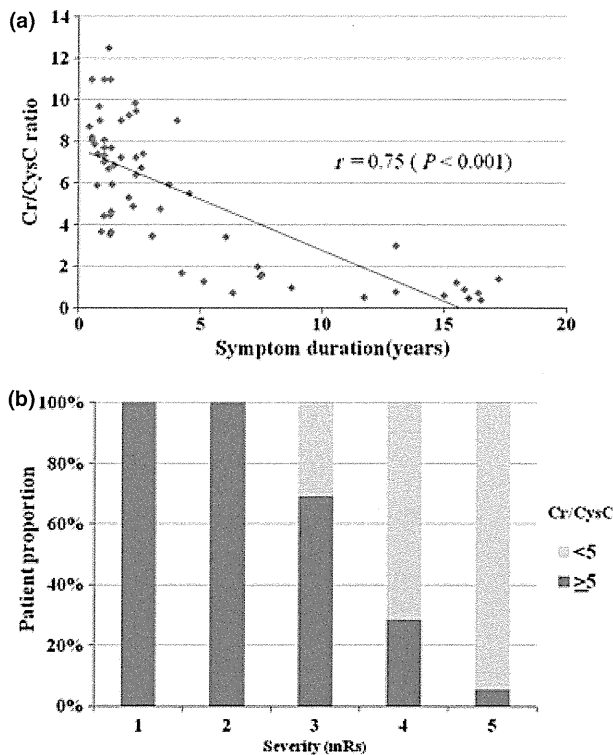
Figure 3 (a) The relationship between the creatinine/cystatin C (Cr/CysC) ratios and amyotrophic lateral sclerosis (ALS) duration is shown in the scatter plot; the plot shows that the Cr/CysC ratio decreased with an increase in the duration of ALS. (b) The percentage of patients with the Cr/CysC ratios of ≥ 5 and those with ratios of <5 as classified by disease severity (modified Rankin scale [mRs]) are shown. The percentage of patients with ratios of <5 was markedly higher in patients with grade 4 and grade 5 disease severity (71% and 94%, respectively).

at different rates, these methods are not considered to be capable of accurately determining residual muscle mass in ALS patients. Thus, we propose that measurement of the serum Cr/CysC ratio in ALS patients, which might account for residual muscle mass of the entire body, might be superior to these methods and eliminate the effect of renal function on serum Cr levels. However, under certain circumstances, the serum Cr/CysC ratio might not be a reliable surrogate marker of the residual muscle mass. For example, if ALS patients have a mild renal dysfunction, the ratio might be erroneously decreased. Thus, we have to interpret the data carefully, and should use only serum CysC levels for monitoring renal function accurately in ALS patients in such a case.A limitation of the present study was the lack of data measuring the actual residual muscle mass; however, as aforementioned, it is challenging to determine residual muscle mass of the entire body in ALS patients. Thus, taking previous studies together with our data and theory of the Cr/CysC ratio, it might be reasonable to suggest that serum Cr/CysC ratio might be a suitable candidate for a surrogate marker of residual muscle mass.

In addition, the use of the serum Cr level as a marker of renal function might be underestimated in diseases such as ALS that are characterized by reduced muscle mass. Thus, it is difficult to accurately assess renal function using serum Cr levels in these patients. This is clearly shown by the result shown in Table 3; eGFR determined by serum Cr was markedly higher in ALS patients than that determined by serum CysC. The latter is, therefore, considered to be a more accurate indicator for the assessment of renal function in ALS patients.

In conclusion, the present results show that the Cr/CysC ratio might be a suitable candidate for a surrogate marker of residual muscle mass of the entire body in ALS patients. Furthermore, the Cr/CysC ratio might also be a useful marker for assessing the response to therapy and determining the progression of ALS.

Acknowledgments

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Pseudobulbar Dysarthria in the Initial Stage of Motor Neuron Disease with Dementia: A Clinicopathological Report of Two Autopsied Cases

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Abstract
We retrospectively analyzed the clinical features of two cases of neurodegenerative disease, whose initial symptoms were motor speech disorder and dementia, brought to autopsy. We compared the distributions of pathological findings with the clinical features. The main symptom of speech disorder was dysarthria, involving low pitch, slow rate, hypernasality and hoarseness. Other than these findings, effortful speech, sound prolongation and initial difficulty were observed. Moreover, repetition of multisyllables was severely impaired compared to monosyllables. Repetition and comprehension of words and sentences were not impaired. Neither atrophy nor fasciculation of the tongue was observed. Both cases showed rapid progression to mutism within a few years. Neuropathologically, frontal lobe degeneration including the precentral gyrus was observed. The bilateral pyramidal tracts also showed severe degeneration. However, the nucleus of the hypoglossal nerve showed only mild degeneration. These findings suggest upper motor neuron dominant motor neuron disease with dementia. We believe the results indicate a subgroup of motor neuron disease with dementia whose initial symptoms involve pseudobulbar palsy and dementia, and which shows rapid progression to mutism.

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Introduction

Apraxia of speech and dysarthria are two major components of motor speech disorder in speech production [1, 2]. Recently, progressive apraxia of speech has attracted much attention in the diagnosis of progressive nonfluent aphasia [2, 3]. However, only a few cases of progressive dysarthria have been reported so far. Although almost half of the patients with amyotrophic lateral sclerosis exhibit pseudobulbar affects [4], and the frontal lobe area is suggested to play a role in this disorder [5], the clinicopathology remains to be resolved.

Herein, we describe two patients with motor neuron disease with dementia exhibiting progressive dysarthria due to pseudobulbar palsy in the initial stage. In motor neuron disease with dementia, almost all patients show a

predominantly bulbar pattern of involvement [6]. We will discuss the unique clinical and pathological relationship observed in the patients.

Case Presentation

Case 1 is a right-handed female patient, who died at 75 years of age. At 73 years of age, she became forgetful of dates. At about the same time, she began to behave in a self-centered manner. For example, when playing cards, she continued to take cards ignoring the other players. Then, her speech became impaired.

Neurological examination disclosed a diminished soft palate reflex, dysarthria, spasticity of the bilateral lower extremities, brisk tendon reflexes and jaw jerk, pathological grasp of both hands, and frontal lobe type dementia.

Her voice was coarse and her speech was effortful. She had sequencing errors, poor intonation, hypernasality, and consonant distortions. Movement of mouth, lips, and tongue was normal. No atrophy or fasciculation of the tongue was observed. Sequential diadochokinesis was impaired in multisyllables. Auditory comprehension, completion of sentences and conversation, naming objects, and repetition of words were largely unimpaired.

Brain magnetic resonance imaging (MRI) revealed atrophy of the anterior temporal lobes and frontal convexity. Single photon emission computed tomography (SPECT) revealed decreased uptake in the frontal and temporal lobes, predominately on the left side.

About 9 months after onset, she had gait disturbance. After 11 months, she became unable to take food and was bedridden. Percutaneous endoscopic gastrostomy was performed. However, she died of respiratory failure 18 months after the onset. Final clinical diagnosis was upper motor neuron dominant type motor neuron disease with frontal lobe dementia.

The fixed brain weighed 1,020 g. Macroscopically, mild atrophy of the frontal lobe was observed. The histopathology of the hypoglossal nucleus revealed preserved numbers of neurons and gliosis was not observed (fig. 1a, b). In the left precentral gyrus, superficial spongiosis, severe neuronal loss with reactive gliosis, and many ubiquitin and TDP-immunoreactive neuronal intracytoplasmic inclusions were observed (fig. 1c). These inclusions were also observed in the hippocampal dentate gyrus. Numerous macrophages were observed in the white matter of the precentral gyrus. However, no degenerative change or TDP-immunoreactive neuronal intracytoplasmic inclusions were observed in the posterior part of the left inferior frontal gyrus, i.e. Broca's area. In the pyramis medullae oblongatae, almost complete loss of the large myelinated fibers and infiltration of macrophages was revealed. Other nuclei of lower motor neurons (the ambiguous nucleus, trigeminal motor nucleus and anterior horn of the spinal cord) were relatively preserved. The pathological diagnosis of case 1 is compatible with amyotrophic lateral sclerosis (ALS) with dementia, or frontotemporal lobar degeneration (FTLD)-TDP (type B by Mackenzie's classification) [7]. Although severe degenerative findings were observed in the upper motor neuron system (precentral gyrus and pyramis medullae oblongatae), pathological findings suggesting bulbar involvement, or Broca's area lesion, were not detected.

Case 2 is a right-handed female patient, who died at 59 years of age. At 52 years of age, she had speech disorder. At about the same time, she became slovenly and indifferent to her surroundings. At 53 years of age, she had immobility of the mouth and tongue and her speech disorder worsened.

Neurologically, she was oriented, but indifferent with poor facial expression. Dysarthria, immobility of the face, tongue and soft palate, brisk tendon reflexes, and jaw jerk were observed. The results of the Wechsler Adult Intelligence Scale-Revised (WAIS-R) revealed diffuse impairment of intelligence.

Her speech was strained with a coarse and effortful voice. She had sound distortions, slow speech rate, poor intonation and hypernasality. No atrophy or fasciculation of the tongue was observed. Sequential diadochokinesis was impaired in multisyllables. Auditory comprehension, reading letters and sentences, and completion of sentences were almost unimpaired. She had reduced verbal output, literal paraphasia, and syntactic errors in the use of particles and verbs; however, naming and repetition were unimpaired. Mild paragraphia and syntactic errors were observed in writing letters and sentences.

Brain MRI, performed 1 year after the onset, revealed atrophy of the caudate head and frontal convexity. SPECT revealed severely decreased uptake in the frontal and temporal lobes.

At 53 years of age, she had frontal lobe impairment and postural instability. At 54 years of age, she developed dysphagia and percutaneous endoscopic gastrostomy was performed. At about the same time, she had tetraplegia and fell into akinetic mutism. She died of pneumonia after a clinical illness of 7 and a half years. The final clinical diagnosis was frontotemporal dementia (FTD) with motor neuron disease.

The fixed brain weighed 730 g. Macroscopically, severe atrophy of the frontal and temporal lobe was observed. In the hypoglossal nucleus, neurons were relatively preserved in number considering the duration of the disease, and gliosis was not observed (fig. 2a, b). In the frontal and temporal lobes including the precentral gyrus, almost complete loss of neurons and severe gliosis were observed. In the remaining neurons, basophilic inclusions which were immunoreactive for anti-FUS antibody were observed (fig. 2c). These inclusions were not immunoreactive for anti-tau and anti-α-synuclein antibodies. In the pyramis medullae oblongatae, almost complete loss of the large myelinated fibers was revealed. Pathological diagnosis of case 2 is compatible with basophilic inclusion body disease, or FTLD-FUS.

Discussion

Ogar et al. [1] suggested that features of spastic dysarthria are strained and strangled voice, low pitch, hypernasality, imprecise consonants, slow rate, and harshness. Common linguistic features of the two patients were coarse and effortful voice, slow speech rate, reduced intonation, hypernasality, and impaired sequential diadochokinesis in multisyllables. Considering that they showed no tongue atrophy or no fasciculation, these linguistic features suggest that they had dysarthria due to pseudobulbar palsy and some features of aphasia. As neither a comprehension deficit nor repetition disorder was ob-
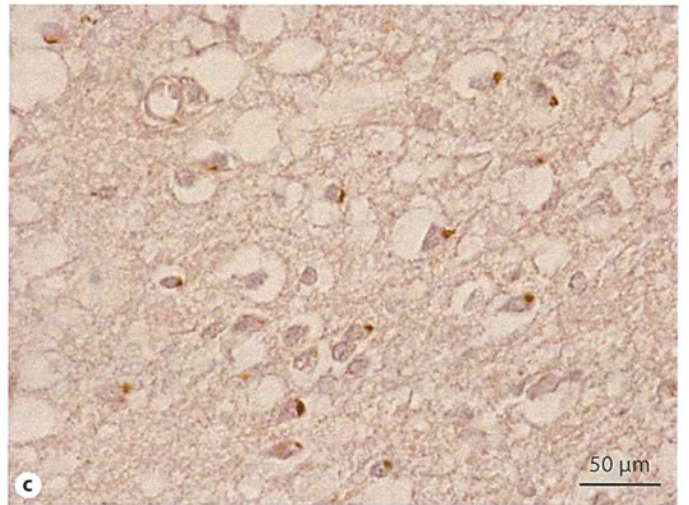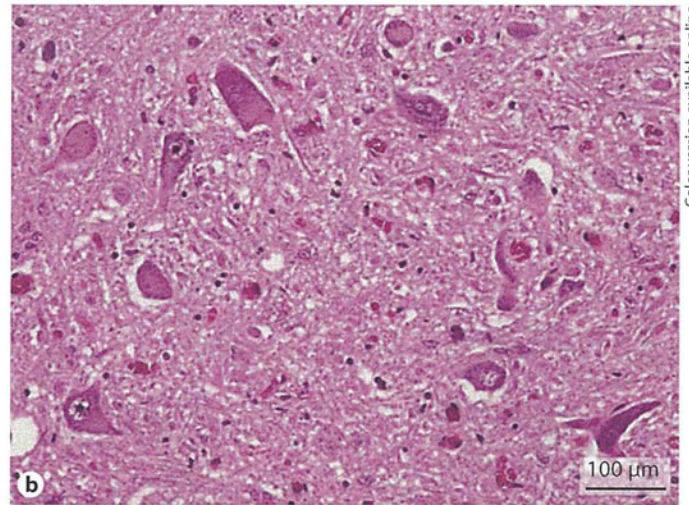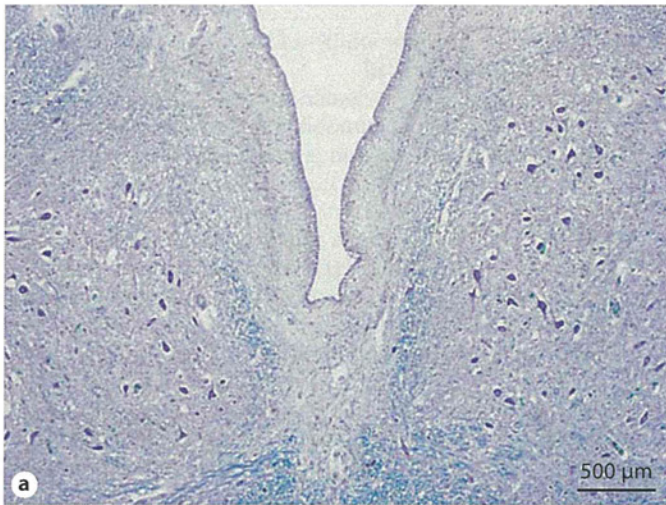
Fig. 1. Pathological findings of case 1. Neurons of the hypoglossal nucleus are relatively preserved in number (**a**) and show minimal gliosis (**b**). Numerous TDP-43 immunoreactive intracytoplasmic inclusions are observed in the frontal lobe cortex (**c**). Klüver-Barrera staining (**a**), hematoxylin and eosin staining (**b**), and anti-TDP-43 immunostaining (**c**; Sigma-Aldrich; 1:200).

served in both cases, we considered that their main speech disorder was dysarthria due to pseudobulbar palsy.

Clinically, in both cases, FTD symptoms and pyramidal tract signs, such as brisk tendon reflexes and pathological reflexes, and brisk jaw jerk, were observed from the onset. Both cases fell rapidly into mutism within 1 or 1 and a half year after the onset. Such rapid progression is another characteristic feature.

Pathologically, the hypoglossal nucleus was relatively preserved in both cases. On the other hand, both cases showed severe degeneration of upper motor neurons (precentral gyrus and pyramis medullae oblongatae). Other nuclei of lower motor neurons (the ambiguous nucleus, trigeminal motor nucleus, and anterior horn of the spinal cord) were relatively preserved in case 1. Although severe degeneration of the entire frontal and anterior

temporal lobes was observed in case 2, the left inferior frontal gyrus (Broca's area) was preserved in case 1. These pathological findings support the idea that the speech disorders observed in the initial phase were mainly due to pseudobulbar palsy, and not due to bulbar palsy or progressive nonfluent aphasia.

Seilhean at al. [8] reported 60 cases with clinical diagnosis of FTD. Of 60 cases, 27 revealed ALS, suggesting a high frequency of the coexistence of the two conditions. Pathologically, 40 cases were diagnosed as FTLD-TDP and 3 cases as FTLD-FUS. In FTLD-FUS, age at onset was relatively young and disease progression was rapid relative to FTLD-TDP. These findings do not contradict the findings in our two cases.

Recently, gene mutation of chromosome 9 open reading frame 72 (C9ORF72) was reported in familiar and

Ishihara/Araki/Ihori/Suzuki/Shiota/Arai/Nakano/Kawamura
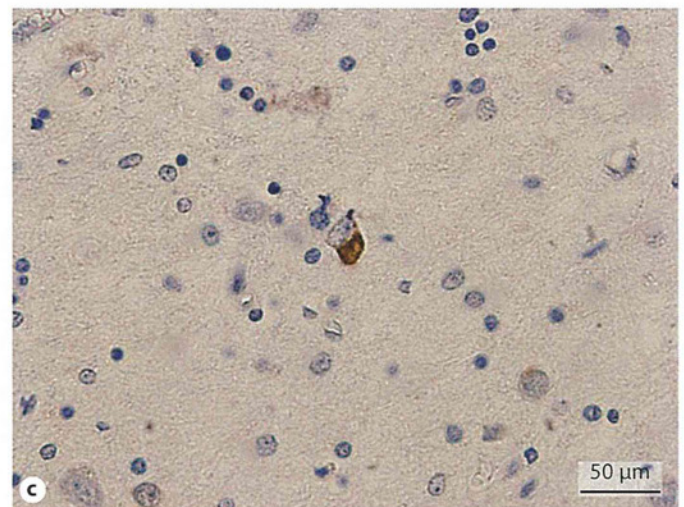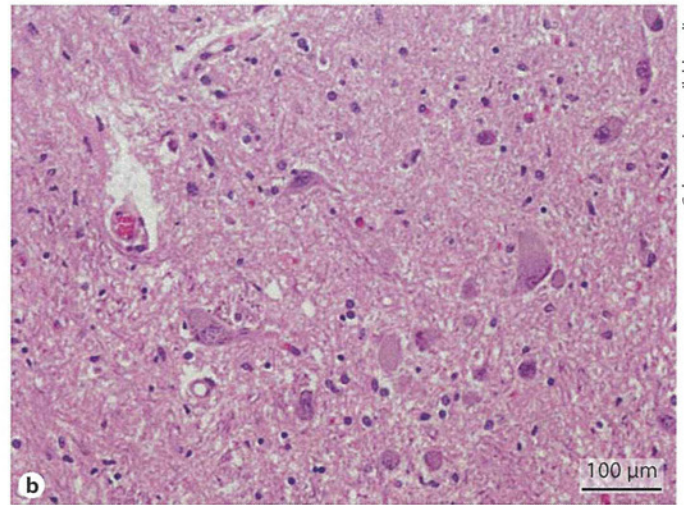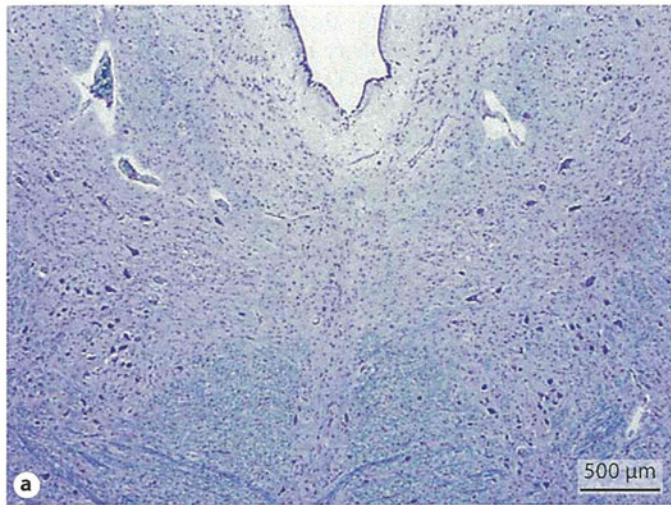
Fig. 2. Pathological findings of case 2. Neurons of the hypoglossal nucleus are relatively preserved in number (**a**) and show minimal gliosis (**b**). FUS-immunoreactive intracytoplasmic neuronal inclusions are observed in the temporal lobe cortex (**c**). Klüver-Barrera staining (**a**), hematoxylin and eosin staining (**b**), and anti-FUS antibody immunostaining (**c**; p403/404, Cosmo Bio, Calif., USA; 1:1,000).

sporadic cases of FTLD-TDP [9, 10]. The major clinical diagnosis of such cases was FTD with or without motor neuron disease. Although gene analysis was not performed in our two cases, degeneration of both upper and lower neurons was shown in such cases; therefore, the clinicopathology was different from our cases.

There are few reported cases of progressive dysarthria. De Koning et al. [11] and Becker et al. [12] reported cases of primary lateral sclerosis [11, 12]. Santens et al. [13] reported 2 cases of progressive dysarthria. Soliveri et al. [14] reported 2 corticobasal degeneration cases, 1 FTD case, 1 progressive aphasia case, 1 ALS case, 1 motor neuron disease plus dementia case, and 1 ALS plus aphasia case. However, these are clinical diagnoses and pathological examinations were not performed.

Broussole et al. [15] suggested a unique syndrome called 'slowly progressive anarthria'. In this syndrome, apraxia of speech in the initial stage and dysarthria, aprosodia and oral-facial apraxia in the advanced stage are core clinical features. Other features are mute, pseudobulbar palsy, frontal lobe impairment, ultimately evolving into anterior opercular syndrome. The lesions responsible, based on the neuroradiological and neuropathological findings, are thought to be in the posterior part of the left frontal lobe. The distribution of these lesions and their slow progression are different from our cases.

Moreover, several autopsied cases of rapidly progressive motor aphasia and motor neuron disease have been reported [16–18]. Most of these cases present anarthria, dysgraphia, and FTD after the initial stage. The left frontal lobe is a common site of lesions, and not all cases

showed hypoglossal lesions. Although symptom chronology differs in our two cases, they have a common mechanism of partial speech disorder. To conclude, we believe a subgroup of motor neuron disease with dementia, initially presenting with pseudobulbar dysarthria due to bilateral upper motor neuron dominant degeneration, may exist and should be considered as part of the diagnosis.

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Disclosure Statement

The authors report no disclosures relevant to the manuscript.

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