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Original article

Relationships between long-term observations of motor milestones and genotype analysis results in childhood-onset Japanese spinal muscular atrophy patients

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Abstract

Aim: To clarify the long-term natural history of SMA in Japanese patients by investigating the peak motor milestones of cases 7 months through 57 years of age, in efforts to contribute to evaluating outcomes of new therapeutic interventions.

Methods: We sub-classified 112 SMA type I-III cases into type Ia, type Ib, type IIa, type IIb, type IIIa and type IIIb, according to peak motor milestone achieved, and analyzed the *SMN1, SMN2* and *NAIP* genes in relation to clinical subtypes.

Results: In type I cases, there was a significant difference ($p < 0.0001$), depending on whether or not head control was obtained, in the time of ventilation support being required. In type II cases as well, the time at which the ability to maintain the sitting position independently was lost also differed significantly ($p < 0.01$) between those acquiring the ability to sit unaided within eight months after birth and those acquiring this ability after eight months of age. In type III cases, being able versus unable to climb stairs was associated with a significant difference ($p = 0.02$) in the median time until loss of walking independently. Positive correlations were also seen between copy numbers and the clinical severity of SMA.

Conclusion: Our long-term results show peak motor milestone evaluations distinguishing between subtypes to be useful not only as outcome measures for assessing treatment efficacy in clinical trials but also for predicting the clinical courses of Japanese SMA patients.

Keywords: Spinal muscular atrophy; Motor milestones; Gene copy number; Clinical severity; *SMN2*; *NAIP*

1. Introduction

Spinal muscular atrophy (SMA), an autosomal recessive disorder characterized by degeneration and deficits of motor neurons in anterior horn cells of the spinal cord, shows progressive muscular atrophy and weakness of affected proximal muscles [1].

SMA is classified into four types on the basis of age at onset and the achievement of motor milestones. Type I (Werdnig-Hoffmann disease; OMIM 253300) has an onset before the age of six months and floppiness is evident in affected infants. It is impossible for these children to sit without support, and difficulty with nursing and inability to swallow, aspiration, respiratory failure, and tongue fasciculation are seen. These children require

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feeding support measures such as nasogastric tube feeding. The average life expectancy is 8 months, and mortality is 75–95% within 24 months in the absence of respiratory support [2]. Type II (Dubowitz disease; OMIM 253550) cases also have an onset in infancy but are able to maintain a sitting position, though they never gain the ability to stand or to walk without support, and tongue fasciculation is seen. Arthrogryposis and scoliosis become increasingly prominent with growth, and respiratory failure is likely to develop after an airway infection. Type III (Kugelberg-Welander disease; OMIM 253400), with childhood onset, is characterized by the ability to walk independently at first, with gradual deterioration, as individuals fall easily, lose the abilities to walk and get up, and elevating the arms becomes more difficult. Individuals with type IV (OMIM 271150), a slowly progressive lower motor neuron disorder, have their onset in adulthood [2–4]. Muscle weakness and atrophy are seen in all SMA types, along with diminution and, ultimately, disappearance of deep tendon reflexes [2,3]. The incidence of childhood SMA is 1–2 in 10,000 live births and the number of patients in Japan is presumed to be approximately 1000 [5,6].

SMA is caused by homozygous deletion, point mutation or gene conversion of the survival motor neuron 1 (*SMN1*) gene located on chromosome 5q13 [5,7,8]. A highly homologous gene named *SMN2* is present in this region, containing five discrepancies allowing *SMN1* to be distinguished from *SMN2*. The single base pair difference, a C to T transition in *SMN2* exon7, is responsible for alternative splicing of exon 7 and producing a high percentage of truncated *SMN2* transcripts lacking exon 7, and thereby in a lower abundance of the full-length transcript. Thus, this difference causes individuals with SMA to have low levels of both the full-length transcript and the functional SMN protein [9]. The neuronal apoptosis inhibitory protein (*NAIP*) gene potentially exerts an influence on the SMA phenotype. The number of copies of the *SMN2* gene and the existence of the *NAIP* gene are thought to be related to the clinical severity of SMA symptoms [10–12].

A major goal of disease-modifying therapy is to increase SMN protein levels. Fundamental therapeutic strategies have not yet been established for SMA. There has, however, been remarkable progress in the development of new therapeutic approaches for SMA. Examples include acceleration of the *SMN2* gene transcript by histone deacetylase (HDAC) inhibitor [13–18] and splicing modification of the *SMN2* gene by antisense oligonucleotides, which have shown promising efficacy in clinical trials [19,20]. These therapeutic trials are ongoing in Japan and several other countries.

As outcome measures for these clinical trials, it is important to document the clinical courses, in terms of motor milestones, of children with SMA. We investigated and analyzed the acquisition and loss of peak motor milestones, changes in respiratory function, and feeding support requirements, over the long-term clinical course, according to a sub-classification of SMA types. Our aim was to clarify the natural histories of the different SMA types in affected Japanese patients. We also examined the relationships of copy numbers of *SMN2* gene exons and the *NAIP* gene with phenotypic features of Japanese SMA cases.

2. Subjects and methods

2.1. Subjects

One-hundred and ninety-six individuals, ranging in age from 7 months to 57 years, with SMA were enrolled by questionnaire. One hundred and eighty subjects were from the Spinal Muscular Atrophy Research & Treatment: SMART Consortium, http://www.sma-rt.org while 16 were outpatients at the Institute of Medical Genetics, Tokyo Women's Medical University. Participants were enrolled from July 2014 through July 2016. One-hundred and fifty-one (77.0%) cases agreed to participate and 112 (57.1%) completed the questionnaire. Thirty-nine cases were excluded for the following reasons: no *SMN1* deletion or mutation, onset age over 20, administered valproic acid, and/or motor milestone information was insufficient.

Table 1
Characteristics of subjects.

Type	Maximum motor function	Subtypes		Number of subjects			Age at entry: median (range)
				M	F	Total	
I	Never sit independently	I a	Head control (−)	19	19	38	56.0m (0y7m-16y9m)
		I b	Head control (+)	4	5	9	32.0m (1y7m-35y7m)
			Total	23	24	47	
II	Never stand independently	II a	Sit independently >8mo	6	4	10	71.0m (2y5m-39y10m)
		II b	Sit independently ≦8mo	14	18	32	89.0m (1y9m-44y)
			Total	20	22	42	
III	Stand & walk independently	III a	Climb stairs (−)	6	4	10	198.5m (5y2m-52y11m)
		III b	Climb stairs (+)	8	5	13	185.0m (4y9m-57y6m)
			Total	14	9	23	

2.2. Methods

2.2.1. Clinical classification of SMA and subtypes, respiratory and feeding support

Individuals with SMA types I-III were classified into subtypes for this study, based on individual motor milestone changes, assessed according to a spectrum of features. As shown in Table 1, type I was divided into type Ia without head control and type Ib in which head control was possible, even if only temporarily. Type II was divided into type IIa, cases able to sit independently after 8 months of age, and type IIb who could sit independently before or at age 8 months. Type III was divided into type IIIa, cases able to walk independently but never climb stairs, and type IIIb who walked and climbed stairs independently. In addition, information pertaining to support for respiration and feeding are presented in Table 2.

2.2.2. Courses of motor milestones achieved

Motor milestones were classified into nine levels (Table 3) [21]. These levels, pertaining to the Japanese lifestyle, ranged from 0 to 8; level 0 is designated as no head control, level 1 as head control possible, level 2 as sitting independently, level 3 as turning on the buttocks, level 4 as shuffling in the sitting position, level 5 as standing with support, level 6 as walking with support, level 7 as walking independently, level 8 as climbing stairs. In addition, level -1 was established to allow examination of changes in the states of respiratory support with tracheostomy positive pressure ventilation (TPPV).

2.2.3. Evaluation of current subtype classification

The current classification of SMA was presented at The 209th ENMC International Workshop [22]. Subtypes in the classification of type I were based on not only peak motor function but also age at diagnosis: 1-A meant that the subjects had been diagnosed in the first 2 weeks of life, 1-B by age 3 months, 1-C 3–6 months. Type III cases were classified, as described by Zerres et al. [3], into IIIa and IIIb based on whether their onsets were before or after three years of age, respectively. Herein, we use the designations 3-A and 3-B to avoid confusion.

2.2.4. SMN2 and NAIP copy numbers

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The *SMN1* exon 7 deletion was detected employing the PCR-restriction fragment length polymorphism technique [10,26]. The multiplex ligation-dependent probe amplification (MLPA) method had already been used in 66 SMA cases to confirm copy numbers of *SMN1*, *SMN2*, and *NAIP*. The assay was performed using the commercially available SALSA®

Table 2
Respiration and feeding support.

Subtypes	Number of subjects	Respiratory support					Feeding support		
		Support (−)	NPPV		TPPV		Support (−)	No. with support (%)	Age at start: median (range)
			No. with support (%)	Age at start: median (range)	No. with support (%)	Age at start: median (range)			
I a	38	0	3 (7.9%)	14.0m (5m–4y3m)	35 (92.1%)	6.0m (2m–4y3m)**	2	36 (94.7%)	4.0m* (1m–40m)
I b	9	4	2 (22.2%)	23.0m (1y3m–2y7m)	3 (33.3%)	12.0m (11m–10y2m)**	7	2 (22.2%)	12.5m* (12m–13m)
	47	4	5		38		9	38	
II a	10	4	6 (60.0%)	105.5m (2y3m–31y7m)	0	-	10	0	–
II b	32	16	16 (50.0%)	37.0m (2y–13y4m)	0	-	32	0	–
	42	20	22		0		42	0	
III a	10	8	2 (20.0%)	39y, 28y	0	-	10	0	–
III b	13	12	1 (7.6%)	50y	0	-	13	0	–
	23	20	3		0		23	0	

NPPV: Noninvasive Positive Pressure Ventilation, TPPV: Tracheostomy Positive Pressure Ventilation.
* $p = 0.04$.
** $p = 0.01$.

Table 3
Definitions of motor milestone levels.

Level	Motor milestone
8	Climbing stairs
7	Walking independently
6	Walking with support
5	Standing with support
4	Shuffling in sitting position
3	Turning on buttocks
2	Sitting independently
1	Head control possible
0	No head control
−1	Respiratory support with tracheostomy positive pressure ventilation (TPPV)

MLPA kit, P021-A2 (MRC Holland, Amsterdam, Netherlands). The products were detected with an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and data were collected employing GeneMapper ver. 4.1 (Applied Biosystems) [23–25]. The data were then analyzed using Coffalyser (MRC-Holland) software. Three control samples with two copies of *SMN1* exon 7, *SMN1* exon 8, *SMN2* exon 7, *SMN2* exon 8 and *NAIP* exon 5 were chosen. All samples were analyzed at least twice.

The droplet digital PCR (ddPCR) assay was applied to cases showing discordancy between *SMN2* exon 7 and *SMN2* exon 8 copy numbers. The gene copy number assays were performed with the ddPCR *SMN2* Copy Number Determination Kit (Bio-Rad, Hercules, CA, USA) for *SMN2* exon 7. The copy number of *SMN2* exon 8 was analyzed with a forward primer (5′-GGA CTC TAT TTT GAA AAA CCA-3′), a reverse primer (5′-CCA CCG TGC TGG C-3′) and a TaqMan MGB labeled FAM probe (5′-AAG ACT GAG GTG GGG G-3′). All primers and probes were purchased from Integrate DNA Technologies (Coralville, Iowa, USA). The *SMN2* exon 8 copy number assays were performed in 22 μl reaction mixtures containing 2x ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 20 μM gene specific primers, 5 μM gene specific probe, 20x RPP30 Primers/Probe mixture and 20 units of the restriction enzyme HindIII HF (New England BioLab, Ipswich, Massachusetts, U.S.A). The *SMN2* exon 7 and the *SMN2* exon 8 reaction solutions were both mixed with 70 μl of Droplet Generation Oil for Probes (Bio-Rad) and then divided into approximately 18,000 droplets on the QX200 Droplet Generator (Bio-Rad). The PCR reactions were performed with the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 55 °C for 1 min, then 98 °C for 10 min with a 2 °C/s ramp rate. After thermal cycling, the fluorescence intensity of the individual droplets was measured using the QX200 Droplets Reader (Bio-Rad). The data were analyzed using QuantaSoft Software. Moreover, the 5 cases showing discordancy were assessed for hybrid *SMN* gene presence by LR-PCR amplification of a region that includes exons 1–8 of *SMN1* and direct DNA sequencing of intron 6, exon 7, and intron 7 [26]. This procedure was carried out because *SMN1* specificity can be confirmed by the presence of intron 6, exon 7 and intron 7 sequences.

2.2.5. Statistical analysis

The peak motor milestones of the study subjects and their relationships with SMA subtypes were analyzed using Kaplan–Meier curves. The relationships of clinical courses with subtypes, based on previous and current data, were also analyzed using Kaplan–Meier curves. Relationships between clinical types and copy numbers were confirmed by applying the Jonckheere-Terpstra test, Wilcoxon rank sum test and Steel–Dwass test. Differences were considered to be statistically significant at $p < 0.05$. All statistical analyses except the Jonckheere-Terpstra test were performed using JMP software ver.11.2 (SAS Institute Inc., Cary, NC, USA). The Jonckheere-Terpstra test was performed using SAS software ver.9.4 (SAS Institute Inc., USA).

Ethics statement

When the questionnaire form was distributed, a document explaining the research outline and methods used to meet the objective was simultaneously given to the patient and/or the patient's legal guardian, and cooperation was requested. This study was approved by the Ethics Committee of Tokyo Women's Medical University (TWMU; Shinjuku, Tokyo, Japan) and was performed with informed consent from all participants, in accordance with TWMU Institutional Review Board Approved protocols No.316, No.2556 and No.3154. All procedures were conducted according to the principles described in the Declaration of Helsinki. Written informed consent was obtained from the subjects and their legal guardians as per our institutional guidelines.

3. Results

3.1. Clinical evaluation based on the classification of SMA subtypes

3.1.1. Motor milestones

The peak motor milestones according to subtypes are shown in Table 3 and Fig. 1A. The peak motor milestone levels showed a wide distribution, from 0 through 8 [21].

As shown in Fig. 1B, in type Ib, the median age at head control acquisition was 4.0 months, range 3–6 months. Four cases still had head control at the time of answering the questionnaire (mean ± standard deviation (SD) months: 68.5 ± 71.1 months), with the oldest being more than 14 years of age. As shown in Fig. 2A,

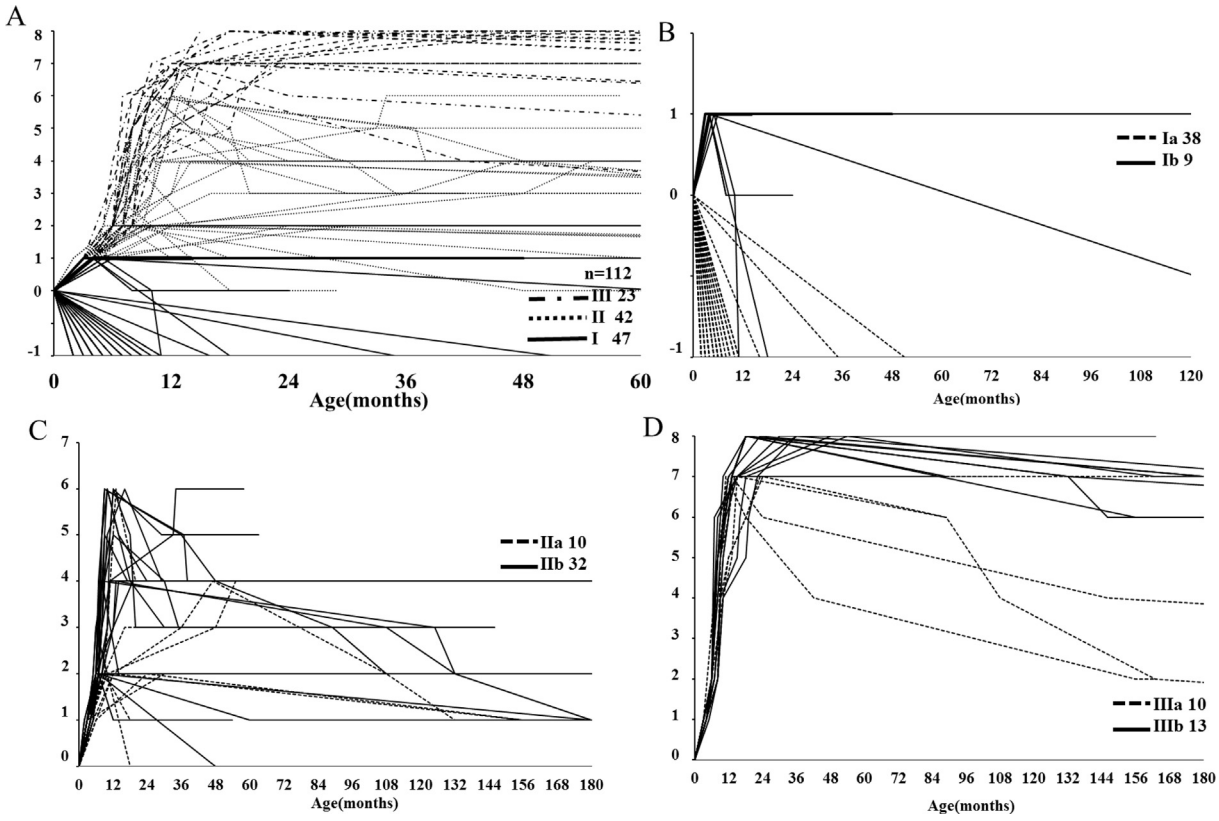


Fig. 1. (A) Changes in the courses of motor milestones according to SMA phenotype. Motor abilities were classified into nine levels, from 0 to 8, corresponding to no head control, head control possible, sitting independently, turning on the buttocks, shuffling in the sitting position, standing with support, walking with support, walking independently, and able to climb stairs. In addition, level -1 was designated as a means of examining changes in the states of respiratory support with TPPV. (B) Differences in motor milestones between SMA type Ia and type Ib. The median time of acquiring head control in type Ib cases was 4.0 months, range 3–6 months. Four cases still had head control at the time of answering the questionnaire (mean age, 68.5 ± 71.1 months). (C) Differences in motor milestones between type IIa and type IIb. Type II showed a wide range of clinical features classified from level 2, sitting independently, through level 6, walking with support. The numbers of cases at each level according to peak motor milestones were as follows. The level 2 group included 21 cases (21/42 = 50.0%, IIa/IIb = 6/15), level 3 two cases (4.8%, IIa/IIb = 1/1), level 4 eight cases (19.0%, IIa/IIb = 2/6), level 5 two cases (4.8%, IIa/IIb = 0/2), and level 6 nine cases (21.4%, IIa/IIb = 1/8). There was a difference in the median time at which level 2 was obtained between type IIa and type IIb [sitting independently after 8 months ($n = 10$, 10 months, range 9–30 months) versus within 8 months ($n = 32$, 7 months, range 4–8 months)]. Ten of the 42 type II cases (23.8%), showed a decrease in function to level 1, meaning that they reached a functional level consistent with that of type I. (D) Differences in motor milestones between type IIIa and type IIIb. The time to acquisition of level 7, walking independently, did not differ markedly between type IIIa cases who could walk independently but never climbed stairs at the time of answering the questionnaire ($n = 10$, median 13 months, range 11–24 months) and type IIIb cases who could walk and climb stairs independently ($n = 13$, median 13 months, range 10–22 months).

those with type Ia who had not acquired head control showed a shorter median time until the introduction of respiratory support with TPPV than those with type Ib who had acquired head control (Ia/Ib $n = 38/9$, median; 6/122 months, $p < 0.0001$).

Type II cases showed a wide range of motor milestones from level 2, sitting independently, through level 6, walking with support (Fig. 1C). The numbers of cases according to peak motor milestones were as follows. Twenty-one cases (21/42 = 50.0%, IIa/IIb = 6/15) were level 2, two cases (4.8%, IIa/IIb = 1/1) level 3, eight cases (19.0%, IIa/IIb = 2/6) level 4, two cases (4.8%, IIa/IIb = 0/2) level 5, and nine cases (21.4%, IIa/IIb = 1/8) level 6. There was a difference in the median age at acquiring level 2 between type IIa, sitting independently after age 8 months ($n = 10$, median; 10 months, range 9–30 months), and type IIb, sitting independently at or before age 8 months ($n = 32$, median; 7 months, range 4–8 months) ($p < 0.001$). There were no significant differences in the median ages at acquisition of other motor milestone levels between type IIa and type IIb. Ten of the 42 type II cases (23.8%) showed decreases in motor milestones to level 1, reaching the same clinical condition as type I cases. As shown in Fig. 2B, there was a significant difference in median time from getting to sit independently until loss of the ability to sit independently between type IIa and type IIb (IIa/IIb $n = 10/32$, median; 132 months/indeterminable, $p < 0.01$).

As shown in Fig. 1D, the age at acquisition of level 7, walking independently, did not differ between type IIIa cases who could walk independently but had never

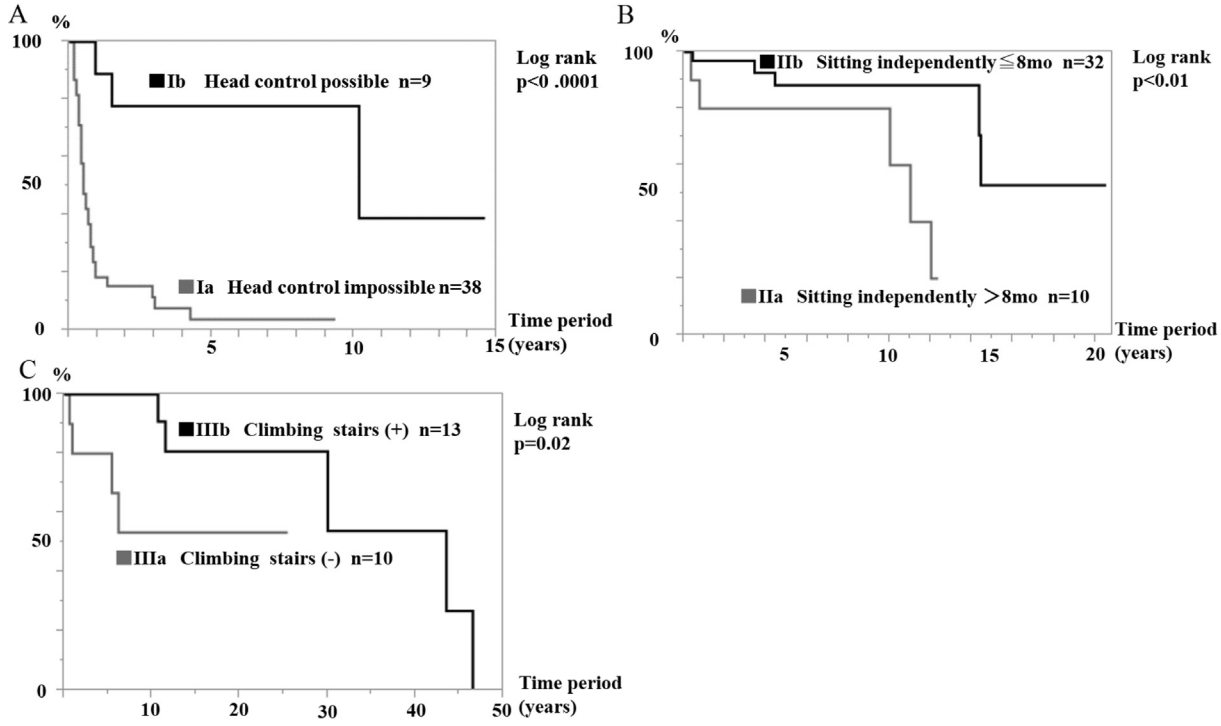


Fig. 2. (A) Kaplan-Meier curve of the timing of TPPV introduction: comparison between type Ia and type Ib. The type Ia cases had a shorter median time ($n = 38$, median 6 months) until the initiation of respiratory support than the type Ib cases ($n = 9$, median 122 months) and the difference was statistically significant ($p < 0.0001$). (B) Kaplan-Meier curve of the time from getting to sit independently until loss of sitting independently: comparison between type IIa and type IIb. There was a significant difference in the median time from getting to sit independently until loss of sitting independently between the type IIa cases ($n = 10$, median 132 months), unable to sit unaided within eight months, and type IIb cases ($n = 32$, median indeterminable) who acquired the ability to sit unaided within eight months ($p < 0.01$). (C) Kaplan-Meier curve of the time from getting to walk independently until loss of walking independently: comparison between type IIIa and type IIIb. The median time from getting to walk independently until walking independently became impossible differed significantly, at the time of answering the questionnaire, between type IIIa cases whose peak motor milestone was walking independently ($n = 10$, median indeterminable) and type IIIb cases whose peak motor milestone was climbing stairs ($n = 13$, median 522 months) ($p = 0.02$).

climbed stairs at the time of answering the questionnaire ($n = 10$, median; 13 months, range 11–24 months), and individuals with type IIIb who could walk and climb stairs independently ($n = 13$, median; 13 months, range 10–22 months). In type IIIb cases, the median age at acquisition of the ability to climb stairs independently was 18.0 months, range 15–54 months. As shown in Fig. 2C, the median time from getting to walk independently until loss of walking independently differed significantly between type IIIa ($n = 10$, median; indeterminable) and type IIIb ($n = 13$, median; 522 months) cases ($p = 0.02$).

3.1.2. Evaluation of current subtype classification

SMA type I was classified into subtypes by not only peak motor function but also age at diagnosis. The classification based on the 209th ENMC International Workshop [22] was followed; 1-A, diagnosed in the first 2 weeks of life, 1-B by age 3 months, 1-C 3–6 months. As shown in Fig. 3A, our dataset revealed a statistically significant difference only between type 1-B and type 1-C in the median age at the initiation of respiratory support ($p < 0.001$). Applying the classification of Zerres et al., SMA type III was subdivided into those with onset age before (3-A) and after or at (3-B) age 3 years [3]. As shown in Fig. 3B, in our dataset there was no significant difference in the median time from getting to walk independently until loss of walking independently between 3-A ($n = 16$, median 360 months) and 3-B ($n = 7$, median 522 months) ($p = 0.36$).

3.1.3. Respiratory support

All patients with type Ia SMA required some form of respiratory support (Table 2). Three cases (3/38, 7.9%) required noninvasive positive pressure ventilation (NPPV), both day and night, and the other 35 (92.1%) required TPPV. Four of the nine cases (44.4%) with type Ib, who still had head control at the time of responding to the questionnaire, did not require respiratory support. Two cases (22.2%) used NPPV at night or intermittently, three (33.3%) TPPV. As shown in Table 2, the ages at starting TPPV differed significantly between type Ia (n = 35, median; 6.0 months, range 2–51 months) and type Ib ($n = 3$, median; 12.0 months, range 11–122 months) cases ($p = 0.01$). One type Ib case had required tracheotomy at age 10 years and mechanical

ventilation assistance was introduced at 18 years of age. Respiratory support was not necessary in 4 cases (4/10, 40.0%) with type IIa and 16 cases (16/32, 50.0%) with type IIb. Night - time NPPV was required in 6 type IIa cases (6/10, 60.0%) and 16 type IIb cases (16/32, 50.0%). One of the type IIb cases also used NPPV intermittently during the daytime. None of the cases with types IIa and IIb needed TPPV support. Eight cases with type IIIa (8/10, 80%) and 12 with (12/13, 92.3%) type IIIb did not need respiratory support. Night - time NPPV was needed by 2 (2/10, 20.0%) type IIIa cases, and one (1/13, 7.7%) with type IIIb. None of the type III cases had any history of TPPV.

3.1.4. Feeding support

Two type Ia cases (2/38, 5.3%) did not require feeding support, by either nasogastric tube or gastrostomy (Table 2). Thirty-six cases (36/38, 94.7%) received tube feeding. Among type Ib cases, seven (7/9, 77.8%) were able to ingest meals orally. Two cases (2/9, 22.2%) required tube feeding. The median times of switching from oral to tube feeding differed significantly between type Ia ($n = 36$, median; 4.0 months, range 1–40 months) and type Ib ($n = 2$, median; 12.5 months, range 12–13 months) cases ($p = 0.04$). None of the type II and type III cases required feeding support.

3.2. Evaluation of subtype classification of SMA in relation to SMN2 and NAIP copy numbers

Among all cases participating in this study, 109 (97.3%) showed homozygous deletions of the *SMN1* gene. Two cases with type I (4.3%) and one case with type III (4.3%) had compound heterozygous mutations [26].

SMN2 exon 7, exon 8 and *NAIP* exon 5 copy numbers were examined in 66 cases (Table 4). All showed *SMN1* deletion. As copy numbers of *SMN2* exon 7, exon 8 and *NAIP* exon 5 increased, the clinical type became milder in the order of SMA type I, II and III, with significance ($p \leq 0.001$) [27,28]. The *SMN2* exon 7 and exon 8 copy numbers differed significantly between type Ia and other subtypes ($p < 0.05$).

In addition, as shown in Table 4, positive correlations were detected between type Ia and Ib ($p < 0.0001$) for exon 7 and exon 8 of *SMN2*.

As the copy numbers of both exon 7 and exon 8 of *SMN2* became larger, the clinical presentations, from type Ia to type IIIb, tended to become milder (Fig. 4).

Hybrid SMN gene analyses were performed for 5 SMA cases showing discordancy between *SMN2* exon 7 and *SMN2* exon 8 copy numbers. The sequences of hybrid *SMN* intron 6, exon 7 and exon 8 were aTggG in all five cases (Table 5).

4. Discussion

We assessed changes over time in the motor milestones of individuals with SMA. The motor milestones were examined according to the method established by Okawa et al. [21], which corresponds to the Japanese lifestyle in which people take off their shoes in the house and often sit on the floor. This method is used for infant medical assessments in Japan. Ages at the acquisitions of head control, sitting independently, and climbing stairs are especially important assessment items. Thus, high accuracy in determining the timing of milestone acquisition is necessary. The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders (CHOP INTEND), Hammersmith Functional Motor

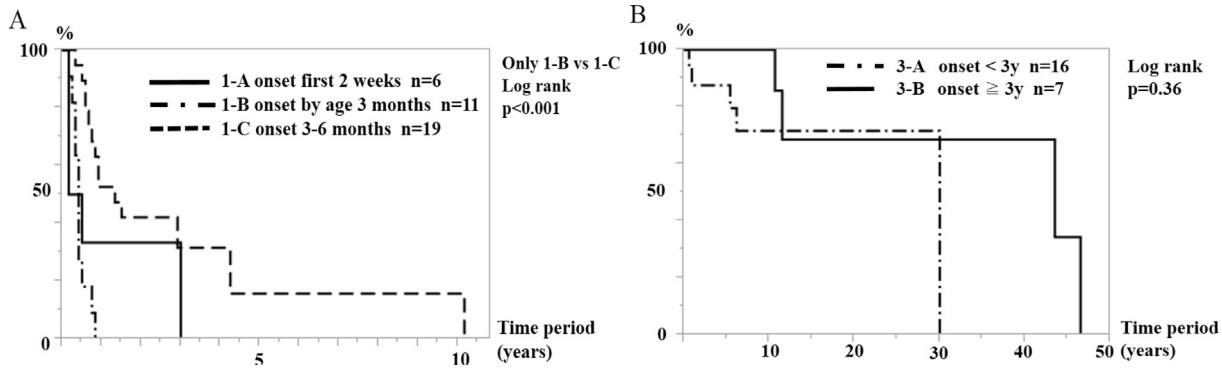


Fig. 3. (A) Kaplan-Meier curve of the age at the TPPV introduction: comparison among types 1-A, 1-B and 1-C. The subtype classification into type I by age at diagnosis was devised by The 209th ENMC International Workshop. SMA type I individuals were classified into 1-A by age at diagnosis within the first 2 weeks of life ($n = 6$, median 4 months), 1-B by 3 months ($n = 11$, median 5 months), and 1-C by 3–6 months ($n = 19$, median 16 months). In our dataset, the only statistically significant difference in the median age at respiratory support was initiated was between 1-B and 1-C ($p < 0.001$). (B) Kaplan-Meier curve of the time from getting to walk independently until the loss of walking independently: comparison between type 3-A and type 3-B. Zerres et al. previously subdivided type III into 3-A, with disease onset before three years of age, and 3-B, with onset after age three years. In our dataset, the median time from getting to walk independently until walking independently became impossible did not differ between the 3-A ($n = 16$, median 360 months) and 3-B ($n = 7$, median 522 months) groups of patients ($p = 0.36$).

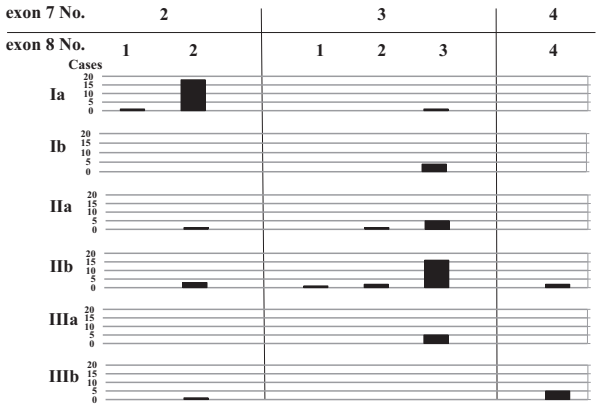


Fig. 4. Tendencies associated with *SMN2* exon 7 and exon 8 copy numbers. There were marked tendencies for larger copy numbers of both exon 7 and exon 8 of the *SMN2* gene to be associated with milder clinical presentations, and this held true from type Ia through type IIIb ($p < 0.0001$).

Scale-Expanded and other tools have utilized the natural history of SMA for motor functional analysis. Our study was based on the acquisition and loss of motor milestones over an extended time period. Analysis of changes in the motor milestone state revealed continuity among clinical types, as shown in Fig. 1A. It was clear that some subjects never attained head control, while others acquired some motor milestones within or below normal limits. For this study, we subdivided SMA types I, II and III into types Ia, Ib, IIa, IIb, IIIa and IIIb based on motor milestones. Type Ia means no acquisition of head control, which is expressed by Okawa et al. [21] as level 0, while type Ib cases acquire head control, which is level 1. Type IIa cases acquire level 2 motor milestones, meaning that they can sit independently after 8 months of age, while type IIb acquire level 2 within the first 8 months of life. Type IIIa corresponds to level 7, i.e. these cases could walk but not climb stairs at the time of responding to the questionnaire, and type IIIb corresponds to level 8, i.e. these cases could both walk and climb stairs (Fig. 1B, C, D).

Among previous studies providing survival data on SMA type I, that by Finkel et al. described the median age at reaching the combined endpoint of death or requiring at least 16 h/day of respiratory support with noninvasive ventilation or intubation leading to tracheostomy as being 13.5 months in patients with SMA type I [29]. Cobben et al. reported the median age at death to be 176 days [30]. According to Oskoui et al., rates of death or ventilation for more than 16 h/day in type I SMA patients born during the decade from 1995 to 2006 had improved significantly, as reflected by increased survival, when compared with those born in the 1980 to 1994 period. Respiratory assistance and gastrostomy reduced the risk of death [31]. In this study, as revealed by the comparison between type Ia and type Ib, acquiring head control played a major role in maintaining respiratory function (Fig. 2A).

The ability to maintain a sitting position is related to quality of life. As shown in Fig. 2B, acquisition of sitting independently after age 8 months versus within 8 months correlated with time from getting to sit independently until the loss of the level 2 motor milestone, i.e. sitting independently.

In the same way, there was a significant difference between type IIIa, unable to climb stairs, and type IIIb, able to climb stairs, in time from getting to walk independently until the loss of walking independently (Fig. 2C).

The classification of SMA used in The 209th ENMC International Workshop was devised by combining age at onset, age at diagnosis and peak motor milestones achieved [22]. In our dataset, subjects whose age at onset or diagnosis could not be determined precisely, made it difficult to classify subtypes, which may explain why there were no statistically significant differences in the median time until the initiation of respiratory support except between 1-B and 1-C (Fig. 3A) [22]. Zerres et al. subdivided type III into 3-A, with disease onset before three years of age, and 3-B, with onset after age three years [3]. In our dataset, there were no significant differences in the median time from getting to walk independently until walking independently became impossible between 3-A and 3-B (Fig 3B).

Bach et al. reported 56 SMA type I cases who developed respiratory failure before age 2 years, of whom 16 underwent tracheostomy at 10.8 ± 5.0 months (mean $\pm$ SD months) of age, and 33 used noninvasive ventilation and assisted coughing [32]. Hachiya et al. described three Japanese cases with SMA type I who required permanent artificial respiration starting around the age of 7 months. [33]. In our study, 80.9% of type I cases used TPPV (median 6 months, range 2–122 months).

Ioos et al. subdivided SMA type I into true type I cases never able to raise their heads and intermediate type I cases with the ability to raise the head. Tracheostomy was performed at 19 ± 20 months (mean $\pm$ SD months) in 33% of true type I and at 5 years $\pm$ 4 years in 57% of intermediate type I cases [34]. As shown in Table 2, in our study, 92.1% of type Ia cases required respiratory support at a median age of 6 months, 33.3% of type Ib cases at a median age of 12 months. Before tracheostomy, NPPV was used in 2 cases with type Ia and the periods were 3 and 34 months, respectively. Almost 50% of type II cases and 3 with type III required NPPV (Table 2). Our observations suggest that respiratory support tends to be introduced earlier for SMA patients in Japan than for those in other countries. This study was performed with the cooperation of the SMART Consortium as well as the enrollment of outpatients at the Institute of Medical Genetics, Tokyo

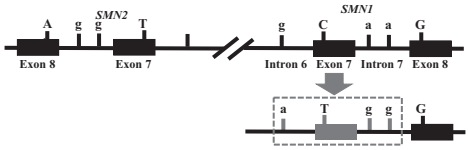
Table 4
SMN2 exon7/8, *NAIP* exon5 copy numbers in SMA clinical types.

Clinical types	n	Copy number													
		SMN2 exon 7				SMN2 exon 8					NAIP exon 5				
		2	3	4		1	2	3	4		0	1	2	3	
I a	20	19	1	0	p<0.0001*	1	18	1	0	p<0.0001*	8	10	2	0	p=0.13
I b	4	0	4	0	p=0.57	0	0	4	0	p=0.32	0	3	1	0	p=0.21
II a	7	1	6	0	p=0.63	0	2	5	0	p=0.71	1	6	0	0	p=0.34
II b	24	3	19	2	p=0.86	1	5	16	2	p=0.48	3	16	4	1	p=0.75
III a	5	0	5	0	p=0.46	0	0	5	0	p=0.47	0	4	1	0	p=0.17
III b	6	1	0	5		0	1	0	5		0	2	4	0	
total	66														

*p<0.001

Table 5
SMA type and copy number in 5 cases with gene conversion.

Case	SMA type	Copy number			
		SMN2 exon 7	*SMN2* exon 8	*SMN1* exon 7	*SMN1* exon 8
1	Ia	2	1	0	1
2	IIa	3	2	0	1
3	IIb	3	1	0	2
4	IIb	3	2	0	1
5	IIb	3	2	0	1



Women's Medical University. There is thus a potential treatment policy bias.

Finkel et al. reported that nutritional support was initiated at a median age of 8 months after birth for SMA I patients [29]. Tassie et al. stated that enteral feeding by nasogastric tube was provided for 85.7% of Australian SMA I cases [35]. In this study, support with either nasogastric tube feeding or gastrostomy was initiated in 80.9% of type I cases (median age 4 months; range 1–40 months). As shown in Table 2, nasogastric tube feeding or gastrostomy support was initiated in 94.7% of type Ia cases (median age 4 months) and 22.2% of type Ib cases (median age 12.5 months).

The survival of patients with type I SMA has improved markedly in recent years. In particular, the early morbidity and mortality associated with type I are attributable mainly to respiratory problems. Introductions of tracheostomy and artificial respiratory support for breathing remain controversial. There are also intercultural and international differences in the applications of these modalities [36]. Oskoui et al. reported that respiratory assistance and feeding support reduce the risk of death [31]. From an ethical perspective, it is important that clinicians present care options in a consensus statement regarding SMA, such as that published in 2007 by Wang et al. [37], providing a uniform standard of care, until more definitive therapeutic strategies can be established.

Copy numbers of *SMN2* exons 7 and 8, and that of *NAIP* exon 5, were examined in 66 of our cases (Table 4). The higher the copy number, the milder the clinical manifestations were and this held true from types I through type III, and this association was statistically significant. These relationships between copy numbers and clinical types were similar to those described in previous reports [27,28]. Our results support current treatment strategies, particularly those targeting *SMN2*.

Based on the results obtained with the Jonckheere-Terpstra test, we hypothesized that copy numbers of the *SMN2* and *NAIP* genes differ significantly between SMA types Ib and IIa, and also between types IIb and IIIa. To examine which subtypes showed differences in copy number from all other subtypes, we applied the non-parametric Wilcoxon rank sum test for comparisons between each pair of subtypes. Differences between type Ia and type Ib were significant. However, there were no significant differences between type Ib and type IIa, or between type IIb and type IIIa. In other words, type I does not differ significantly from type II, nor type II from type III. This is despite marked variability in the courses of motor milestones among type I, type II, and type III. Thus, not only *SMN2* and the *NAIP* gene but also other modifiers determining clinical severity must differ between type I and type II, as well as between type II and type III. We identified the hybrid *SMN* gene type in 5 cases. All 5 cases had a deletion in *SMN1* exon 7 combined with an *SMN1* to *SMN2* conversion. Previous studies have described an association between disease severity and conversion [38,39]. Our present results do not provide clear information about the possible relationships between symptoms and conversion.

We focused on the combination of exon 7 and exon 8 of the *SMN2* gene (Fig. 4). There were marked tendencies for larger copy numbers to be associated with a milder clinical presentation and this held true from type Ia through type IIIb. In addition, copy numbers of *SMN2* exon 7 and *SMN2* exon 8 differed strikingly between type Ia and other SMA types. The pathogenesis of type Ia may thus differ from that of other SMA types.

We examined the states of motor milestones, respiratory and feeding support, as well as phenotype and genotype correlations in Japanese SMA type I, II and III cases, employing a new classification of subtypes. Motor milestone evaluation comparing subtypes is useful not only as an outcome measure for clinical trials and assessment of treatment efficacy but also for predicting the clinical courses of individuals with SMA. If treatments targeting the early stage after SMA onset can be developed, it might become feasible to further reduce clinical manifestations. Subdivision based on peak motor milestones is a valid approach to evaluating SMA (Fig. 1A). The relationship between clinical classi-

fication and the SMA genotype has been demonstrated in Japan.

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