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SMN2 gene copy number affects the incidence and prognosis of motor neuron diseases in Japan

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Abstract

Background The copy number status (CNS) of the survival motor neuron (*SMN*) gene may influence the risk and prognosis of amyotrophic lateral sclerosis (ALS) and lower motor neuron diseases (LMND) other than spinal muscular atrophy (SMA). However, previous studies of this association, mainly from Europe, have yielded controversial results, suggesting possible regional differences. Here, we investigated the effect of the *SMN* gene in Japanese patients with ALS and LMND.

Methods We examined the *SMN* copy numbers and clinical histories of 487 Japanese patients with sporadic ALS (281 men; mean age at onset 61.5 years), 50 with adult LMND (50 men; mean age at onset 58.4 years) and 399 Japanese controls (171 men; mean age 62.2 years). Patients with pathogenic mutations in ALS-causing genes were excluded. *SMN1* and *SMN2* copy numbers were determined using the droplet digital polymerase chain reaction.

Results The frequency of a copy number of one for the *SMN2* gene was higher in patients with ALS (38.0%) than in healthy controls (30.8%) (odds ratio (OR) = 1.37, 95% confidence interval (CI) = 1.04–1.82, $p < 0.05$). The *SMN2* copy number affected the survival time of patients with ALS (median time: 0 copies, 34 months; 1 copy, 39 months; 2 copies, 44 months; 3 copies, 54 months; log-rank test, $p < 0.05$). Cox regression analysis revealed that the *SMN2* copy number was associated with increased mortality (hazard ratio = 0.84, 95% CI = 0.72–0.98, $p < 0.05$). Also, null *SMN2* cases were significantly more frequent in the LMND group (12.0%) than in the control group (4.8%) (OR = 2.73, 95% CI = 1.06–6.98, $p < 0.05$).

Conclusions Our findings suggest that *SMN2* copy number reduction may adversely affect the onset and prognosis of MND, including ALS and LMND, in Japanese.

Keywords ALS, SMA, LMND, SMN, Copy number status

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Background

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset upper and lower motor neuron disease (MND) with a diverse genetic background; 5–10% of ALS case are familial, and more than 30 genes are involved, including *TARDBP*, *FUS*, *TBK1*, *c9orf72*, *and SOD1* [1–3]. In addition, several related genes have been reported to influence disease development, one being *SMN*, whose copy number status (CNS) appears to be of importance in this context [3, 4].

SMN has two homologs, *SMN1* and *SMN2*, which are the causative and disease-modifying genes of spinal muscular atrophy (SMA), a lower motor neuron disease that develops in early childhood [5, 6]. SMA is caused by a deficiency of *SMN1*, and the severity is reduced as the copy number of *SMN2* increases [6]. Differences in *SMN1* and *SMN2* messenger RNA (mRNA) splicing are the major factor in the pathomechanism of SMA. *SMN1* mRNA produces normal SMN protein, whereas mRNA derived from *SMN2* produces mostly unstable SMN protein, although a small amount undergoes normal splicing [7]. The amount of normal SMN protein produced plays a major role in determining the severity of SMA [6].

ALS and SMN are also associated with dysregulation of nuclear function. Nucleolar gemini bodies (GEM), in which the SMN protein is a major component, are involved in the maturation of functional small nuclear RNAs (snRNAs) and play an important role in mRNA splicing [8]. In SMA-affected tissues, the levels of SMN protein are markedly reduced, resulting in GEM depletion and impairment of mRNA splicing function [9]. Interestingly, in ALS, GEM are decreased in affected tissues, and snRNA expression is also altered [10, 11], suggesting that *SMN* CNS could be a noteworthy factor in ALS.

Table 1 Differences in *SMN1* and *SMN2* copy number in control cases by country/region

***SMN1* CNS**	**China [23]**	**Taiwan [24]**	**France [13]**	**Netherlands [14]**	**Mali [25]**
0 (%)	0.0	0.0	0.0	0.0	0.0
1 (%)	2.4	2.1	2.1	2.3	0.5
2 (%)	89.7	90.3	95.5	94.1	47.0
3 (%)	7.0	7.5	2.4	3.6	39.6
4 (%)	1.0	0.2	0.0	0.0	12.9
Total n=	1712	107,611	621	984	628
***SMN2*CNS**	**China** [23]	**Taiwan** [24]	**France** [13]	**Netherlands** [14]	**Mali** [25]
0 (%)	5.2	4.6	8.4	7.9	23.9
1 (%)	29.0	31.9	38.5	37.8	43.9
2 (%)	61.1	60.5	51.7	49.4	27.4
3 (%)	3.9	2.8	1.4	4.7	1.8
4 (%)	0.9	0.2	0.0	0.2	0.6
Total n=	1712	107,611	621	984	613

Country names followed the notation in the referenced papers

A number of studies have examined the association between *SMN* CNS and ALS [12–18], and a recent large-scale investigation found no link between *SMN* CNS and the development and prognosis of the disease [19]. That study, however, and most of the previous ones, were conducted in Europe, suggesting the need for a wider survey of regional differences in genetic background [4].

The frequency of genetic mutations often varies widely by region and can influence the diagnosis of ALS and formulation of treatment strategies. For example, the most frequent ALS-causing variant in Caucasians is the hexanucleotide repeat expansion of *C9orf72* [20, 21]; however, this mutation is very rare in Asians [22]. Furthermore, *SMN* CNS in controls varies widely in Europe [13, 14], Asia [23, 24], and Africa [25] (Table 1). Regarding the association between *SMN* CNS and MND in East Asia, two studies from Korea have reported that deletion of the *SMN2* gene is involved in the development of ALS [16] and lower motor neuron disease (LMND) [26], although each of those studies involved only a small number of cases.

As the effects of *SMN* CNS on ALS and LMND may differ between Europe and Asia, a larger study is warranted. Here, we examined the association between *SMN* CNS and the onset and outcome of ALS or LMND in Japanese patients.

Methods

This study included 487 Japanese patients with sporadic ALS (SALS) (281 men, 206 women; mean age at onset 61.5 years; bulbar onset 121 patients), 50 Japanese patients with adult lower motor neuron disease (LMND) (50 men; mean age at onset 58.4 years) and 399 Japanese controls (171 men, 228 women; mean age at sampling 62.2 years) (Table 2). Of the 487 ALS cases, 440 were registered in the Japanese Consortium for Amyotrophic Lateral Sclerosis (JaCALS) data bank, and 47 were autopsy cases at Niigata University. The LMND patients were 50 adults who were negative for spinal and bulbar muscular atrophy (SBMA) and SMA by genetic testing among 100 consecutive patients who had requested genetic testing for SBMA at Niigata University. For this reason, all of the LMND patients were male. As controls, we also included 299 spouses of patients with ALS registered in the JaCALS. Another 100 of the controls were patients with other diseases, including 41 with spinocerebellar ataxia(SCA) (SCA3: 3 cases, SCA6: 10 cases, SCA31: 7 cases, DRPLA: 1 case, undetermined: 20 cases), 5 with early onset SCA (EAOH: 3 cases, undetermined: 2 cases), 4 suspected of Huntington's disease (HD: 1 case, undetermined: 3 cases), 42 with leukoencephalopathy or cerebral small vessel disease (CADASIL: 2 cases, HDLS: 1 case, undetermined: 39 cases), 5 with parkinsonism (undetermined: 5 cases), 2 with dementia (undetermined: 2

Table 2 Characteristics and *SMN1* or *SMN2* copy number groups in this study

	Control (*n* = 399)	ALS (*n* = 487)	LMND (*n* = 50)	Control vs. ALS OR (95% CI)	Control vs. LMND OR (95% CI)
Characteristics					
Age (years) Mean (SD)	62.2 (11.0)	61.5 (11.1)	58.4 (12.6)	-	-
M/F	171/228	281/206	50/0	-	-
SMN1 copies					
1 copy	5 (1.3%)	4 (0.8%)	0 (0%)	0.65 (0.20–2.17)	-
2 copies	364 (91.2%)	457 (93.4%)	47 (94.0%)	1.46 (0.88–2.42)	1.51 (0.46–4.82)
3 copies	28 (7.0%)	23 (4.7%)	2 (4.0%)	0.66 (0.37–1.13)	0.55 (0.13–2.10)
4 copies	2 (0.5%)	3 (0.6%)	1 (2.0%)	1.23 (0.25–6.96)	4.05 (0.27–35.2)
SMN2 copies					
0 copy	19 (4.8%)	35 (7.2%)	6 (12.0%)	1.55 (0.88–2.75)	2.73 (1.06–6.98)*
1 copy	123 (30.8%)	185 (38.0%)	14 (28.0%)	1.38 (1.04–1.81)*	0.87 (0.46–1.68)
2 copies	251 (62.9%)	260 (53.4%)	29 (58.0%)	0.68 (0.52–0.89)*	0.81 (0.45–1.47)
3 copies	6 (1.5%)	7 (1.4%)	1 (2.0%)	0.96 (0.35–2.87)	1.34 (0.11–8.43)

* $P < 0.05$

cases), and 1 normal control seen at Niigata University. The age at onset and the prognosis of the ALS cases were investigated on the basis of the JaCALS registry information and autopsy summaries. The LMND patients were not followed up and only age at onset was investigated based on the order sheets for genetic testing. The date when individual patients had first noticed symptoms was denoted as the onset of ALS or LMND. The date of death from any cause, or the introduction of invasive tracheostomy ventilation was set as the endpoint of ALS.

The JaCALS method for extraction of DNA has been reported previously [27]. DNA samples from autopsy cases were also collected from central nervous system (occipital lobe, motor cortex, and cerebellum) tissue using a DNA extraction kit (QIAamp® DNA Mini Kit Cat. No. 56304; Qiagen, Venlo, Netherlands). DNA of LMND patients was extracted from blood samples.

We examined the causative genes of ALS and excluded those with pathological mutations to assess the effects of *SMN* copy number more accurately. For 344 of the JaCALS-registered patients, a comprehensive analysis had been conducted previously [27], and the remaining 96 had undergone analysis of repeat expansions in *C9orf72* [28] and high-frequency causative gene mutations (*SOD1*, *TDP-43* and *FUS*) using high-resolution melting (HRM) [29]. For the 47 autopsy cases, we performed comprehensive Illumina NovaSeq 6000 exome analysis by outsourcing (Takara Bio, Shiga. Japan) and excluded patients with non-synonymous or truncated variants of specific genes (*TARDBP*, *OPTN*, *FUS*, *SOD1*, *TBK1*, *SQSTM1*, *MATR3*, *TUBA4A*, *NEK1*, *HNRNPA2B1*, *VCP*, *ELP3*, *SETX*, *HNRNPA1*, *CCNF*, *VAPB*, *C21orf2*, *CHCHD10*, *NEFH*, *ANG*, *DCTN1*, *CHMP2B*, *UBQLN2*, *Fig. 4*, *PFN1*, *ARHGEF28*, *EWSR1*, *TAF15*, *ANXA11*, *DAO*, *ERBB4*, *MAPT*, *TIA1*, *GLE1*, *PRPH*, *C9orf72*, *ALS2*, *SPG11*, *SIGMAR1* and *DNAJC7*) and *C9orf72* repeat expansions. ALS causative gene mutations were observed in five JaCALS cases and three Niigata University cases. These 8 cases are not included in the 487 cases analyzed in this paper. In the LMND group, the number of CAG repeats in the *AR* gene was determined in order to exclude SBMA, and cases with 0 copies of the *SMN1* gene, i.e., SMA cases, were also excluded.

SMN1 and *SMN2* copy numbers were determined using TaqMan® droplet digital PCR (ddPCR) on a QX200 system (Bio-Rad Laboratories, Hercules, CA, USA) and ddPCR™ Supermix for Probes (No dUTP) (Cat. No. 1863024; Bio-Rad Laboratories). *BCKDHA* was used as a reference gene [30]. Primer and TaqMan probe sequences were designed for distinguishing between single base differences in exon 7 of the *SMN1* and *SMN2* genes [30]. The thermal cycler settings were as follows: (1) 94°C for 10 min, (2) 94°C for 30 s, (3) 50°C for 2 min, (4) return to step 2) 49 times, (5) 90°C for 10 min, and (6) maintain at 4°C. The copy number of the target gene was determined as the ratio of the number of droplets positive for the target genes to those positive for *BCKDHA*.

To test for differences in the frequency of each *SMN* CNS between ALS or LMND patients and controls, the Fisher exact test was applied. Kaplan-Meier survival curves for the *SMN1* or *SMN2* copy number groups in ALS were compared, and a log-rank test was performed. Cox regression analysis was used to determine independent prognostic factors for survival, after adjusting for *SMN1* or *SMN2* copy number, sex, type of onset (bulbar or other), and age at onset. Each Cox regression analysis was performed independently, since the copy numbers of *SMN1* and *SMN2* are related. GraphPAD Prism 10 was used for all statistical analyses.

Results

The distribution of *SMN1* and *SMN2* copy numbers is summarized in Tables 2 and 3. Most control cases had two copies of *SMN1*: more than 90% of individuals had two copies, 7.0% had three copies, and only 1.3% had one copy of *SMN1*. The frequency of *SMN1* copy numbers did not differ significantly between ALS patients and the control group. In contrast, genetic variations were observed in the copy numbers of *SMN2* in both groups. In the control group, 4.8% had null alleles, 30.8% had one copy, 62.9% had two copies, and 1.5% had three copies. The presence of one *SMN2* copy was more common in patients with ALS (38.0%) than in the controls (OR=1.38, 95% CI=1.04–1.81, $p<0.05$).

We classified ALS patients based on their *SMN1* and *SMN2* copy number and investigated their survival time (Fig. 1a, b). This revealed no significant differences among the groups classified according to *SMN1* copy number (Fig. 1a). However, classification by *SMN2* copy number revealed significant differences in survival time. Patients with the *SMN2* null allele showed shorter survival than the other patients [median/mean survival: null *SMN2* ($n=35$)=34/38.6 months; one copy ($n=185$)=39/62.0 months; two copies ($n=260$)=44/61.0 months; three copies ($n=7$)=54/66.6 months; log-rank test, $p<0.05$] (Fig. 1b). Cox regression analysis revealed that age at onset ($p<0.001$) and *SMN2* copy number (hazard ratio=0.84, 95% CI=0.72–0.98, $p<0.05$) were independently associated with the mortality rate.

SMN2 copy number had no effect on the age at onset of ALS (mean 62.9 years for null cases, 61.7 years for one copy, 61.2 years for two copies, and 63.9 years for three copies; one-way ANOVA, $p=0.77$). There was no significant difference in *SMN2* copy number between patients with bulbar onset and those with non-bulbar onset (43.0% and 36.3%, respectively, for one *SMN2* copy; chi-squared test, $p=0.19$). There was also no significant difference in *SMN2* copy number between JaCALS-registered control patients and the control patients from Niigata University (30.4% versus 32.0%, respectively, for one *SMN2* copy).

In addition, null *SMN2* cases were significantly more frequent in the LMND group than in the control group (12.0% versus 4.8%, respectively; OR=2.73, 95% CI=1.06–6.98, $p<0.05$) (Table 2). There were no statistically significant differences in *SMN1* copy number. As no prognostic data were available for the LMND group, prognostic evaluation was not performed.

Discussion

Our present study of 487 Japanese ALS patients revealed that the CNS of *SMN2* differed significantly from that in the controls: ALS patients with one *SMN2* copy were significantly more frequent, and *SMN2*-null ALS patients had a significantly worse outcome. Furthermore, in the LMND group (excluding SBMA), *SMN2*-null patients were significantly more frequent than in the control group. These findings suggest that, at least in Japanese patients with adult-onset MND, including ALS and LMND, *SMN2* copy number reduction adversely affects the onset and prognosis of MND.

However, a recent large European study showed that *SMN* copy number did not affect the risk for development of ALS or its prognosis [19]. This discrepancy may be attributable to differences in genetic background. *SMN* CNS in healthy subjects varies by region (Table 1). For example, normal individuals with only one copy of *SMN2* are reportedly more frequent among Caucasians (37.9 −42.2%) [13–15] than among Asians (29.0 −31.9%) [16, 23, 24]. A study from South Korea, located in the East Asian region as Japan, yielded results similar to those of the present study, in which *SMN2* gene deficiency was associated with the development of ALS [16] and LMND [26], although the results were based on only

Table 3 Details of *SMN1* or *SMN2* copy number groups

SMN1:SMN2 copies	Control ($n=399$)	ALS ($n=487$)	LMND ($n=50$)	Control vs. ALS OR (95% CI)	Control vs. LMND OR (95% CI)
1:1	3 (0.8%)	1 (0.2%)	0	0.27 (0.02–1.83)	-
1:2	1 (0.3%)	3 (0.6%)	0	2.46 (0.37-32.0)	-
1:3	1 (0.3%)	0	0	-	-
2:0	14 (3.5%)	29 (6.0%)	4 (8.0%)	1.74 (0.90–3.28)	2.39 (0.83–7.33)
2:1	103 (25.8%)	168 (34.5%)	13 (26.0%)	1.51 (1.13–2.03)*	1.01 (0.51-2.00)
2:2	242 (60.7%)	254 (52.2%)	29 (58.0%)	0.70 (0.54–0.93)*	0.90 (0.50–1.62)
2:3	5 (1.3%)	7 (1.4%)	1 (2.0%)	1.15 (0.40–3.22)	1.61 (0.13–12.1)
3:0	4 (1.0%)	3 (0.6%)	1 (2.0%)	0.61 (0.15–2.29)	2.02 (0.16–12.5)
3:1	17 (4.3%)	16 (3.3%)	1 (2.0%)	0.76 (0.40–1.57)	0.46 (0.04–2.76)
3:2	7 (1.8%)	3 (0.6%)	0	0.35 (0.10–1.24)	-
4:0	1 (0.3%)	3 (0.6%)	1 (2.0%)	2.47 (0.37–32.1)	8.12 (0.42–154.5)
4:2	1 (0.3%)	0	0	-	-

* $P<0.05$

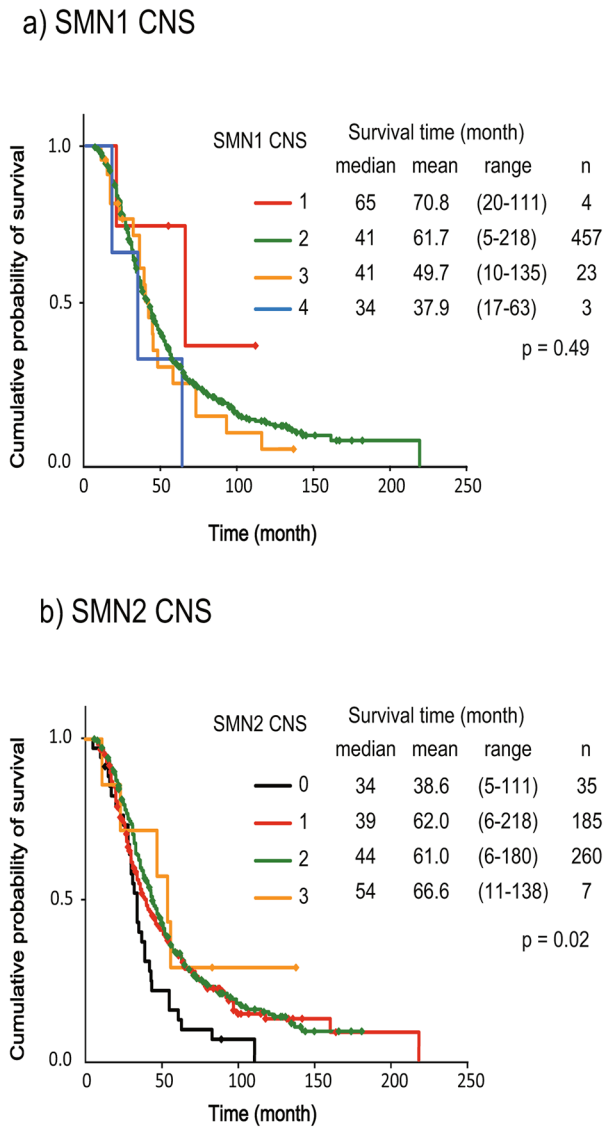

Fig. 1 Kaplan-Meier analysis of survival time in the (**a**) *SMN1* and (**b**) *SMN2* copy number groups. Survival time differed between the *SMN2* copy number groups (Cochran Mentel Haenszel test; $p = 0.02$)

a small number of cases. These results suggest that *SMN2* copy number reduction may adversely affect the incidence and prognosis of adult-onset MND in East Asia, including Japan and South Korea.

The main issue raised by this study is why the copy number of *SMN2*, and not that of *SMN1*, affects the development and prognosis of ALS and LMND, even though most of the SMN protein is expressed from the *SMN1* gene. A simple decrease in the SMN protein level due to *SMN2* gene reduction cannot explain the development of MND and its prognostic impact.

There are several hypotheses that could explain this situation: the first is that the *SMN2*-derived defective SMN protein has physiological importance. There are several rare isoforms of SMN in addition to the normal type, and axonal SMN with intron 3 retention plays an important role in mammalian brain [31]. However, the Δ7 SMN protein, which is produced mainly from *SMN2*, is less stable because it lacks the exon 7-derived amino acid [31] and no important physiological function has been reported for it to date.

A second possibility is that *SMN2* may have tissue-specific importance, as the splicing patterns of *SMN2* mRNA vary from tissue to tissue. In the testes of mice experimentally expressing *SMN2*, the splicing pattern of *SMN2*-derived mRNA is greatly altered and full-length SMN protein is produced in large amounts [32, 33]. *SMN2* CNS changes would be important if *SMN2* mRNA splicing is altered and involved in normal SMN protein expression in the human nervous system, muscle, and cardiopulmonary tissues, and would influence ALS pathogenesis or prognosis, as in the mouse testis model. Currently, however, there is no such evidence, and therefore this possibility remains merely speculative.

A third possibility is simultaneous loss of genes near *SMN2*. *NAIP*, located close to *SMN1*, is reportedly associated with ALS [34], whereas *SERF1B*, located close to *SMN2*, has not yet been linked to any neurological disease. Thus, the mechanism by which the CNS of *SMN2* influences the onset and prognosis of adult MND remains unclear and requires further investigation.

One of the limitations of this study was the small number of cases examined in comparison to recent large-scale studies. The proportion of SMA carriers in our reported control cohort does not differ significantly from previously reported data in Japan [35], suggesting that the analysis group in this study reflects the *SMN* CNS status in Japan to a certain extent. However, there are limitations to what can be concluded from an analysis based on several hundred cases. Additional studies of East Asian populations are therefore warranted. In addition, as the LMND group comprised only male patients, and prognosis evaluation has not been conducted. Future studies should also include female patients and investigate the relationship between the prognosis of LMND cases and *SMN* CNS. Additionally, several mutations in the *SMN1* and *SMN2* genes are known, and these may also impact the disease prognosis of SMA [36]. The influence of these genetic mutations on ALS and LMND is an important subject for future investigation.

This study has shown that the CNS of *SMN2* affects the incidence and prognosis of ALS and LMND in Japanese patients, contrary to the results of previous studies in Europe, suggesting regional differences in genetic background.

Abbreviations

CNS	Copy number status
SMN	Survival motor neuron

ALS	Amyotrophic lateral sclerosis
LMND	Lower motor neuron diseases
SMA	Spinal muscular atrophy
OR	Odds ratio
CI	Confidence interval
MND	Motor neuron disease
mRNA	Messenger RNA
snRNAs	Small nuclear RNA
SALS	Sporadic ALS
JaCALS	Japanese Consortium for Amyotrophic Lateral Sclerosis
SBMA	Spinal and bulbar muscular atrophy
SCA	Spinocerebellar ataxia
DRPLA	Dentatorubral-Pallidoluysian Atrophy
EAOH	Early onset ataxia associated with hypoalubuminemia and oculomotor apraxia
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
HDLS	Hereditary diffuse leukoencephalopathy with axonal spheroids
HRM	High-resolution melting
ddPCR	Droplet digital PCR

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Author contributions
TI, AKo, AY, and OO contributed to the conception and design of the study. TI wrote the main manuscript text and prepared all figures and tables. TI, AKo, ST, and KK analyzed the genomic data for SMN CNS. TI, NA, SH and YH performed ALS-related gene mutation analysis of the target case genes. NA, RN, GT, YI, RK, MM, AT, OK, MA, SK and GS contributed to the data collection and management of cases from JaCALS. MT and AKa contributed to the data collection and management of autopsy cases. TI, SH and OO contributed to the data collection and management of LMND cases. Statistical review was performed by TI, AY, YH and NA. All authors reviewed the manuscript.

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Data availability
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval was obtained from the research ethics committees of Niigata University (G2015-0781, G2020-0031) in accordance with the Declaration of Helsinki. ALS cases and their spouses participating in JaCALS have given written consent for genetic samples and clinical data to be provided for JaCALS and its collaborations. In the patients of genetic testing at Niigata University, written consent was obtained to use genetic information for research purposes. Written informed consent for autopsy cases, including the use of tissues for research purposes, was obtained from the families of the patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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