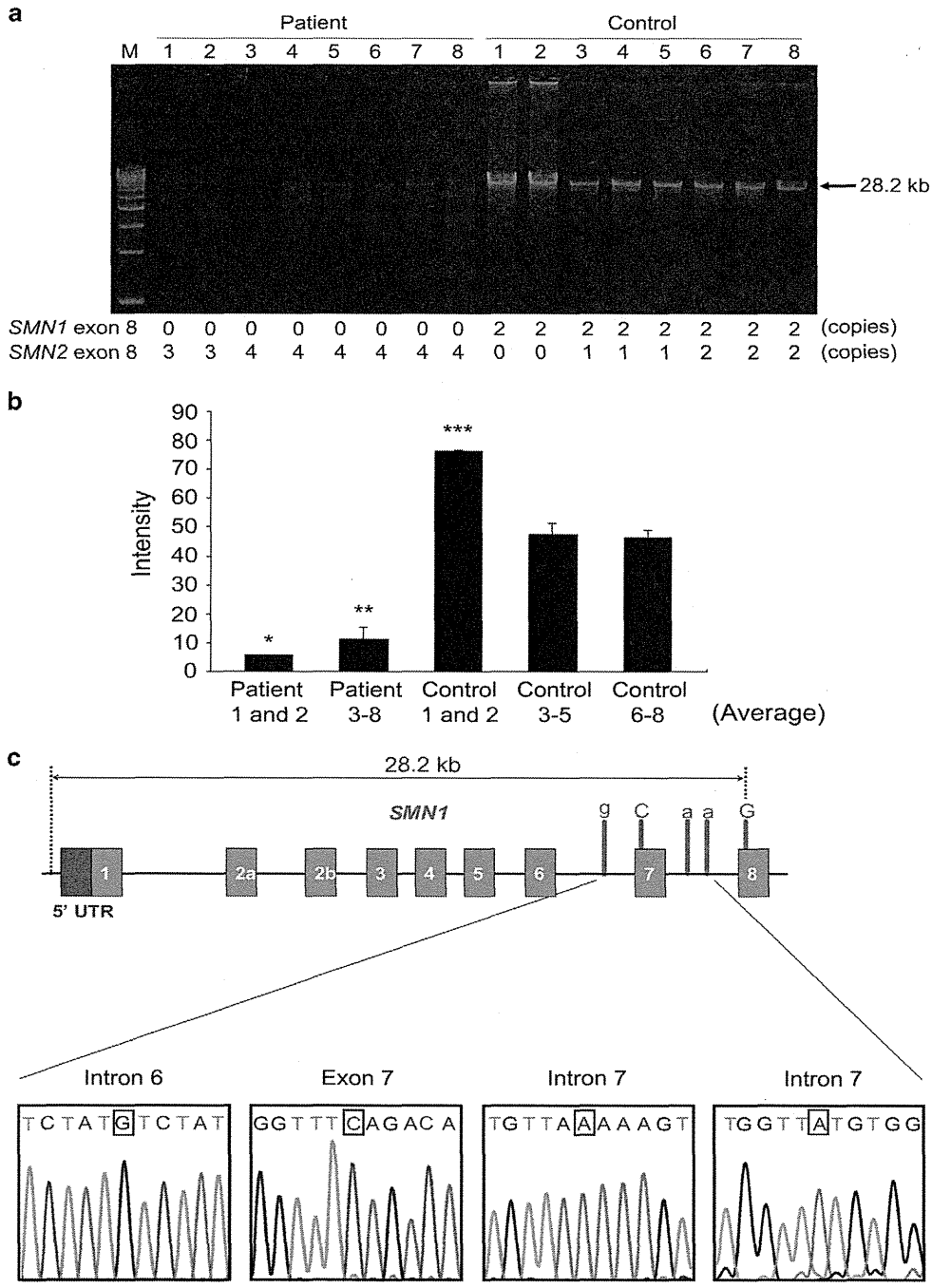

Figure 2 Evaluation of new method. *SMN1*-specific amplifications from exon 1(-654) to exon 8 (28.2 kb) are shown. (**a**) Controls 1–8 yielded 28.2-kb amplicons, whereas there were few signs of amplification in patients 1–8. Copy numbers of *SMN1* and *SMN2* exon 8 determined by MLPA are shown at the bottom of each line. M, molecular weight marker (TAKARA 2.5-kb DNA Ladder). (**b**) Quantification of nLR-PCR products. Average intensities of samples with the same *SMN2* exon 8 copy number are presented. *P*-value: Student's *t*-test. *Patients 1 and 2 versus controls 6–8; $P=0.001$, **Patients 3–8 versus controls 6–8; $P=0.000$, ***controls 1 and 2 versus controls 6–8; $P=0.002$. (**c**) *SMN1* specificity was confirmed by the presence of intron 6, exon 7 and intron 7 sequences. A full color version of this figure is available at the *Journal of Human Genetics* online.

DISCUSSION

We developed an efficient and broadly applicable LR-PCR method to detect intragenic mutations in *SMN1* (Figure 1). Without the need for complementary DNA cloning, this new method makes it possible to analyze all exons and introns of *SMN1*, the 5′- and 3′-untranslated regions, the promoter region, small or large insertions and deletions and hybrid *SMN* genes. Differences between controls and patients were clear ($P<0.05$), and the specificity was verified (Figure 2b). The absence of *SMN2*, which inhibits *SMN1*-specific PCR, yielded an increase in nLR-PCR products (controls 1 and 2). Even when there are more copies of *SMN2* than of *SMN1*, specific *SMN1* regions can be amplified using our nLR-PCR method (Figure 3).

We identified a novel mutation in exon 1 of *SMN1*, c.5C>T, in three unrelated patients (patients 10–12) with SMA type III (Table 1).

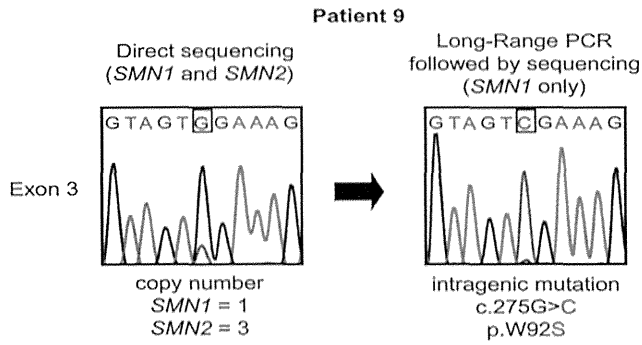

Figure 3 Detection of an intragenic mutation in a patient with type I SMA. Patient 9 was compound heterozygous for *SMN1*, with one deleted *SMN1* allele and an intragenic mutation (c.275G>C, p.W92S) in the other allele.[19] This patient had three *SMN2* copies. (Left) Direct sequencing of *SMN1* and *SMN2*; (Right) Sequencing of *SMN1* exon 3 isolated by the new long-range PCR technique is shown.

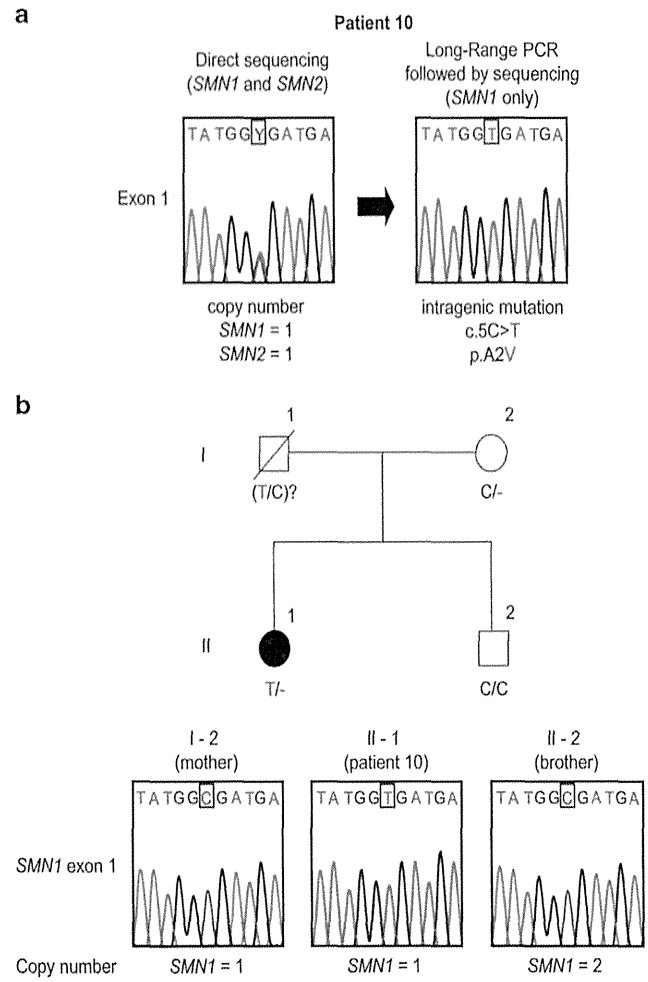

Figure 4 Identification of an intragenic mutation in *SMN1*. (**a**) Patient 10 had one copy each of *SMN1* and *SMN2*. (Left) Direct sequencing for *SMN1* and *SMN2* results are shown; (Right) direct sequencing of *SMN1* (right) exon 1 isolated by the new long-range PCR technique. The sequence revealed a c.5C>T mutation (red signal), leading to an alanine-to-valine substitution (p.A2V). (**b**) Patient 10 family analysis. The mutation in patient 10 (II–1) was absent from I–2 and II–2.

With the currently available methods, it was difficult to isolate only *SMN1* mRNA from the peripheral blood leukocytes of patient 11 (data not shown). We attribute this to low *SMN1* mRNA expression in these cells. Although family members of patient 10 (II–1) were shown by sequencing analysis to have the c.5C>T mutation, the intragenic mutation in patient 10 (II–1) was absent in both her mother (I–2) and her brother (II–2; Figure 4b). Patient 10 (II–1) had inherited the allele deletion from her mother, whereas the intragenic mutation had either been inherited from her father or occurred *de novo*.

The c.5C>T mutation was evaluated as a hazardous change based on *in silico* analysis results. The c.5C>T mutation was not registered in dbSNP, the 1000 Genome Project database or the Human Genetic Variation Database and might be a Japanese-specific variant. Consistent with these results, one patient with SMA type II and two with SMA type III with c.5C>G (p.A2G, dbSNP: rs75030631) mutations were reported previously.[23] These patients had only one *SMN2* copy and presented with similar mild symptoms. There are also reports of SMA associated with the c.5C>G mutation. Although *SMN* knockout mice with low *SMN2* copy numbers have severe SMA, phenotype rescue could be achieved in a transgene SMN A2G missense mutant.[24] Although SMN knockout is lethal in mouse embryos,[25] SMN(A2G) SMA mice exhibit the onset of motor neuron loss, resulting in mild SMA. The SMN A2G mutation inhibits self-association and affects SMN binding, probably by disrupting the formation of SMN oligomers. Because the effect of p.A2G is mild, it is associated with a later age of onset and relatively mild symptoms. The p.A2V variation is likely similar to p.A2G in its phenotypic effect.

Phenotypic effects might differ among intragenic mutation positions. For example, despite patient 9, with W92S(c.275G>C) and *SMN1* deletion, having three copies of *SMN2*, the relatively severe SMA type I phenotype was evident.[19] This mutation was located in exon 3, corresponding to the Tudor domain, an essential region for interaction of SMN with fundamental components of multiple nuclear RNA-protein complexes. This mutation impaired the interaction of SMN with various proteins. Therefore, mutations of this type may have a critical impact on SMN function.

Furthermore, the positions of intragenic mutations seemed to have more profound effects on phenotype than the size of the deletion in one allele. Although patients 10–12 had a large deletion including *NAIP* and *H4F5* in one allele (Supplementary Table 2), their phenotype was mild. On the other hand, although patient 9 had a small deletion including only *SMN1*, the SMA phenotype was severe.

We identified three hybrid *SMN* gene types in eight patients. Our method enables the direct isolation and sequencing of the entire hybrid *SMN* gene. We identified large (Type A), complex (Type B) and small conversions (Type C; Figure 5). SMA in patients 13–17 was associated with a deletion in *SMN1* exon 7 combined with an *SMN1-to-SMN2* conversion. SMA in patients 17–20 was associated with a homozygous *SMN1-to-SMN2* conversion. Cusco *et al.*[26] reported milder symptoms in patients with a homozygous conversion than in those with a combination of deletion and conversion. An association between disease severity and conversion has been described[27] but other reports suggest no such association.[28] Increased copy numbers of hybrid *SMN* genes and *SMN2* have also been reported to be associated with disease severity.[26] In this study, similar to a report by *Cusco et al.*,[26] symptoms were found to be milder in patients 18–20, who carry a homozygous conversion. Patient 15 had late onset of disease compared with patients 13, 14, 16 and 17, and could walk,

Table 1 Detected mutations, genotypes and phenotypes

Patient	SMA type	Onset (year)	Mutation	Site of mutation	*SMN2* copy number	Phenotype	Reference
9	I	<6 m	c.275G>C, p.W92S	Exon 3	3	Japanese male severely floppy infant, muscular hypotonia, depression of tendon reflexes. At 5 months, he exhibited poor sucking. At 8 months, ventilator support was required.	Kotani *et al.*[19]
10	III	12	c.5C>T, p. A2V	Exon 1	1	Japanese female showing motor function regression with symmetrical muscle weakness in the limbs. Walked until age 32; wheelchair-bound since age 32. Positive Gowers sign and waddling gait; muscle biopsy showed neurogenic changes.	—
11	III	11	c.5C>T, p. A2V	Exon 1	1	Japanese male with muscular atrophy and muscle weakness of the quadriceps. Walking at age 11; easily tired by non-strenuous exercise. Progressive muscle weakness of the limbs starting at age 13. Electromyography showed a neurogenic pattern. Muscle biopsy showed neurogenic changes.	Yamamoto *et al.*[30]
12	III	13	c.5C>T, p. A2V	Exon 1	1	Japanese female with mild proximal lower limb weakness and plantar muscular atrophy. Walking and swimming at age 13. Waddling gait; gradually lost ability to run. Electromyography showed a neurogenic pattern; muscle biopsy showed neurogenic changes.	Yamamoto *et al.*[30]

Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron.

Table 2 Hybrid *SMN* gene analysis in eight SMA patients with homozygous deletion of *SMN1* exon 7 but not exon 8

Patient	SMA Type	Onset (year)	Highest function	Copy number: *SMN2* E7	*SMN2* E8	*SMN1* E7	*SMN1* E8	Hybrid SMN gene sequence I6, E7, I7, E8	Hybrid type
13	III	6 m[a]<	Walk	3	2	0	1	*aTa***gG**	B
14	III	12 m[a]	Stand	3	2	0	1	*aTgg***G**	A
15	III	8	Walk	3	2	0	1	**g***Taa***G**	C
16	III	14 m[a]	Stand	3	2	0	1	*aTgg***G**	A
17	III	9 m[a]	Stand	3	2	0	1	*aTgg***G**	A
18	III	3	Walk	4	3	0	1	*aTgg***G**	A
19	III	15	Walk	4	2	0	2	*aTgg***G**	A
20	IV	40	Walk	4	2	0	2	*aTgg***G**	A

Bold face: sequence (**gCaaG**) derived from *SMN1*; italics: sequence (*aTggA*) derived from *SMN2*.
[a]We assigned SMA type by giving priority to evaluating each patient's highest function over age of onset.

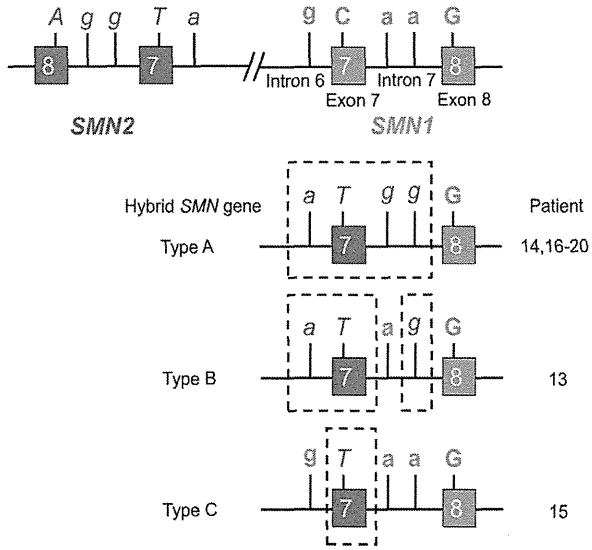

Figure 5 Schematic illustration of the three hybrid *SMN* gene types. Dotted line frames indicate *SMN2* sequences and show the *SMN1-to-SMN2* gene conversion. The type A hybrid was most common. The sequences of intron 6, exon 7 and intron 7 were of *SMN2* origin, whereas that of exon 8 was of *SMN1* origin. Type B was a complex form. The sequences of intron 6, exon 7 and intron 7 (only one base) were of *SMN2* origin, whereas those of intron 7 (the other base) and exon 8 were of *SMN1* origin. Type C had the fewest changes: the exon 8 sequence was of *SMN2* origin, whereas intron 6, intron 7 and exon 8 were of *SMN1* origin. A full color version of this figure is available at the *Journal of Human Genetics* online.

thereby showing disease severity similar to that of patients 18–20. We speculate that milder symptoms might correspond to small conversion regions, like Type C.

Patients with a missense mutation or hybrid *SMN* gene, identified in this study, showed relatively mild SMA symptoms. As to possible mechanisms underlying such mild symptoms, Prior *et al.*[29] reported that the c.859G>C substitution in the *SMN2* gene is a positive modifier of the SMA phenotype. Although we tested for the c.859G>C change in the *SMN2* gene, neither the missense mutation nor the hybrid *SMN* gene (patients 9–20) carried this change.

Our method for detecting intragenic mutations of *SMN1* by nLR-PCR (28.2 kb) is more efficient and has broader applications than the currently available methods. In three patients for whom current methods yielded no results, we identified a c.5C>T mutation in *SMN1* exon 1. In eight patients with a hybrid *SMN* gene, we identified three hybrid types. This new method allows analysis of previously undetectable regions, including all introns and exons of *SMN1* and all *SMN* genes. Furthermore, we identified three distinct hybrids.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to the patients who agreed to participate in this study. We thank Drs T Yamamoto, S Morikawa, S Sawai, H Nakajima, T Oshita and T Kurashige for their valuable contributions to this study. This work was supported by Grants-in-Aid from the Research Committee of Spinal Muscular Atrophy, the Ministry of Health, Labour and Welfare of Japan (to KS). This

work was also supported by the Global COE program, Multidisciplinary Education and Research Center for Regenerative Medicine (MERCREM) from the Ministry of Education, Culture, Sports Science, and Technology (MEXT), Japan (to YK and KS).

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

DIAGNOSTIC USE OF SURFACE EMG IN A PATIENT WITH SPINAL MUSCULAR ATROPHY

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Running title: SEMG in the trapezius muscle

Word Count: 499 words

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/mus.24643

We recently documented that surface EMG (SEMG) successfully discriminated between neurogenic and myopathic disorders using the newly devised "Clustering index" (CI) method.[1,2] Here we report on a patient with spinal muscular atrophy (SMA), who had long been misdiagnosed with Duchenne muscular dystrophy (DMD), but in whom SEMG led to a correct diagnosis.

A 44-year-old man was admitted to our hospital with dyspnea. Developmentally, he could sit but did not acquire ambulation. He was diagnosed with DMD at age 2 years without further genetic confirmation and had received home care without a ventilator. His skeletal muscles, including the tongue were extremely atrophic, and only slight voluntary movements were noticed in the finger flexors, trapezius and gastrocnemius muscles. We doubted the diagnosis of DMD because of failure to acquire ambulation, preserved cardiopulmonary function, normal creatine kinase, and tongue atrophy instead of macroglossia. SMA was thought to be the more likely, although a congenital myopathy was also considered. He refused to undergo even a brief needle EMG examination. Therefore, we attempted an SEMG evaluation to differentiate a neurogenic from a myopathic disorder.

SEMG was performed on the trapezius muscle. Five age- and gender-matched controls (39–51 years) were also examined. All subjects gave informed consent, and the study was approved by the local ethics committee. Two Ag-AgCl cup electrodes were placed on the muscle belly 3 cm apart. The bandpass filter was set at 50 to 1000 Hz. The subject was asked to perform a sustained contraction of the trapezius muscle, and 10 to 20 SEMG epochs of 1-s length from weak to maximal contractions were recorded from each subject.

Eleven SEMG epochs from the patient, and 91 epochs from the controls were collected. Upon visual inspection, single MUPs firing up to 27 Hz were identified in the patient during weak contractions (Fig. 1A), which corresponded to a reduced

recruitment pattern on needle EMG. In contrast, a complete interference pattern was observed from the weakest contraction in control subjects (Fig. 1B). The CI method[1] using a window width of 7.5 ms was also applied to these signals. In the CI vs. area plot, the epochs from the patient and the controls were well segregated (Fig. 1C). SEMG showed no MUPs in the tibialis anterior muscle and also suggested a neurogenic pattern in the gastrocnemius muscle. These results indicated a neurogenic disorder. Genetic analysis of the survival motor neuron gene subsequently confirmed the diagnosis of SMA type 2.[3]

We previously demonstrated that individual MUPs can be recorded by SEMG using appropriate electrode placements.[1,2] In the trapezius muscle of this very weak patient, ordinary bipolar SEMG recordings revealed unequivocal neurogenic features. The CI method quantitatively supported this conclusion. SEMG might be considered as an alternative examination when needle examination is not possible, and would be especially promising for the examination of children.

Acknowledgement: This study was partly supported by Health and Labour Sciences Research Grants for Research on Intractable Diseases (Neuroimmunological Disease Research Committee) from the Ministry of Health, Labour and Welfare of Japan.

Abbreviations:

CI, clustering index; DMD, Duchenne muscular dystrophy; MUP, motor unit potential; SEMG, surface electromyography; SMA, spinal muscular atrophy

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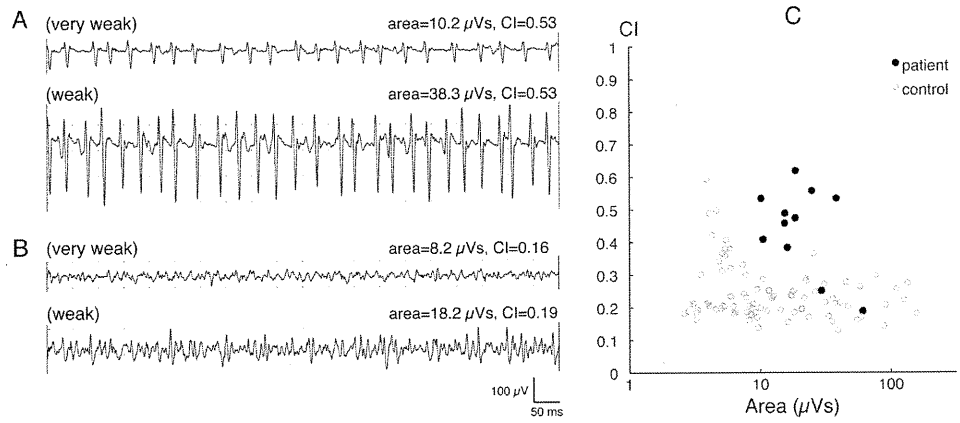
FIGURE LEGEND

FIGURE 1. (A) Raw SEMG signals from the trapezius muscle of the patient. Single MUPs firing at 23 or 27 Hz are observed during weak contractions, which correspond to the reduced recruitment pattern characteristic of neurogenic changes seen on needle EMG. (B) Raw SEMG signals from a control subject (44-year-old man). A complete interference pattern is observed from the weakest contraction. (C) The CI vs. area plot using a window width of 7.5 ms. Closed circles are epochs from the patient, and the open circles are those from controls. The 2 are well segregated, with the patient exhibiting higher CI values during weaker contractions.



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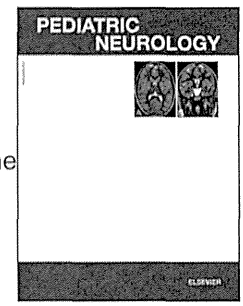

Two Japanese patients with SMA type 1 suggest that axonal-SMN may not modify the disease severity

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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.

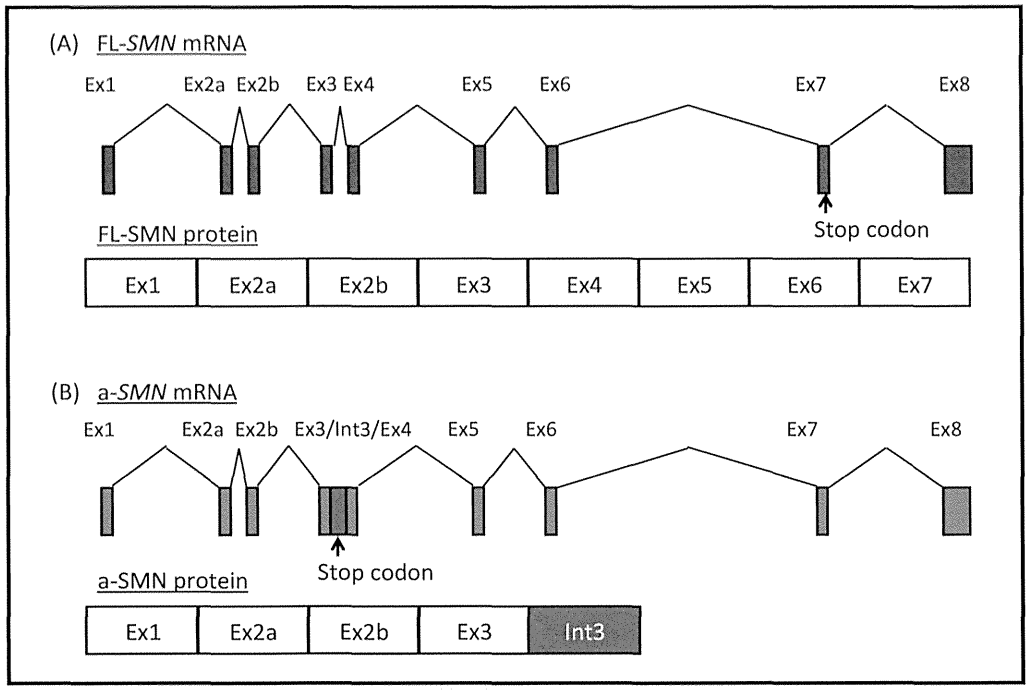

Figure 1.

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).

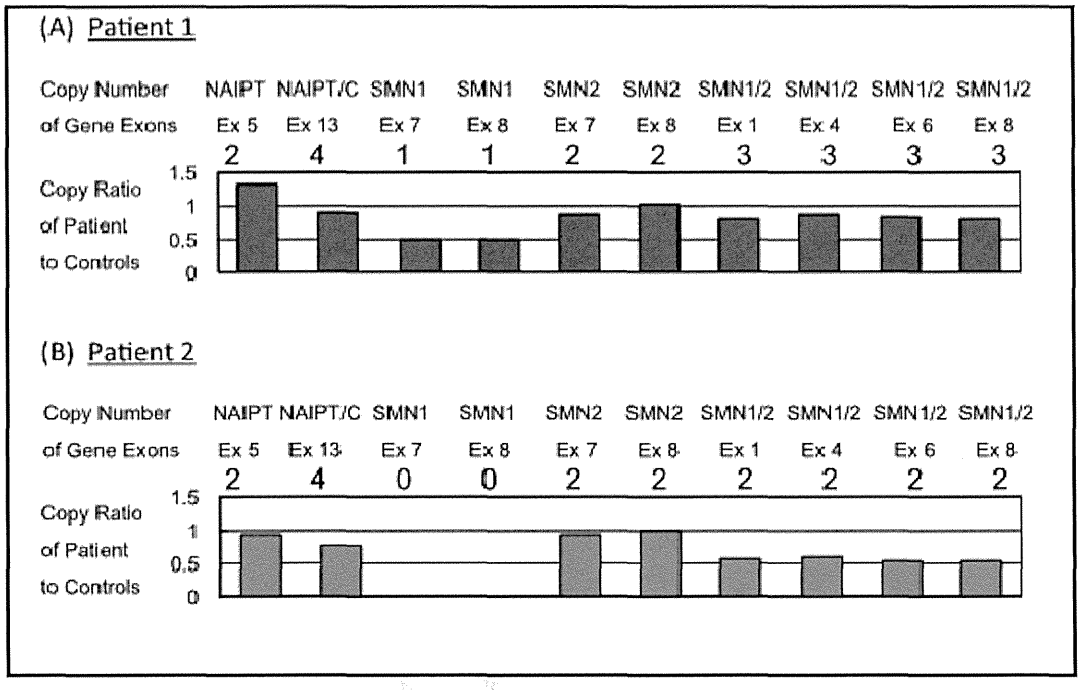

Figure 2.

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.

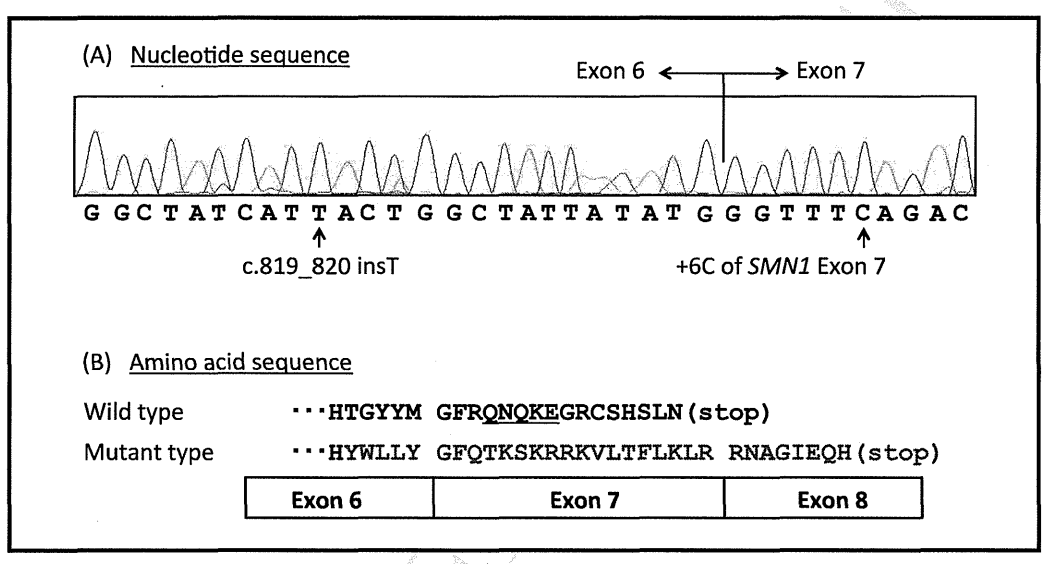

Figure 3.

***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).