Survival motor neuron protein in the spinal anterior horn cells of patients with sporadic amyotrophic lateral sclerosis

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Abbreviations: ALS, amyotrophic lateral sclerosis; AHC, anterior horn cell; SMA, spinal muscular atrophy; SMN, survival motor neuron; IOD, integrated optical density; SOD1, superoxide dismutase; TDP-43, TAR DNA-binding protein

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease involving mainly the upper and lower motor neurons of adult humans. With regard to the pathomechanism of spinal anterior horn cell (AHC) degeneration in ALS, copy number abnormalities of the survival motor neuron (SMN) genes have been reported in sporadic (s) ALS. SMN protein is the protein responsible for the pathogenesis of spinal muscular atrophy (SMA), an autosomal recessive disease characterized by lower motor neuron loss and muscle atrophy. The disease is caused by deficiency of SMN protein induced by mutation of one of the SMA-associated genes, SMN1. To clarify the role of SMN protein in the degeneration of spinal AHCs in sALS, we examined the amount of cytoplasmic SMN protein in individual AHCs using cytofluorophotometry in 9 patients with sALS and 10 control subjects. It was found that: 1) SMN protein was present in

the cytoplasm, nucleus and nucleolus of AHCs and in the nucleus of glial cells, 2) expression of SMN protein in AHCs was significantly associated with cell size in both sALS patients and controls, 3) expression of SMN protein per unit area in AHCs was similar in sALS patients and controls, These findings suggest that: 1) the amount of SMN protein in the cytoplasm of AHCs is strictly controlled in accordance with cell size, in both sALS patients and controls, 2) the amount of SMN protein in the AHCs of sALS patients may be reduced when the AHCs are atrophic, and 3) decrease of SMN protein in the AHCs of sALS patients may be a secondary, and not primary, phenomenon according to their sizes.

Key words: amyotrophic lateral sclerosis, anterior horn cell, survival of motor neuron protein

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration of lower motor neurons and muscle atrophy (Lefebvre et al., 1995). The majority of cases result from homozygous deletions or mutations of the survival motor neuron 1 (SMN1) gene. In humans, the SMN gene exists as two highly homologous copies, the telomeric copy (SMN1), which encodes the full-length protein, and the centromeric copy (SMN2), which encodes a truncated isoform (Lorson et al., 1999; Monani et al., 1999). The SMN1 gene is homozygously deleted in approximately 95% of SMA patients (Bussaglia et al., 1995; Velasco et al., 1996) and the SMN2 gene plays a role in modulating the severity of the phenotype (Anderson and Talbot, 2003).

Furthermore, the level of SMN protein is markedly reduced in the spinal cords of patients with SMA (Coovert et al., 1997; Lefebvre et al., 1997). It has been considered that defective SMN protein disrupts normal cellular RNA metabolism, thus causing motor neuron degeneration (Kolb et al., 2007).

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease involving mainly the upper and lower motor neurons of adult humans. Among patients with ALS, 5-10% have the familial form, 20% of such cases being associated with mutation in the Cu/Zn superoxide dismutase (SOD1) gene on chromosome 21 (Rosen et al., 1993). The remaining 90-95% of ALS cases are sporadic, and do not have an obvious family history (Schymick et al., 2007). The pathological hallmarks of sporadic (s) ALS are loss of spinal anterior horn cells (AHCs) and degeneration of the corticospinal tract, and the presence of Bunina bodies and ubiquitinated skein-like inclusions in the spinal cord (Kato et al., 2003; Piao et al., 2003; Tomonaga et al., 1978; Kato et al., 1989). Although the cause of sALS remains unknown in the great majority of cases, it is believed to be a multifactorial disease (Figlewicz and Orrell, 2003). With regard to the pathomechanism of AHC degeneration in ALS, Corcia et al. (2002a) reported that within three families ALS and SMA were concurrent. More recently, copy number abnormalities of the SMN genes have been reported in sALS. The frequency of patients with one or three abnormal copies of the SMN1 gene was reported to be significantly increased in sALS cases, although many control subjects show two copies (Corcia et al., 2002b; Corcia et al., 2006), suggesting that the gene may be involved in sALS (Corcia et al., 2009). In addition, homozygous deletions of SMN2 are suspected to act as a susceptibility factor for

ALS mainly involving lower motor neurons in adults (Moulard et al., 1998; Echaniz-Laguna et al., 2002, Kim et al. 2010), as well as a prognostic factor affecting survival time in patients with sALS (Veldink et al., 2001). Veldink et al. (2005) speculated that a smaller SMN protein might be expressed, based on a formula that takes into account the SMN1 and SMN2 gene copy numbers in sALS. Recently it has also been reported that reduction in the level of SMN protein in the spinal cord contributes to the pathogenesis of motor neuron death in transgenic SOD1 mice with G93A mutation, which has been used as a model of SOD1-linked ALS (Turner et al., 2009).

In addition, TAR DNA-binding protein (TDP-43) has recently been identified as the major disease protein of sALS (Neumann et al., 2006, Arai et al., 2006). Bose et al. (2008) have reported that TDP-43 overexpression enhances exon 7 inclusion during SMN2 pre-mRNA splicing, although global splicing in the cells remains to be investigated. Thus, SMN protein is becoming an increasing focus of attention in sALS research. However, no previous studies have examined the level of SMN protein in individual spinal AHCs in sALS patients.

In the present study, we examined the amount of SMN protein in individual spinal AHCs from sALS patients and controls using cytofluorophotometry of sections immunostained for SMN.

2. Results

2.1. Expression of SMN protein in the lumbar spinal cord

SMN protein was observed in both AHCs and glial cells in controls as well as sALS patients. SMN immunoreactivity was observed in the cytoplasm, nucleolus

and nucleoplasm of the AHCs, the expression corresponding to that seen by immunohistochemistry using bright field and immunofluorescence microscopy (Fig. 1A (MO1 antibody), Figs. 2E-2H (H-195 antibody)). Absorption by the SMN1 recombinant protein (P01: H00006606, Abnova) was tried for MO1 antibody, and it failed to show the positive immunostainability (Fig. 1B). In the cytoplasm, SMN protein was localized in the soma and proximal portion of neurites. In the nucleus, SMN immunolabeling was observed at the nuclear membrane, the nucleolus, and fine dot-like structures in the nucleoplasm, as reported previously (Liu and Dreyfuss, 1996). On the other hand, expression of SMN protein was also noted in glial cell nuclei in the spinal cord in the present study (Fig. 1A, Figs. 2E and 2H).

As in the controls (Figs. 2A and 2E), SMN protein expression was observed in the cytoplasm, nucleolus, nucleoplasm and nuclear membrane of chromatolytic (Figs. 2C and 2G) and shrunken (Figs. 2D and 2H) AHCs, and also in normal-looking AHCs (Figs. 2B and 2F) in ALS patients.

2.2. Amount of cytoplasmic SMN protein in AHCs of the lumbar spinal cord

We examined the amount of cytoplasmic SMN protein using immunofluorophotometry. We plotted in Figure 3 the integrated optical density (IOD) of SMN protein against the area of cytoplasm in the AHCs of the lumbar spinal cord based on data for 129 AHCs from sALS patients and 144 AHCs from controls. The IOD of the SMN protein in each AHC showed a cell size-dependent tendency. The amount of SMN protein per sectional area of the cytoplasm in

each AHC did not differ between sALS patients and controls. To elucidate the similarity or difference among the large and small AHCs, we categorized 2 types of neurons by size: small (cytoplasmic sectional area between 500 μm2 and 1500 μm^2) and large (more than1500 μm^2). Table 2 shows the IOD of SMN protein per unit area and the amount of IOD of SMN protein. A two-way analysis of variance (ANOVA) was performed for the IOD of SMN protein per unit area. However, the main effects and interaction did not reach statistical significance. We also performed a two-way ANOVA for the amount of IOD of SMN protein. There were significant main effects of group ($F(1/269) = 12.92$, $p < 0.01$) and neuron size ($F(1/269) = 448.43$, $p < 0.01$). An interaction was also significant ($F(1/269) = 24.70$, $p < 0.01$). *Post hoc* test revealed that, in small neurons, the amount of IOD in the sALS group were significantly lower than that in the control group ($p < 0.05$). This was considered to be due to the larger number of small AHCs in sALS patients, which was induced by shrinkage of AHCs (Figs. 2B-D, and 2F-H), than in controls.

2.3. Comparison between SMN protein amount and clinical features including disease duration, ages at the onset and death, and clinical symptoms

Amount of SMN protein in the AHCs in sALS was not correlated with disease duration, ages at the onset and death, and onset symptoms such as upper or lower motor neuron sign (Figs. 4A-D).

3. Discussion

The SMN protein is a 294-amino-acid polypeptide that is expressed in various human tissues and cell types (Coovert et al., 1997; Lefebvre et al., 1997). Consistent with previous immunohistochemical analyses of adult and fetal human spinal cord (Francis et al., 1998; Briese et al., 2006), our present study revealed SMN expression in the cytoplasm and nucleus of AHCs in the adult human spinal cord of controls and patients with sALS. Within the cytoplasm, SMN was localized in the soma and the proximal portion of neurites, whereas in the nucleus, SMN immunolabeling was observed at the nuclear membrane, nucleolus, and dot-like structures in the nucleoplasm. It was of interest that prominent astrocytic and oligodendroglial cell nuclei in the spinal cord were immunopositive for SMN protein. Lefebvre et al. (1997) reported no significant staining for GFAP, an astrocyte marker, in SMN-containing cells in spinal cord sections from control and SMA fetuses. However, a figure shown by Briese et al. (2006) suggests the presence of SMN protein expression in glial cells of the spinal cord from a human fetus. Our present study confirmed that SMN protein was expressed in the nuclei of spinal cord glial cells from sALS patients and controls.

Previous studies have reported that mutations in the SMN1 gene result in reduced production of functional SMN protein in autosomal recessive SMA (Lefebvre et al., 1997; Coovert et al., 1997). Several lines of evidence support the view that SMN protein is essential for the assembly and regeneration of spliceosomal small nuclear ribonucleoproteins in all cell types (Rossoll et al., 2003; Meister et al., 2002). Knockout mice with deletion of SMN exon 7 show

postnatal neuronal death (Frugier et al., 2000), suggesting that SMN expression is necessary for cell survival.

With regard to the correlation between SMN and sALS, the French ALS Study Group studied the SMN1 and SMN2 genes in 600 patients with sALS and 621 controls, and found an association of sALS with copy number abnormality (one or three copies) of the SMN1 gene, but no such association for SMN2 copy number (Corcia et al., 2006). In contrast, Veldink et al. (2005) reported that sALS patients carried fewer SMN2 copies. These previous studies suggest that the SMN gene is likely linked to sALS. Figure 5 in Coovert et al. (1997) showing a blot of SMN protein indicates a possible reduction in the level of the protein in the spinal cord of a sALS patient. These findings suggest that SMN in motor neurons may play an important role in sALS. However, the expression of SMN protein in the central nervous system of sALS patients has not been clarified.

In the present study, we demonstrated that the amount of SMN protein per unit sectional area of cytoplasm in individual AHCs was similar in sALS patients and controls, and that in both groups the expression of SMN protein was cell size-dependent. Concerning the measurement of the amount of SMN protein in the nucleus, SMN protein locates mainly in the nucleolus. The fact that nucleoli are small in size and irregular in appearance causes quite incoherent data depending on sectioning. Thus the present authors exclude the data of nucleus in the present study.

Examination of cells of different sizes revealed that large AHCs expressed a larger amount of SMN protein, whereas small AHCs expressed a small amount. All of the neurons in the anterior horn are shrunken in ALS (Kusaka and Hirano.

1985a, b). The relatively larger proportion of small neurons in sALS may be the result of atrophy of diseased AHCs, as indicated in previous studies (Oyanagi et al., 1991), and this change in the relative proportion of large and small AHCs may lead to a decrease in the total amount of SMN protein in the anterior horn of sALS patients. As represented in Figure 3, AHCs contain SMN protein according to their sizes, whether they are normal-looking, chromatolytic or simple shrinkage in sALS.

The present study revealed that: 1) SMN protein was present in the cytoplasm, nucleus and nucleolus of AHCs, as well as the nucleus of glial cells, 2) expression of SMN protein in AHCs was significantly correlated with cell size in both sALS patients and controls, 3) expression of SMN protein per unit area in AHCs was similar between sALS patients and controls, 4) none of the large AHCs exhibited any alteration in the average amount of SMN protein, in both sALS patients and controls, and 5) a relatively larger proportion of small (possibly atrophic) AHCs contained a small amount of SMN protein in sALS patients than was the case in the controls. These findings suggest that: 1) the amount of SMN protein in the cytoplasm of AHCs is strictly controlled in accordance with cell size, in both sALS patients and controls, 2) the amount of SMN protein in the AHCs of patients with sALS may be reduced when these cells become atrophic, and 3) decrease of SMN protein in the AHCs of sALS patients is a secondary, and not a primary, phenomenon according to their sizes.

In conclusion, 1) expression of SMN protein in AHCs is significantly associated with cell size in both sALS patients and controls, and 2) expression of SMN protein per unit cell size in AHCs is similar between sALS patients and

controls.

4. Materials and methods

4.1. Subjects

This study was performed in accordance with the provisions of the Declaration of Helsinki (1995), and was approved by the Ethics Committees of Tokyo Metropolitan Institute for Neuroscience, Brain Research Institute of Niigata University, Shinrakuen Hospital, and Tokyo Metropolitan Neurological Hospital. Brains and spinal cords were collected postmortem from nine patients with sALS (aged 50–84 years, mean 69.3 years) and from ten age-matched controls (aged 50–87 years, mean 64.9 years). The ages of the patients at disease onset ranged from 48 to 87 years (mean 66.9 years) and the duration of the illness ranged from 18 to 61 months (mean 28.4 months). In each patient, the diagnosis of sALS was confirmed clinicopathologically. In these patients, there was no family history of genetic disorders or pathological features suggesting complications arising from Alzheimer’s disease, Parkinson’s disease, dementia with Lewy bodies, or other neurodegenerative diseases. None of the patients had been maintained on an artificial respirator. The clinical findings in these patients are listed in Table 1 (the neuropathological features were reported previously by Oyanagi et al. (2008).

4.2. Light microscopic examination and immunohistochemistry

For light microscopic examination, the spinal cords were fixed with 10% formalin, and multiple tissue blocks were embedded in paraffin. Histological examinations

were performed on 6-μm-thick sections. These sections were stained with hematoxylin and eosin, Klüver-Barrera (K–B), or Bodian, and immunostained for ubiquitin or phosphorylated neurofilaments for neuropathological diagnosis. For SMN immunohistochemistry, sections taken at the lumbar spinal cord level were deparaffinized, rinsed, and pretreated by autoclaving in 10 mM sodium citrate buffer (pH 6.0) for 20 min at 121 ℃, followed by blocking in PBS containing 5% normal goat serum at room temperature for 30 min. The sections were then incubated with antibodies against SMN (H-195, rabbit polyclonal, dilution 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or SMN1 (M01, mouse monoclonal, dilution 1:100, Abnova Corp., Taipei, Taiwan) overnight at 4 °C. The sections were then rinsed in PBS and incubated for 2 hours with biotinylated goat anti-rabbit or horse anti-mouse IgG (dilution 1:200; Vector, Burlingame, CA, USA). Labeling was detected using the avidin-biotinylated HRP complex (ABC) system (Vector) coupled with the diaminobenzidine (DAB) reaction. Absorption by the SMN1 recombinant protein (P01: H00006606, Abnova) was tried for MO1 antibody. In addition, immunofluorescence examinations for SMN, with the same pretreatment, were performed using the rabbit polyclonal antibody against SMN (H-195, 1:25). The secondary antibody used was Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:200). The sections were then immersed in autofluorescence eliminator reagent for 1 min at room temperature to delete any of the autofluorescent pigment lipofuscin that had accumulated. AHCs were identified by the presence of a prominent nucleolus and Nissl substance and/or lipofuscin.

4.3. Quantitative examination of SMN protein in AHCs

AHCs with nucleoli, and whose maximum short diameter measured more than 20 μm perpendicular to the long diameter, and had an area exceeding 500 μm^2, being located in the 8th and 9th layers of Rexed (Rexed, B., 1952; Coimbra et al., 1986), were counted bilaterally in one section at the level of the 4th or 5th lumbar segment. Measurements were performed randomly for 129 AHCs in sALS patients and 144 AHCs in control subjects using cytofluorophotometry. The IOD for SMN immunoreactivity in the cytoplasm, and the sectional area of the cytoplasm, were measured using a Zeiss Axiovert 135 12-bit camera (Carl Zeiss, Oberkochen, Germany) and the MetaMorph software package (Universal Imaging Co., West Chester, PA, USA) (Anamizu et al., 2005; Nagasao et al., 2008). Epifluorescent microscopic observation and the analysis of the findings by MetaMorph have been used for protein quantification (Nishijima et al. 2007, Jiang et al. 2010). The IOD of SMN protein determined by cytofluorophotometry using immunostained sections was expressed as the “amount of SMN protein” in the present study. IOD per sectional area of the cytoplasm of AHCs was also determined.

4.4. Statistical analysis

Amount of cytoplasmic SMN protein and the ratio between the IOD of immunopositive SMN and sectional area of cytoplasm in the AHCs of sALS patients and controls were analyzed statistically by two-way analysis of variance (ANOVA) and Tukey-Kramer’s *post hoc* test using the Excel software package (Microsoft Ltd.). Correlations between the amount of SMN and duration of illness,

ages at onset and death, and clinical symptoms were analyzed statistically by Peason's correlation coefficient test. All values are presented as mean and standard deviation (S.D.).

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Legends

Table 1: Examined sporadic amyotrophic lateral sclerosis (sALS) patients and control subjects

Nine Japanese patients who had been clinically diagnosed and pathologically verified as having classic sALS and ten Japanese control subjects without neurological symptoms or unusual neuropathological findings were studied. None of the patients had been maintained using an artificial respirator. The site of initial symptoms from the onset of weakness in the upper and lower extremities, and the disease period were noted. Bars represent no neurological symptoms or no duration of the neurological illness in the subjects.
Abbreviations; F, female; M, male; Upper, upper extremities; Lower, lower extremities.

Table 2: Integrated optical density (IOD) of SMN (survival motor neuron) protein in AHCs (anterior horn cells) of the lumbar spinal cord with different cell sizes.

Regarding the amount of SMN protein in small (cytoplasmic sectional area between 500 μm^2 and 1500 μm^2) and large (more than1500 μm^2) AHCs, comparison between 95 small AHCs from sALS patients and 72 small AHCs from controls demonstrated no significant inter-group difference in the IOD of SMN protein per unit area . Similarly, there was no difference in the amount of SMN protein per unit area in large AHCs between the sALS patients and controls. However, in AHCs from sALS patients, which are smaller than 1500 μm^2, the average amount of SMN protein was significantly lower than in those from

controls (p <0.05), but the amount of SMN protein in AHCs with a sectional cytoplasmic area exceeding 1500 μm^2 was similar in the controls and sALS patients (p =0.63). This was considered to be due to the larger number of small AHCs in sALS patients, which was induced by shrinkage of AHCs than in controls.

Fig. 1. Immunohistochemistry of SMN (survival motor neuron) protein using MO1 antibody in the anterior horn of the lumbar spinal cord from a control subject using the diaminobenzidine (DAB) reaction. A: SMN immunoreactivity is evident in the cytoplasm, proximal portion of neuritis, nuclear membrane, nucleoplasm and nucleus of an anterior horn cell (AHC). Nuclei of glial cells are also labeled. B: Immunohistochemistry of SMN protein in the anterior horn of the lumbar spinal cord from a control subject after absorption by the SMN1 recombinant protein (P01). Scale bar; 50 μm for A and B.

Fig. 2: Histological findings of the AHCs in the lumbar spinal cord revealed by immunofluorescence. A and E; an AHC from a control subject. B and F; serial sections of a normal-looking AHC from a sALS patient. C and G; chromatolytic AHC from a sALS patient. D and H; shrunken AHC from a sALS patient. A and E, B and F, C and G, D and H, are serial sections of same AHCs. A-D; Klüver-Barrera staining, E-H; SMN protein immunofluorescence. SMN protein was present in the AHCs as well as glial cells of controls and sALS patients. Autofluorescence eliminator was performed. Scale bar for all of Figure 2; 10 μm**.**

Fig. 3: Correlation between integrated optical density (IOD) of SMN protein and area of cytoplasm in AHCs of the lumbar spinal cord. The content of SMN protein was measured in terms of IOD using SMN-immunostained 6-µm-thick sections. The IOD of SMN protein in the AHCs was cell size-dependent in both controls and sALS patients.

Fig. 4: Correlation between integrated optical density (IOD) of SMN protein/cell area of the AHCs and duration of illness (A), ages at onset (B) and death (C), and onset symptoms such as upper or lower motor neuron sign (D). Each ALS patient was distinguished by the colors in A. Dots represented means and bars were +/-S.D.

Table 1

	No. of patients/subjects	Age at death (years)	Gender	Duration of illness (months)	Site of initial symptom
ALS	1	84	M	18	Lower
	2	58	F	20	Upper
	3	50	M	22	Upper
	4	83	F	23	Upper
	5	52	M	24	Upper
	6	83	F	24	Upper
	7	74	F	29	Upper
	8	71	M	35	Lower
	9	69	F	61	Lower
Control	1	50	M	-	-
	2	54	M	-	-
	3	60	M	-	-
	4	63	M	-	-
	5	73	M	-	-
	6	55	F	-	-
	7	61	F	-	-
	8	63	F	-	-
	9	83	F	-	-
	10	87	F	-	-

Table 2

	IOD/area		Total IOD	
	Control	ALS	Control	ALS
1500 μm^2 ≤Neuron area	40.29 ± 4.17 (n=72)	41.44 ±3.97 (n=34)	76914.39 ±13936.05 (n=72)	79047.02 ±16453.70 (n=34)
500 μm^2 ≤ Neuron area < 1500 μm^2	42.04 ± 4.09 (n=72)	41.12 ± 4.18 (n=95)	51796.05 ± 7724.56 (n=72)	38521.55 ± 10922.07 (n=95) *

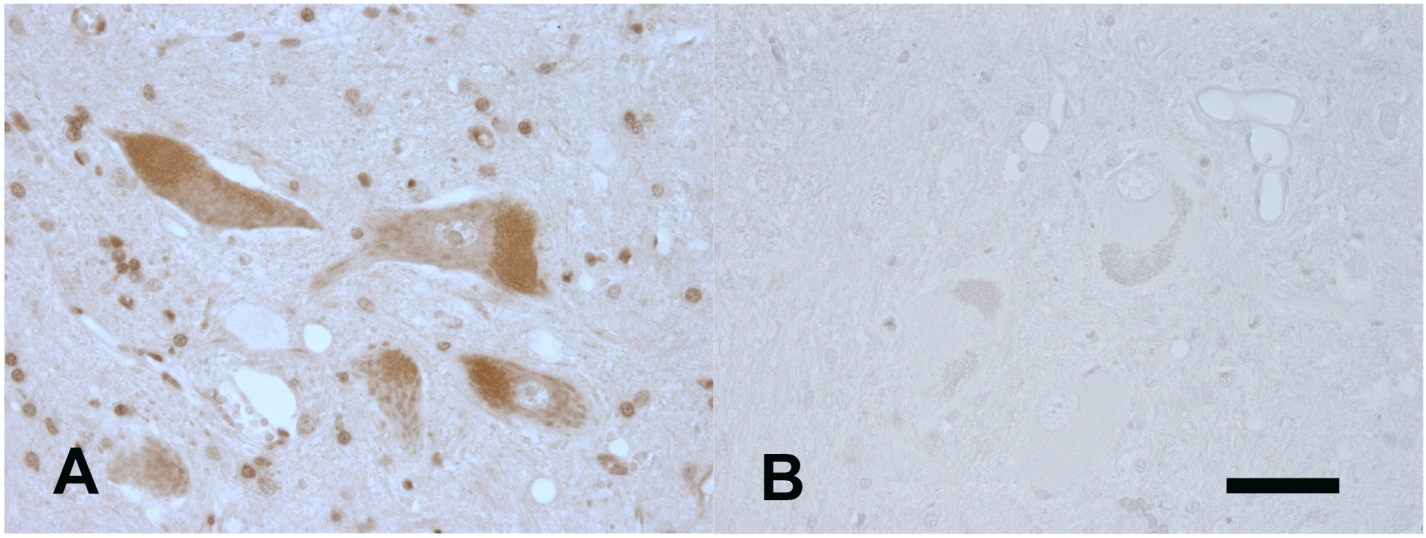

Fig. 1

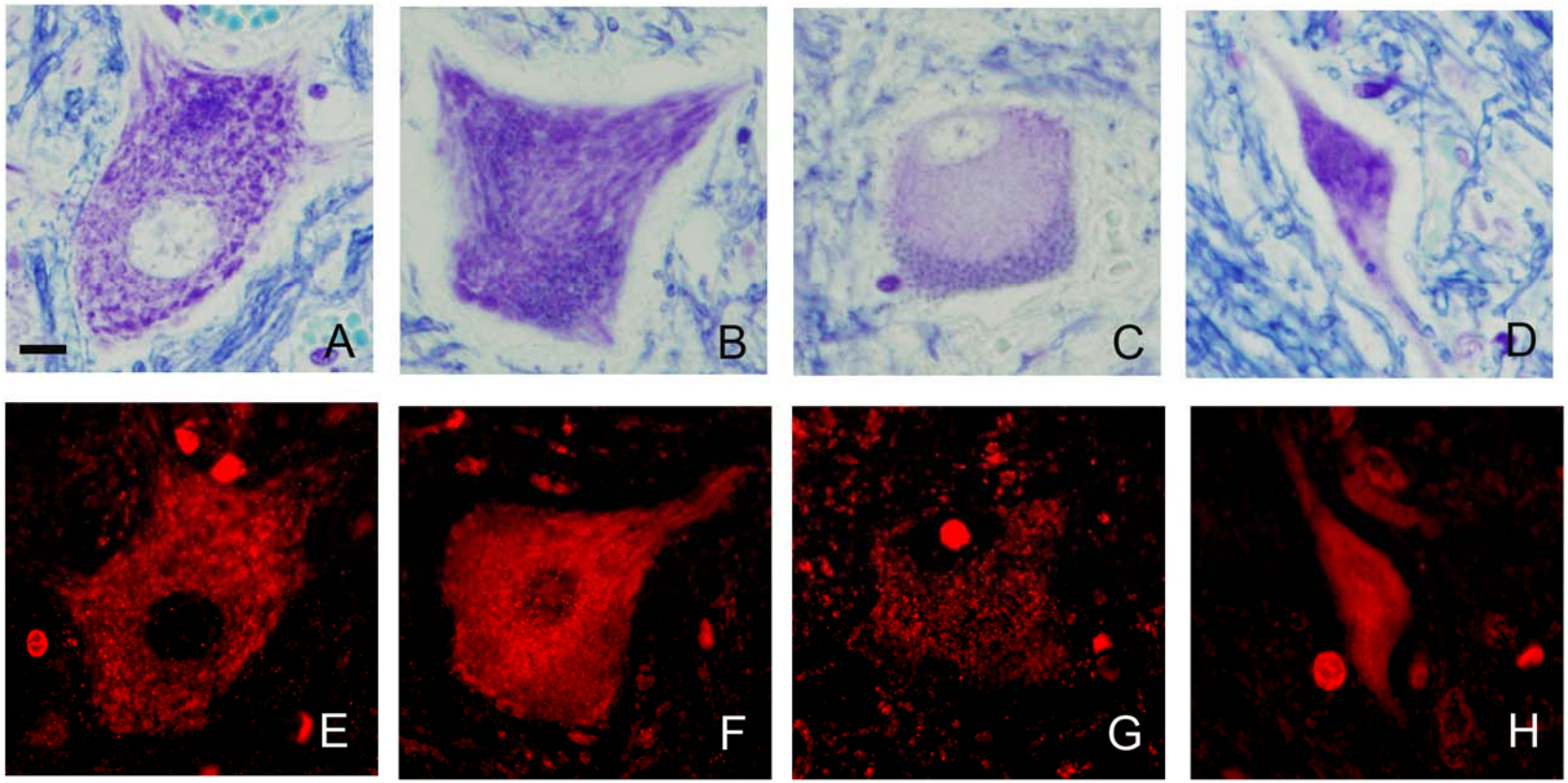

Fig. 2

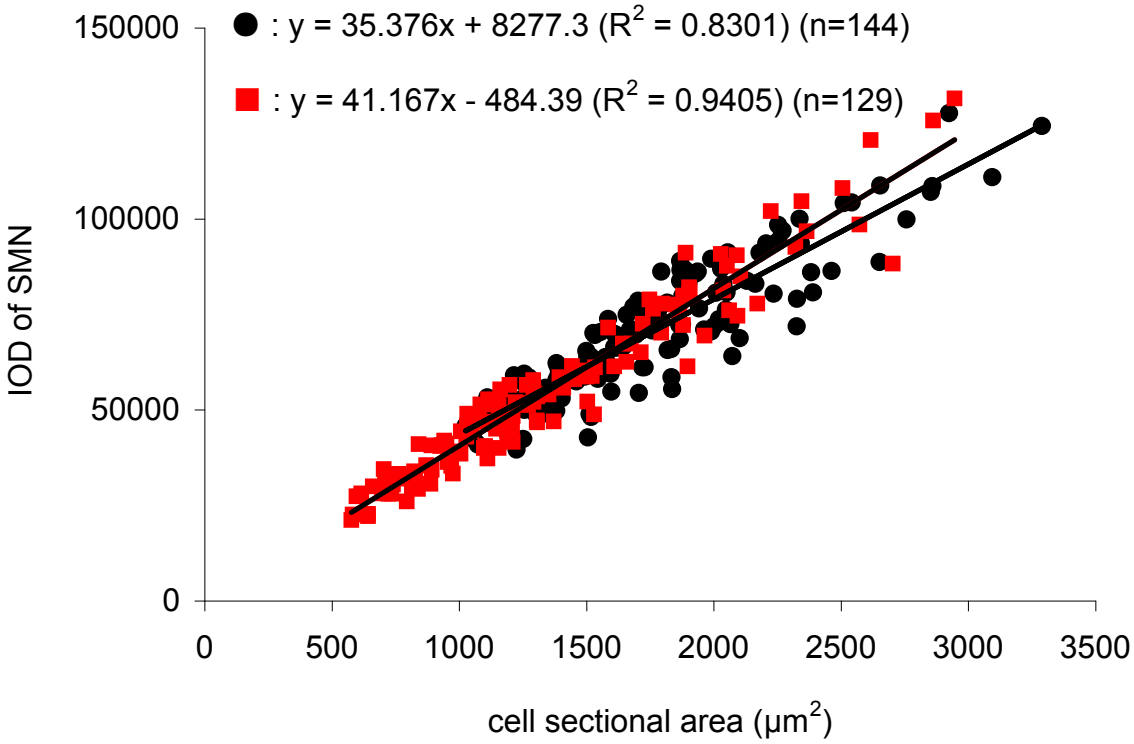
● : y = 35.376x + 8277.3 (R2 = 0.8301) (n=144)
■ : y = 41.167x - 484.39 (R2 = 0.9405) (n=129)
IOD of SMN
150000
100000
50000
0
0
500
1000
1500
2000
2500
3000
3500
cell sectional area (μm2)

Fig. 3

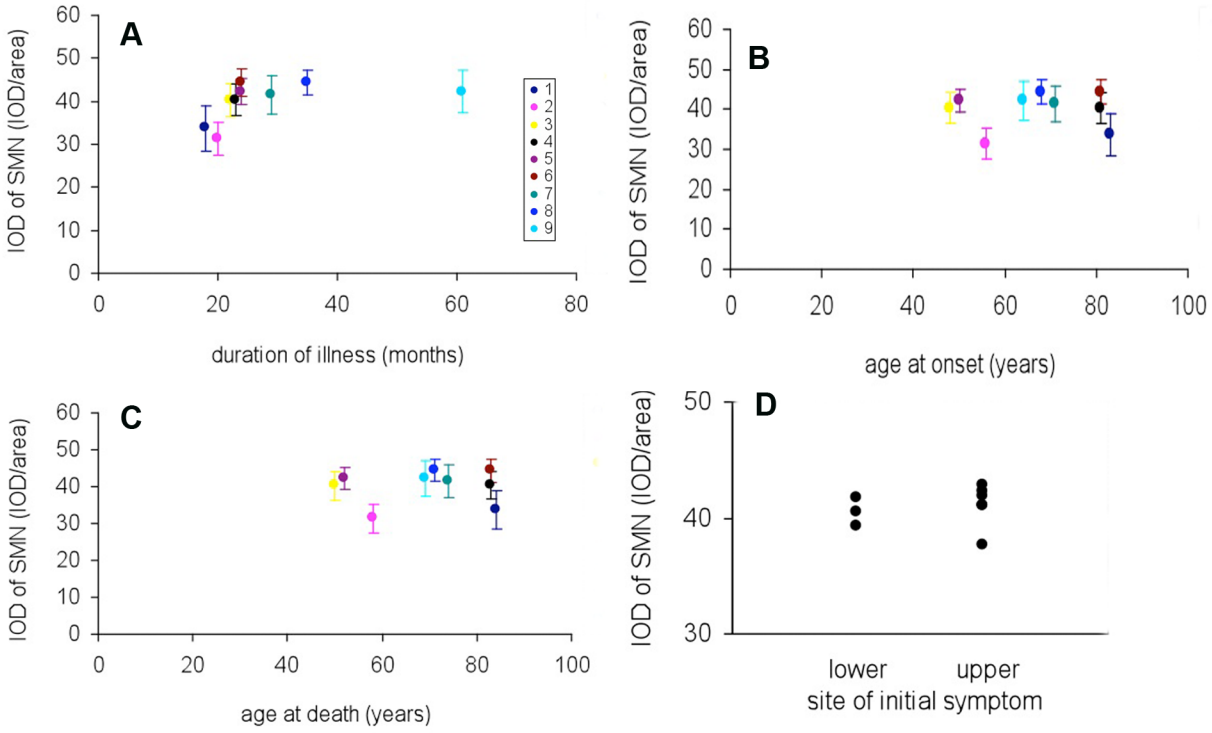
A
IOD of SMN (IOD/area)
duration of illness (months)
1
2
3
4
5
6
7
8
9
B
IOD of SMN (IOD/area)
age at onset (years)
C
IOD of SMN (IOD/area)
age at death (years)
D
IOD of SMN (IOD/area)
lower
upper
site of initial symptom

Fig. 4