260/280 was 1.62. The DNA of this sample was sufficiently deteriorated to prevent amplification with 45 cycle PCR using 50 ng of DNA. However, this was an exceptional case. Other samples, including three 8-year-old DBS samples, were successfully used for PCR-HRMA.

PCR-HRMA data

The melting curves were normalized after subtraction of the post-melting area fluorescence at 81.3–82.5°C from the pre-melting area fluorescence at 69.8–70.8°C. Normalized melting curves of SMA patients with *SMN1* deletion and *SMN2* retention [*SMN1*(−)/*SMN2* (+) curves] were located near the curves of controls with *SMN1* retention and *SMN2* deletion [*SMN1*(+)/*SMN2*(−) curves], but far from the curves of controls with *SMN1* and *SMN2* retention [*SMN1*(+)/*SMN2*(+) curves] (Figure 3). It should be noted in this study that the *SMN1*(−)/*SMN2*(+), *SMN1*(+)/*SMN2*(−) and *SMN1*(+)/*SMN2*(+) curves formed their own groups, but the curves in each group did not converge into one line. In addition, the melting curves of samples with the same genotype did not converge into one line.

Comparison with PCR-RFLP data

The PCR-HRMA data were completely consistent with the data obtained by conventional PCR-RFLP methods (sensitivity = 1.0, specificity = 1.0) (Table 1). However, we failed to determine the relationship between the difference plots and the copy number of the *SMN1* and *SMN2* genes in the control samples. We also failed to determine the relationship between the difference plots and the copy number of the *SMN2* gene in SMA patient samples.

Discussion

We have developed a rapid, accurate and simple method for SMA screening, which consists of DNA extraction from DBSs on filter paper and PCR-HRMA. We previously reported a PCR-RFLP method of detecting SMA using DNA extracted from DBSs [15]. However, when DNA extracted from DBSs is used for PCR-HRMA, the problem of DNA quality/quantity must be confronted. To overcome this problem, others have performed nested PCR before HRMA [10]. Our strategy to overcome the DNA quality/quantity problem was to simply increase the number of PCR cycles. We increased the PCR cycle number by 50%, obtaining successful results. Our method to overcome the quality/quantity problem looks very easy, once it has been done.

The other problem in HRMA is generating allele-specific melting curves. The melting curves of SMA patients with *SMN1* deletion can be distinguished from those of controls with *SMN2* deletion by conducting PCR with a special probe [11], or by adding driver DNA (*SMN1*/*SMN2*=0:3) to the sample DNA prior to PCR [13]. Also used is a method incorporating calibrated short-amplicon melt profiling with an oligonucleotide forming a hairpin structure, which increases the resolution between the curve profiles because of the tightened clustering of curves [14]. However, our method does not require probes, drivers or calibrated short-amplicon melt profiling. Our study showed that the primer set designed by Lefebvre et al (R111 and 541C770) [2] enabled sufficient differentiation of the melting curves in PCR-HRMA. The primer set was firstly reported in the article of cloning *SMN1* in 1995 [2], but has not been used in PCR-HRMA in all laboratories except ours [12].

The primary outcome measure of our screening method was to determine the accuracy of the PCR-HRMA with DBS-DNA. The accuracy of PCR-HRMA with DBS-DNA was evaluated by the rate of concordance between PCR-HRMA with DBS-DNA and PCR-RFLP with DNA from freshly collected blood. Our study demonstrated that the results of PCR-HRMA with DBS-DNA were completely concordant with those of PCR-RFLP with DNA from freshly collected blood (Table 1). The secondary outcome of our study was to determine the length of time a DBS on filter paper will last without deteriorating. PCR amplification cannot be performed using DNA from

deteriorated DBS. In our study, we found only one deteriorated DBS sample among the 70 samples with storage period of 1-8 years. Thus, we concluded that even if stored more than 8 years, the DBS samples can be used for PCR.

We should mention here some limitations of PCR-HRMA using DBS-DNA for SMA screening. First, PCR-HRMA with DBS-DNA cannot determine the copy number of alleles, because the melting curves of samples with the same genotype did not always converge into one line. This limitation may be partly due to the variable deterioration of DNA extracted from DBSs that have been stored for extended periods (years). Second, PCR-HRMA can detect only *SMN1* deletion, and cannot detect any intragenic mutations in *SMN1*.

In conclusion, we demonstrated the strength of our PCR-HRMA method: the rapidity, the accuracy and the simplicity. As to the required time from the start of PCR to the end of HRMA, our method takes only 1.5 hours, while PCR-RFLP takes more than 8 hours. The machine in our study, LightCycler®480 System II, can handle as many as 384 samples, according to the manufacturer's instruction. As to the accuracy, our results of PCR-HRMA with DBS-DNA were completely concordant with those of PCR-RFLP with DNA from freshly collected blood. In addition, our method does not need the second PCR procedures. Our method needs neither special probes nor drivers. Our method needs only "a single PCR with a primer set". Thus, our "PCR-HRMA with DBS-DNA" may be a useful screening method to detect *SMN1* deletion. It should also be noted that DNA from 8-year-old DBSs can be used as template for PCR-HRMA. All of these findings suggest that our method is practicable for a large population study and/or for a long period study.

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Table and Figure legends

Table 1 **HRMA with DBS-DNA vs PCR-RFLP with DNA freshly collected blood.**

Figure 1 **PCR primers.** (A) Positions and directions of the oligonucleotide primers. G and A in intron 6, and C and T in exon 7 indicate nucleotide differences between *SMN1* and *SMN2*. (B) Primer sequences. The expected size of the PCR product is 202 bp.

Figure 2 **Amplification curves and melting curves of PCR products.** (A) Amplification curves of PCR products. (B) Melting curves of PCR products. DBS-DNA was used as template DNA. Blue-colored curves represent amplified products, and orange-colored curves (or lines) represent non-amplified ones. The non-amplified curves were obtained from one DBS-DNA sample (*) and a negative control without DNA (**).

Figure 3 **HRMA data of three genotypes.** (A) Normalized and shifted melting curves. (B) Normalized and temperature-shifted difference plot. HRMA clearly distinguished three genotypes: *SMN1*(+)/*SMN2*(+) (*SMN1* and *SMN2* retention; normal control individuals), *SMN1*(+)/*SMN2*(−) (*SMN1* retention and *SMN2* deletion; normal control individuals), and *SMN1*(−)/*SMN2*(+) (*SMN1* deletion and *SMN2* retention; SMA patients).

Table 1 HRMA with DBS-DNA vs PCR-RFLP with DNA freshly collected blood.

(A)

		PCR-RFLP with DNA from freshly collected blood		Total
		SMN1 (+)	*SMN1* (-)	
HRMA with DBS-DNA	*SMN1* (+)	29	0	29
	SMN1 (-)	0	40	40
Total		29	40	69

sensitivity 1.0, specificity 1.0

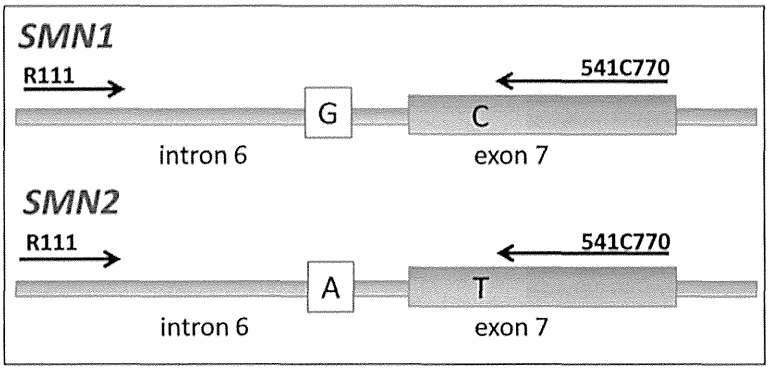
(B)

		PCR-RFLP with DNA from freshly collected blood		Total
		SMN2 (+)	*SMN2* (-)	
HRMA with DBS-DNA	*SMN2* (+)	66	0	66
	SMN2 (-)	0	3	3
Total		66	3	69

sensitivity 1.0, specificity 1.0

Figure 1 PCR primers.

(A)

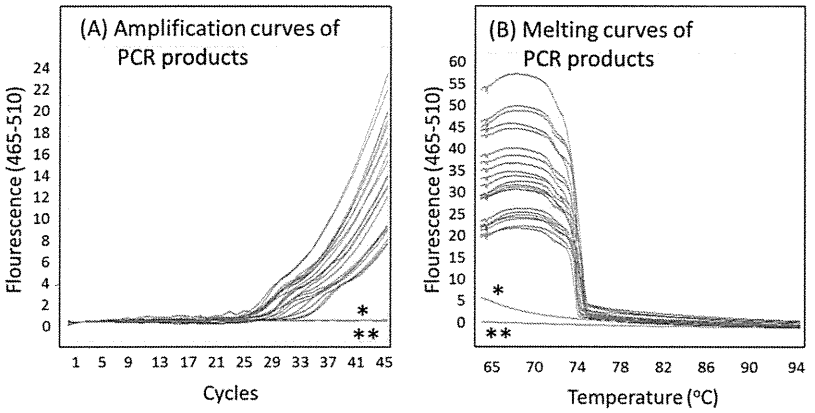


(B)

Primer	Sequence	Size	Reference
R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	202 bp	[2]
541C770	5'-TAA GGA ATG TGA GCA CCT TCC TTC-3'		

(A) Positions and directions of the oligonucleotide primers. G and A in intron 6, and C and T in exon 7 indicate nucleotide differences between *SMN1* and *SMN2*. (B) Primer sequences. The expected size of the PCR product is 202 bp.

Figure 2 Amplification curves and melting curves of PCR products.



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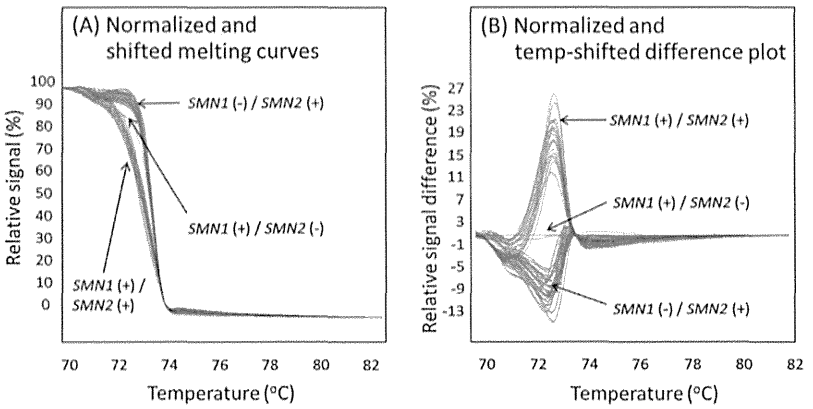

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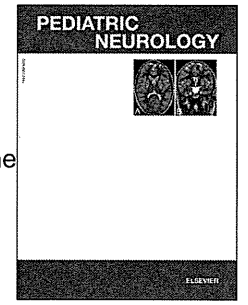

Two Japanese patients with SMA type 1 suggest that axonal-SMN may not modify the disease severity

Hiroyuki Yamada, Yoshinobu Nishida, Toshiro Maihara, Nihayatus Sa'adah, Nur Imma Fatimah Harahap, Dian Kesumapramudya Nurputra, Mawaddah Ar Rochmah, Noriyuki Nishimura, Toshio Saito, Yuji Kubo, Kayoko Saito, Hisahide Nishio

PII: S0887-8994(15)00111-3

DOI: 10.1016/j.pediatrneurol.2015.02.023

Reference: PNU 8609

To appear in: *Pediatric Neurology*

Received Date: 23 December 2014

Revised Date: 18 February 2015

Accepted Date: 23 February 2015

Please cite this article as: Yamada H, Nishida Y, Maihara T, Sa'adah N, Harahap NIF, Nurputra DK, Rochmah MA, Nishimura N, Saito T, Kubo Y, Saito K, Nishio H, Two Japanese patients with SMA type 1 suggest that axonal-SMN may not modify the disease severity, *Pediatric Neurology* (2015), doi: 10.1016/j.pediatrneurol.2015.02.023.

< Clinical Observations >

Two Japanese patients with SMA type 1 suggest that axonal-SMN may not modify the disease severity

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ABSTRACT

Background: Spinal muscular atrophy (SMA) is caused by *SMN1* mutations. *SMN1* produces a full-length SMN1 (FL-SMN1) protein isoform encoded by exons 1–7, and an axonal-SMN (a-SMN) protein isoform encoded by exons 1–3 and intron 3. The a-SMN protein is expressed only in the embryonic period and plays a significant role in axonal growth. However, there has been no report on contribution of a-SMN to SMA severity until now.

Case Descriptions: Two male patients with SMA type 1 in our study presented with generalized muscle weakness and respiratory insufficiency soon after birth, and were bound to an artificial ventilator from early infancy. Patient 1 was compound heterozygous for two *SMN1* mutations, whole gene deletion and an intragenic mutation (c.819_820insT). He retained one copy of *SMN1* producing N-terminal part of SMN1 including a-SMN. On the other hand, Patient 2 was homozygous for *SMN1* deletion. Both of them showed the same copy number of SMA modifying genes, *NAIP* and *SMN2*. These findings suggested that the C-terminal domain of FL-SMN1 determined the severity, irrespective of presence or absence of a-SMN expression.

Conclusion: In Patient 1, the C-terminal domain of FL-SMN1 determined SMA severity, rather than the a-SMN, one copy of which could be present and intact. The presence or absence of a-SMN may not impact disease severity in SMA type 1 patients.

Keywords: spinal muscular atrophy; full-length SMN1; C-terminal domain; axonal-SMN

Conflict of interest: none

Introduction

Spinal muscular atrophy (SMA) is a common neuromuscular disease characterized by lower motor neuron death, leading to muscle weakness and atrophy. SMA is classified into three subtypes depending on age of disease onset and achievement of motor milestones, specifically, type 1 (severe form; onset age 0–6 months, unable to sit unaided), type 2 (intermediate form; onset age < 18 months, unable to stand or walk unaided), and type 3 (mild form; onset age > 18 months, able to stand or walk unaided).[1]

The survival motor neuron gene (*SMN*) and neuronal apoptosis inhibitory protein gene (*NAIP*) were cloned as causative candidate genes of SMA in 1995.[2,3] *SMN* exists as two highly homologous copies, *SMN1* and *SMN2*, and it has been established that SMA is caused by deletions or intragenic mutations of *SMN1*.[2] *SMN1* is homozygously deleted in > 90% of SMA patients and deleteriously mutated in the remaining patients.[2,4] The main protein product of *SMN1* is full-length SMN (FL-SMN), encoded by *SMN1* exons 1–7, while that of *SMN2* is Δ-7 SMN, encoded by *SMN2* exons 1–6 and 8 (*SMN2* exon 7 is usually skipped).[2] *SMN2* is now considered a modifying gene of the SMA phenotype as higher *SMN2* copy numbers are associated with a milder phenotype.[5] *NAIP* is also considered a modifying gene as its absence is more frequently observed in patients with SMA type 1.

In 2007, axonal-SMN protein (a-SMN) was identified as an isoform encoded by *SMN1* exons 1–3 and intron 3 (Fig. 1).[6] The a-*SMN* mRNA is transcribed specifically from *SMN1*, and a-SMN protein expressed during the embryonic period, playing a role in axonal growth.[6,7] The a-SMN protein expression is down-regulated after birth.[6] However, there are no reports on the relationship between a-SMN expression and SMA disease severity.

We had an opportunity to gain insight into the contribution of a-SMN to disease severity through observation of two 1-year-old boys with SMA type 1. One patient carried a

C-terminal-disruptive mutation in *SMN1* exon 6, while the other had lost a whole region of *SMN1*. In this study, we demonstrated that SMA severity in Patient 1 was mainly determined by defective FL-SMN, and not a-SMN.

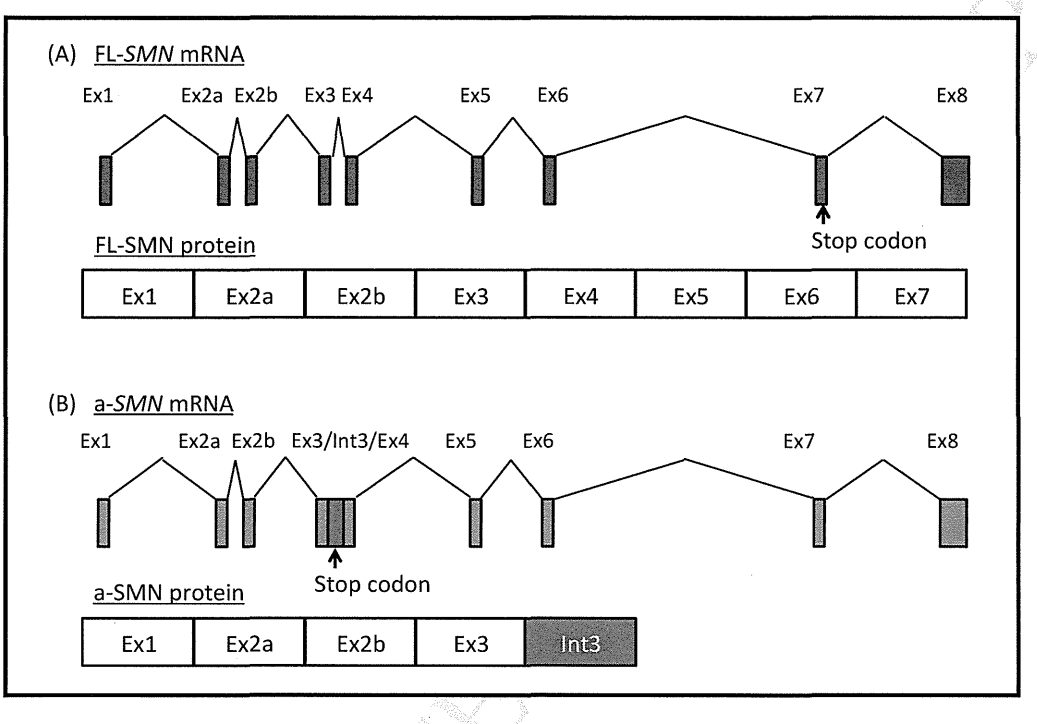

Figure 1.

Schematic presentation of FL-*SMN* and a-*SMN* mRNA and their protein products. (A) FL-SMN; and (B) a-SMN. Boxes indicate exons, and diagonal lines connecting boxes indicate splicing patterns. In a-*SMN* mRNA, exon 3, intron 3, and exon 4 comprises one large exon with a stop codon in intron 3.

Case Descriptions

Patient 1

Patient 1 was a 7-month-old boy. He was born at 41 weeks of gestation, the first child of non-consanguineous healthy parents without any significant family history. His birth weight was 3,256 g and Apgar score was 7/9 (1min/5min). Shortly after birth he showed dyspnea with paradoxical breathing and generalized hypotonia, and was transferred to our hospital. Oral

feeding was started on day 1, but poor sucking became increasingly apparent within the first 10 days, and a nasogastric feeding tube was inserted at age 19 days. From 20 days of age, his dyspnea worsened and was treated with nasal directional positive airway pressure (nDPAP). Neurological examination revealed decreased deep tendon reflexes in the upper and lower limbs. Ocular movement was intact and tongue fasciculation not observed. His respiratory condition gradually deteriorated despite the use of nDPAP, and he became bound to an artificial ventilator at 3 months old, undergoing a permanent tracheostomy at 6 months.

Patient 2

Patient 2 was a 6-year-old boy. He was born at 41 weeks of gestation, the first child of non-consanguineous healthy parents without any significant family history. His birth weight was 2,802 g. He was noticed to have mild hypotonia and thoracic deformity at birth, but his sucking, swallowing, and breathing coordination was almost normal. He left the hospital at age 5 days but was hospitalized again at age 2 months because of difficulties in swallowing and dyspnea with excessive saliva in the mouth. Neurological examination at that time revealed decreased deep tendon reflexes in the upper and lower limbs. Ocular movement was intact and tongue fasciculation not observed. About 3 weeks after the second admission, he presented with frequent apnea related to pneumonia. He was intubated and bound to an artificial ventilator from 3 months, and underwent a permanent tracheostomy 5 days after intubation. He was discharged from the hospital at 7 months with an artificial ventilator and a nasogastric feeding tube.

Molecular genetic analysis

Molecular genetic analysis showed that Patient 1 carried one copy of *SMN1*, while Patient 2 had no copies (Fig. 2). Multiplex ligation-dependent probe amplification

(MLPA) analysis confirmed Patient 2 had completely lost *SMN1*, with no possibility of a-SMN expression in any period of life (Fig. 2). Both patients shared the same genotype for *SMN2* and *NAIP*, modifying genes of the SMA phenotype; they had two copies of *SMN2* and *NAIP* (Fig. 2).

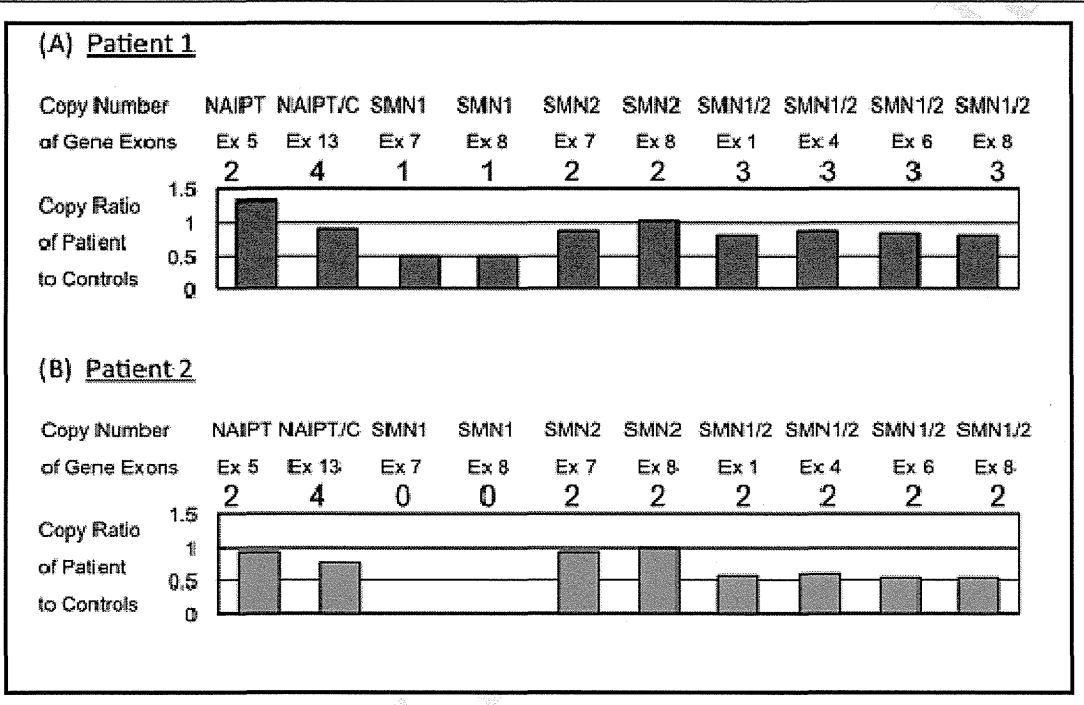

Figure 2.

Copy numbers of *SMN1*, *SMN2*, and *NAIP* exons based on MLPA analysis. (A) Patient 1 and (B) Patient 2. We determined the exon copy number of the genes in each patient using a commercially available Salsa MLPA kit P021 (MRC-Holland, Amsterdam, Netherlands). The vertical axis shows the copy number ratio of patient to control exons. The numbers above histograms indicate the copy number of each exon. Here, NAIPT and NAIPC denote *NAIP* and NAIPψ, respectively.

Nucleotide sequencing of the remaining *SMN1* copy in Patient 1 identified a single nucleotide insertion (c.819_820insT) in exon 6 (Fig. 3).[8] This frame-shifting mutation, which has already been registered in LOVD database (http://www.dmd.nl/nmdb2/home.php?select_db=SMN1), causes disruption of the

C-terminal domain of FL-SMN1 protein (p.Thr274TyrfsX32). The "QNQKE" motif was absent in Patient 1 because of the frame-shifting mutation. Active transport in motor neurons and cytoplasmic localization of FL-SMN in motor neurons are closely related with the presence of a "QNQKE" motif in the domain derived from exon 7.[10] Thus, the mutated FL-SMN1 was likely to be non-functional.

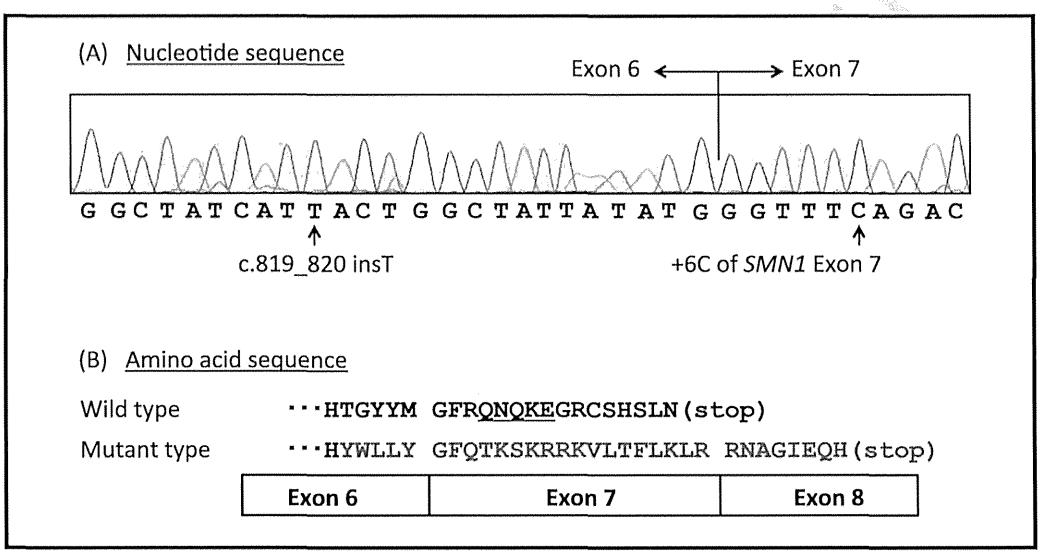

Figure 3.

***SMN1* cDNA exons 6–7 and the C-terminus of FL-SMN1 in Patient 1. (A) Nucleotide sequence. The presence of a *SMN1*-specific nucleotide (+6C in exon 7) confirms the presence of the c.819_820 insT mutation in the *SMN1* gene. (B) Amino acid sequence. The "QNQKE" motif is absent in Patient 1 (see Text).**

In Patient 1, additional mutations were not identified in any exons other than exon 6 or intron 3 of *SMN1*. Thus, Patient 1 carried an intact a-SMN sequence in the remaining *SMN1* allele, suggesting high possibility of a-SMN expression in the embryonic period.

Discussion

Disease course and severity in Patients 1 and 2 were similar. Therefore, our findings indicated that defective FL-SMN function due to the frame-shifting mutation in Patient 1 was compatible with absence of FL-SMN due to complete loss of the *SMN1* gene in Patient 2. In Patient 1, the disrupted C-terminal domain of FL-SMN may have a serious effect on cellular function as the domain is closely associated with self-oligomerization, active transport, and cytoplasmic localization of FL-SMN in motor neurons.[9,10]

Interestingly, the presence or absence of a-SMN did not seem to influence disease severity in the two patients examined. Patient 1 carried an intact sequence coding a-SMN with high possibility of a-SMN expression in the embryonic period, while Patient 2 has no sequence coding a-SMN and no possibility of a-SMN expression. We cannot provide evidence for embryonic expression of a-SMN in material obtained from Patient 1 after birth, but even if enough a-SMN was expressed in the embryonic period, it is unlikely to function efficiently enough to modify SMA disease severity because of the frame-shifting mutation disrupting the C-terminal domain of FL-SMN.

We previously examined two patients with a mutation (c.275G>C) in *SMN1* exon 3, leading to an amino acid substitution (p.Try92Ser) in the Tudor domain.[11] The p.Try92Ser mutation alters a-SMN structure.[7] Locatelli *et al.* clearly demonstrated that the p.W92S mutation hampers axonal growth of NSC34 motor neurons induced by a-SMN.[7] However, from our own observations, the clinical phenotype of the patients with the p.W92S mutation is not as severe as Patient 1 in this study: neither of them required artificial ventilators in the first year of life.[11] In other words, disruption of the C-terminal domain of FL-SMN may have a more serious effect on SMA phenotype compared with a mutation in the Tudor domain of a-SMN (and FL-SMN).

We should mention several limitations of the present study. First, we could not show a-SMN expression in Patient1, not only in the fetal period but also in the postnatal period. The a-SMN expression is down-regulated after birth.[6] According to our experience, it is impossible to detect a-SMN expression in the peripheral blood cells of human infants and children. Second, we examined only a set of SMA patients with the same genotypic background except axonal-SMN. To formulate general concepts about axonal-SMN, we need more sets of SMA patients with the same genotypic background except axonal-SMN. At last, we did test only two genes modifying the SMA phenotype, *SMN2* and NAIP. These genes have already been proved to modify the SMA phenotype. However, it has not been clarified until now what genes are critically involved in modifying mechanism of the SMA phenotype.

In conclusion, in Patient 1, the C-terminal domain of FL-SMN1 determined the severity, rather than the a-SMN, one copy of which could be present and intact, although its generalization may be premature. Our study suggested that SMA disease severity may be determined by C-terminal defects of FL-SMN1, irrespective of presence or absence of a-SMN expression. However, it should be noted that this study focused on disease progress, including respiratory dysfunction, and it is necessary to further study the role of a-SMN in axonal growth of motor neurons, as a-SMN may have roles in fine tuning of neural circuit formation.

Acknowledgments

This study was supported in part by Grants-in-Aid provided from the Research Committee of Spinal Muscular Atrophy, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.