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Diagnosis of Spinal Muscular Atrophy Via High-Resolution Melting Analysis Symmetric Polymerase Chain Reaction Without Probe: A Screening Evaluation for *SMN1* Deletions and Intragenic Mutations

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Aim: Spinal muscular atrophy (SMA) is a well-defined autosomal recessive neuromuscular disorder caused by mutations in the survival motor neuron 1 (*SMN1*) gene. The most frequently observed mutation is a deletion of exon 7, which has been documented in >95% of SMA patients. A novel technique for detecting mutations known as high-resolution melting analysis (HRMA) has rapidly become the tool of choice for screening pathogenic genetic variants. In the present study, we attempt to validate the applicability of HRMA to the detection of exon 7 deletions and other intragenic mutations in *SMN1*. *Results:* Three primer sets were adopted in our HRMA screening for deletion of *SMN1* exon 7. In screening attempts utilizing two primer sets, the results of HRMA were not compatible with those obtained by polymerase chain reaction–restriction fragment length polymorphism. Therefore, we applied a modified protocol using revised primer sets, which resulted in an absolute compatibility of results between HRMA and polymerase chain reaction–restriction fragment length polymorphism. With regard to screenings for intragenic mutations in *SMN1* exon 3, two primer sets were adopted for use in HRMA. In the initial HRMA screening using the first primer set, we failed to identify any intragenic mutations; however, when using a revised primer set, HRMA successfully detected the presence of a c.275G>C mutation. *Conclusion:* HRMA is a simple but versatile tool to add to the existing arsenal of diagnostic techniques that could aid clinicians/researchers in diagnosing SMA. However, as we demonstrate in the present study, the design and selection of primers is of monumental importance in ensuring the successful application of HRMA to screening for pathogenic variants.

Introduction

SPINAL MUSCULAR ATROPHY (SMA) is a neuromuscular disorder characterized by degeneration and loss of lower motor neurons in the anterior horns of the spinal cord. SMA is one of the most prevalent autosomal recessive disorders with an incidence of 1/10,000 (Pearn, 1978). In addition, the SMA carrier ratio in the general population is estimated at 1:35 (Feldkötter *et al.*, 2002). The survival motor neuron (*SMN*) gene located on 5q13 is the most common cause of SMA (Lefebvre *et al.*, 1995). It exists as two nearly identical copies, *SMN1* and *SMN2*—both of which encode the SMN protein. To date *SMN1* remains the most critical gene involved in the development of SMA, as >95% of SMA patients present with homozygous mutations of *SMN1* exon 7. The remaining minority groups of SMA patients carry subtle intragenic mutations in *SMN1* (Lefebvre *et al.*, 1995). In every SMA patient with a homozygous deletion of *SMN1* exon 7, at least one copy of *SMN2* is retained; however, because the majority of *SMN2* transcripts lack the information preserved on exon 7—as a result of alternative splicing (*Δ7-SMN*)—the amount of functional SMN protein produced by *SMN2* is insufficient to compensate for the loss of *SMN1*, which ultimately results in progressive motor neuron degeneration (Lorson and Androphy, 2000).

The two *SMN* genes, *SMN1* and *SMN2*, are virtually identical with exception to a difference of five bases found in the 3′ end of the genes (Bürglen *et al.*, 1996). This difference in nucleotides has been exploited in screening efforts to detect deletions of the *SMN1* gene, used for diagnosing SMA. At present, techniques based on polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP),

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which are capable of differentiating *SMN1* and *SMN2* based on their nucleotide differences, remain the most widely applied methods in detecting homozygous deletions of *SMN1* exon 7 (Ogino and Wilson, 2002). Unfortunately, the results of PCR-RFLP can occasionally be ambiguous due to incomplete enzyme digestion. While there are alternative procedures to detect homozygous deletion of *SMN1* exon 7, such as single strand conformation polymorphism or denatured high-performance liquid chromatography, these methods are often laborious and time-consuming.

In recent years, high-resolution melting analysis (HRMA) has rapidly emerged as the tool of choice among researchers and clinicians for screening pathogenic variants, owing to its inherent simplicity. This method uses a saturating dye to generate an accurate melting curve with high resolution that enables detection of even a single-nucleotide variation in sample amplicons (Wittwer *et al.*, 2003). Another benefit of HRMA is its ability to perform homogeneous genotyping without resorting to fluorescently labeled probes or allele-specific PCR. At present, two differing HRMA methods have been established: the first involves symmetric PCR without a probe (symmetric PCR-HRMA); the second involves asymmetric PCR with a probe (asymmetric PCR-HRMA). In general, the former method is considered a far simpler method.

Recently, Chen *et al.* (2009) evaluated the application of HRMA to screenings for deletion of *SMN1* exon 7, and based on their results, the authors conclude that asymmetric PCR-HRMA is superior to symmetric PCR-HRMA. The difficulty in distinguishing patients lacking *SMN1*, from normal controls lacking *SMN2*, was one of the major factors attributing to the authors' final assessment contending the apparent inferiority of symmetric PCR-HRMA (Chen *et al.*, 2009). In contrast, in the present study, we demonstrate that symmetric PCR-HRMA—with a simple change of primer sets—is an able and dexterous method which allows for distinguishing patients lacking *SMN1* from normal controls, via discernible differences in melting curve patterns. In addition to this, we further applied and evaluated symmetric PCR-HRMA in the scanning of an intragenic *SMN1* mutation, and show that symmetric PCR-HRMA is also capable of identifying an intragenic mutation in *SMN1* exon 3 that we had early identified by direct sequencing.

Materials and Methods

Subject

Forty-three SMA patients and 56 controls were enrolled in this study. Genomic DNA was extracted from whole blood using a DNA extraction kit, SepaGene (Sanko Junyaku). Screening assessments for deletion of the *SMN1* gene were carried out via HRMA. To efficiently detect *SMN1* gene deletions, PCR and enzyme digestion were performed according to the method described by van der Steege *et al.* (1995). Preliminary screenings for variation in *SMN1* and *SMN2* were conducted via PCR-RFLP: 41 patients had an *SMN1* deletion; 47 controls were found to retain both the *SMN1* and *SMN2* genes; and 3 controls were found to lack the *SMN2* gene.

We previously reported a novel mutation in *SMN1* exon 3, 275G>C. This mutation was identified in two patients with SMA type 1, and results in a tryptophan-to-serine substitution at amino acid 92 (W92S), which is found at the N-terminal of the SMN Tudor domain (Kotani *et al.*, 2007).

Regarding the screening evaluation for the W92S mutation, eight individuals were examined via HRMA. Two of the individuals had earlier been identified as having a c.275 G>C mutation by direct sequencing, and in 6 controls, no intragenic mutations in *SMN1* exon 3 could be identified by direct sequencing.

Symmetric PCR-HRMA for SMN1 *deletion test*

Selection of optimal primer sets for PCR is crucial to the successful application of HRMA to *SMN1* deletion tests. In this study, three different primer sets, designated A [R111 (Lefebvre *et al.*, 1995) and SMNR (Chen *et al.*, 2009)], B [R111 and X7Dra (van der Steege *et al.*, 1995)], and C [R111 and 541C770 (Lefebvre *et al.*, 1995)], were examined (Fig. 1). Each PCR product had 2 nucleotide changes between *SMN1* and *SMN2*: c.835-45 G > A and c.840 C > T. PCRs were carried out in 10 μL final volume using 30 ng template DNA and LightCycler®480 High Resolution Melting Master (Roche Diagnostic), which contains DNA polymerase, nucleotides, and the dye ResoLight. Primers and $MgCl_2$ were used at a concentration of 0.3 μM and 3 mM, respectively. HRM assays were performed using LightCycler480 System II (Roche Diagnostic) provided with LightCycler480 Gene Scanning Software (Roche Diagnostic). The PCR program consists of an initial denaturation–activation step at 95°C for 10 min, followed by a 45-cycle program (denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s). The melting program includes three steps: denaturalization at 95°C for 1 min, renaturation at 40°C for 1 min, and then melting, which consists of a continuous fluorescent reading from 65°C to 95°C at 25 acquisition per °C. The melting curve analysis comprises three steps: normalization of melting curves, equaling to 100% the initial fluorescence and to 0% the fluorescence remnant after DNA dissociation, shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA is completely denatured, and, finally, the difference plot analyzes the differences in melting curve shape by subtracting the curves from control samples lacking *SMN2*.

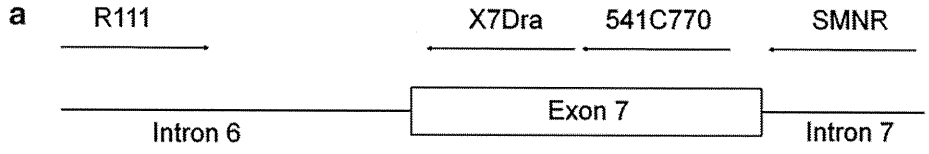
Application of symmetric PCR-HRMA for detection of subtle SMN1 *mutations*

In applying HRMA to the detection of intragenic *SMN1* exon 3 mutations, designing optimal primer sets—which took into consideration the unique polyadenine tract in intron 3—was a crucial factor. Considering this unique feature of intron 3, two different primer sets, D (gEX3F and gInt3R) and E (gEX3F and cEX3R), were examined (Fig. 2). HRMA PCR conditions were as described above, with slight adjustments introduced in annealing temperature to accommodate differing primer sets.

Results

SMN1 *deletion test using symmetric PCR-HRMA*

SMN1 deletion tests were performed via symmetric PCR-HRMA (Figs. 3–5). By using primer set A, which was described by Chen *et al.* (2009), HRMA showed two distinct patterns of melting curves. Samples from normal controls retaining *SMN1* and *SMN2* had a lower melting temperature due to heteroduplex formations of the amplicons. Patient



b

Primer Set	Primer Name	Primer Sequence	Length	Aneealing Temperature	Curve * Separation
A	R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	241bp	56℃	Poor
	SMNR	5'-GAT TCA CTT TCA TAA TGC TGG-3'			
B	R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	183bp	56℃	Poor
	X7Dra	5'-CCT TCT TTT TGA TTT TGT TT-3'			
C	R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	202bp	56℃	Good
	C541770	5'-TAA GGA ATG TGA GCA CCT TCC TTC-3'			

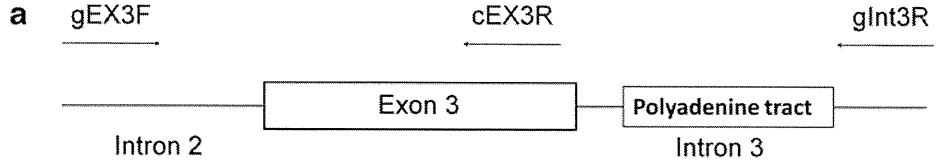
FIG. 1. Primer positions, primer sets, and annealing temperature for the survival motor neuron 1 (*SMN1*) deletion test. **(a)** Primer positions. **(b)** Primer sets and annealing temperature. *Curve separation: "Good" means that high-resolution melting analysis (HRMA) can distinguish melting curve of spinal muscular atrophy (SMA) patients from that of healthy control. "Poor" means that HRMA cannot distinguish melting curve of SMA patients from that of healthy control.

samples lacking *SMN1*, along with control samples lacking *SMN2*, had a higher melting temperature due to homoduplex formations of the amplicons. As shown in Figure 3, under the primer set A protocol, it was nearly impossible to distinguish patient samples lacking *SMN1* from control samples lacking only *SMN2*.

In assessments using primer set B, which includes a reverse primer described by van der Steege *et al.* (1995), HRMA with primer set B also showed two distinct patterns of melting curves (Fig. 4). Unfortunately, as with primer set A, it was impossible to distinguish patient samples lacking *SMN1* and control samples lacking only *SMN2*. Distinguishing patients lacking the *SMN1* gene and controls lacking only the *SMN2* gene was a common issue, as observed by Chen *et al.* (2009).

In assessments using primer set C, which includes a reverse primer described by Lefebvre *et al.* (1995), HRMA showed apparently different results from those of primer sets A and B. HRMA with primer set C showed three discernible patterns of melting curves (Fig. 5). The difference plots of the melting curves with primer set C had a single peak. As shown in Figure 5, when using primer set C, the difference plot manifested distinct variations between patient samples lacking *SMN1*, control samples retaining *SMN1* and *SMN2*, and control samples lacking *SMN2*.

With regard to the detection of *SMN1* deletions, we classified normal controls (controls lacking *SMN2* and controls retaining *SMN1* and *SMN2*) and SMA patients (patients lacking *SMN1*) into the "No *SMN1* deletion," and "*SMN1* deletion" groups, respectively. Controls retaining only *SMN1* were correctly placed in the "No *SMN1* deletion" group by symmetric PCR-HRMA. In contrast, patients lacking the *SMN1* gene were correctly placed into the "*SMN1*



b

Primer Set	Primer Name	Primer Sequence	Length	Aneealing Temperature	Curve * Separation
D	gEX3F	5'-GTG TGC ATG TAT ATA TCT TT-3'	308bp	52℃	Poor
	gInt3R	5'-TGT ATA CTG TTT AAC AGT TT-3'			
E	gEX3F	5'-GTG TGC ATG TAT ATA TCT TT-3'	224bp	50℃	Good
	cEX3R	5'-TCT GTT CTA TAT TAT TAG CTA CT-3'			

FIG. 2. Primer positions, primer sets, and annealing temperature for screening of SMN intragenic mutation in exon 3. **(a)** Primer positions. **(b)** Primer sets and annealing temperature. *Curve separation: "Good" means that HRMA can distinguish melting curve of SMA patients from that of healthy control. "Poor" means that HRMA cannot distinguish melting curve of SMA patients from that of healthy control.

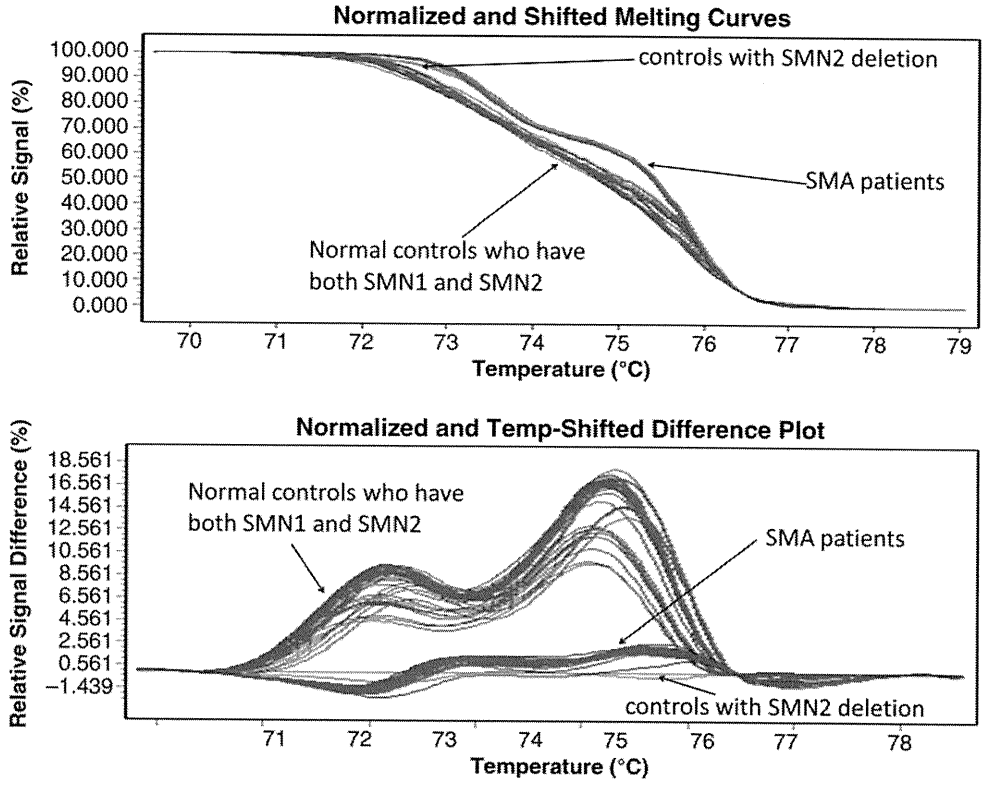


FIG. 3. Results of HRMA by using primer set A. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

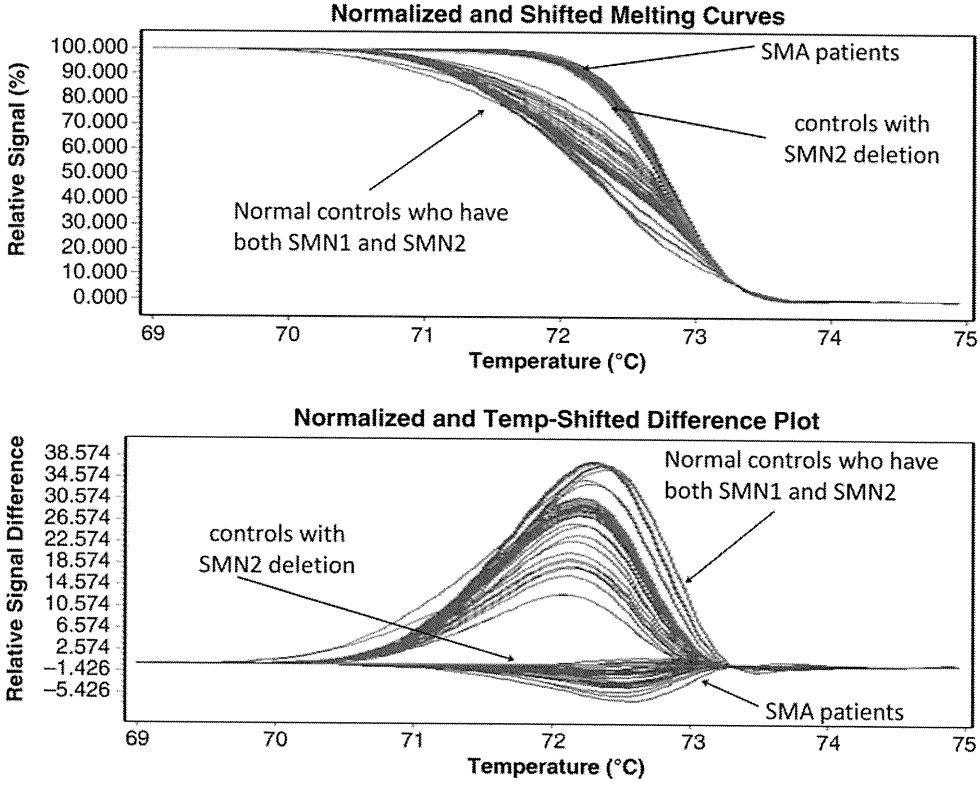


FIG. 4. Results of HRMA by using primer set B. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

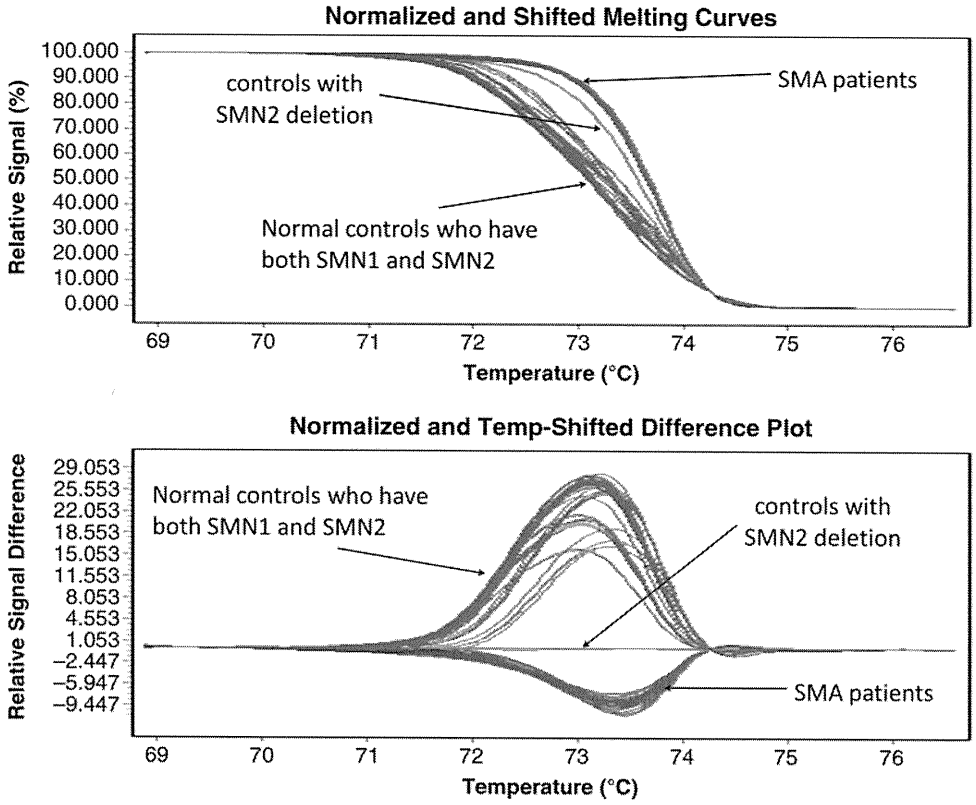


FIG. 5. Results of HRMA by using primer set C. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

deletion" group by symmetric PCR-HRMA. Essentially, the HRMA results obtained using primer set C were completely compatible with the data obtained by conventional PCR-RFLP methods (sensitivity = 1.0, specificity = 1.0) (Table 1). Additionally, we further ventured to determine the relationship between the difference plots and the number of copies of the *SMN1* and *SMN2* genes among control samples (Fig. 6). HRMA showed six variable patterns of difference plot peaks.

Application of symmetric PCR for detection of intragenic SMN *mutations*

For detection of intragenic mutations, screenings were performed by optimized symmetric PCR-HRMA. For mutation screenings covering *SMN1* exon 3, the presence of a polyadenine tract posed a potential of compromising the screening effort. However, this was circumvented with the use of two reverse primers. cEX3R was positioned in exon 3, upstream of the polyadenine tract, and gInt3R was positioned in intron 3, downstream of the polyadenine tract (Fig. 2). In assessments using primer set D, we observed irregular patterns of difference plots, which made the task of distinguishing patient samples having a c.275 G>C mutation from control samples with no mutation a nearly impossible feat (Fig. 7). Only after using primer set E did we observe two patterns of difference plots that correlated with patients and controls (Fig. 7). Samples from patients who had a mutation in exon 3 showed a lower melting temperature due to heteroduplex formation of amplicons (mutated *SMN1* and normal *SMN2*). Samples from controls, on the other hand, showed a higher melting temperature due to homoduplex formation of amplicons. Therefore, with primer set E, samples from patients who had c.275 G>C mutations could clearly be distinguished from control samples.

TABLE 1. SENSITIVITY AND SPECIFICITY OF SYMMETRIC POLYMERASE CHAIN REACTION–HIGH-RESOLUTION MELTING ANALYSIS WITH PRIMER SET C

		PCR-RFLP	
		SMN1 deletion	*No SMN1 deletion*[a]
Symmetric PCR-HRMA	*SMN1* deletion	41	0
	No *SMN1* deletion	0	50

Sensitivity: 41/41 (1.0); specificity: 50/50 (1.0).

[a]No *SMN1* deletion contains both controls lacking *SMN2* and controls retaining *SMN1* and *SMN2*.

HRMA, high-resolution melting analysis; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SMN, survival motor neuron.

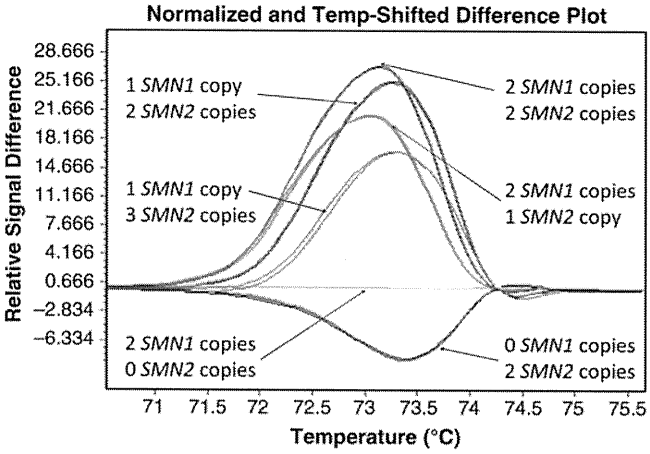


FIG. 6. Copy number analysis of HRMA by using primer set C.

Discussion

HRMA with saturation dye is a new and attractive tool for genotyping and mutation scanning. HRMA has many advantages, such as cost-effectiveness, simplicity, absence of post-PCR treatment, and quickness to identify pathogenic variants (de Juan *et al.*, 2009). Further, HRMA can identify pathogenic variants with high accuracy (Reed and Wittwer, 2004). However, when using HRMA for genotyping or mutation scanning, there are several points to note. Melting temperature is related to external factors (salt concentration and pH) and internal factors (DNA concentration, product length, GC content, and nearest neighbor interactions) (Marziliano *et al.*, 2000).

In an *SMN1* deletion test using HRMA conducted by Chen *et al.* (2009), SMA patients lacking *SMN1* could be readily distinguished from controls lacking *SMN2* by asymmetric PCR with probe. On the other hand, distinguishing patients lacking *SMN1* from controls lacking *SMN2* by symmetric PCR-HRMA without probe was reportedly a daunting and difficult task.

In the present study, we hypothesized that we could distinguish patients lacking *SMN1*, from controls lacking *SMN2* by symmetric PCR-HRMA, simply by applying a modified primer set. The result of HRMA using primer set C on the other hand showed three distinct types of melting curves that specifically correlate with the expected genotypes (controls retaining *SMN1* and *SMN*; controls lacking *SMN2*; and, patients lacking *SMN1*) (Figs. 3–5). Under the difference plots, these differences were fully evident. Further, we succeeded in distinguishing the 41 SMA cases from the controls with a striking 100% sensitivity and specificity as compared with PCR-RFLP analyses (Table 1).

In this study, we further sought to examine the relationship between the difference plots and the copy number of the *SMN1* and *SMN2* genes among control samples (Fig. 6). It should be noted that the difference plot peaks were separated according to the ratio of *SMN1* copies to *SMN2* copies (*SMN1*:*SMN2*). With regard to the formation of heteroduplexes, the difference plot peaks with an *SMN1*:*SMN2* ratio of 2:2 were the highest, simply because *SMN1* and *SMN2* form complete heteroduplexes. Meanwhile, the peaks manifesting under an *SMN1*:*SMN2* ratio of 1:3 were the lowest because varying amounts of *SMN1* and *SMN2* amplicons resulted in the formation of both hetero- and homoduplexes. Taken together, these findings suggest that the height of the difference plot peaks may reflect the proportion of heteroduplex formations in the amplicon.

Our results further suggest the potential of this screening protocol to be able to distinguish not only SMA patients lacking *SMN1*, but also SMA carriers retaining only one copy of *SMN1*, from normal controls retaining two or more copies of *SMN1*. Unfortunately, the method we describe is not without its limitations. Samples having a proportionate *SMN1*:*SMN2* ratio, such as 2:2 and 1:1, may be indistinguishable from one another. However, this limitation could

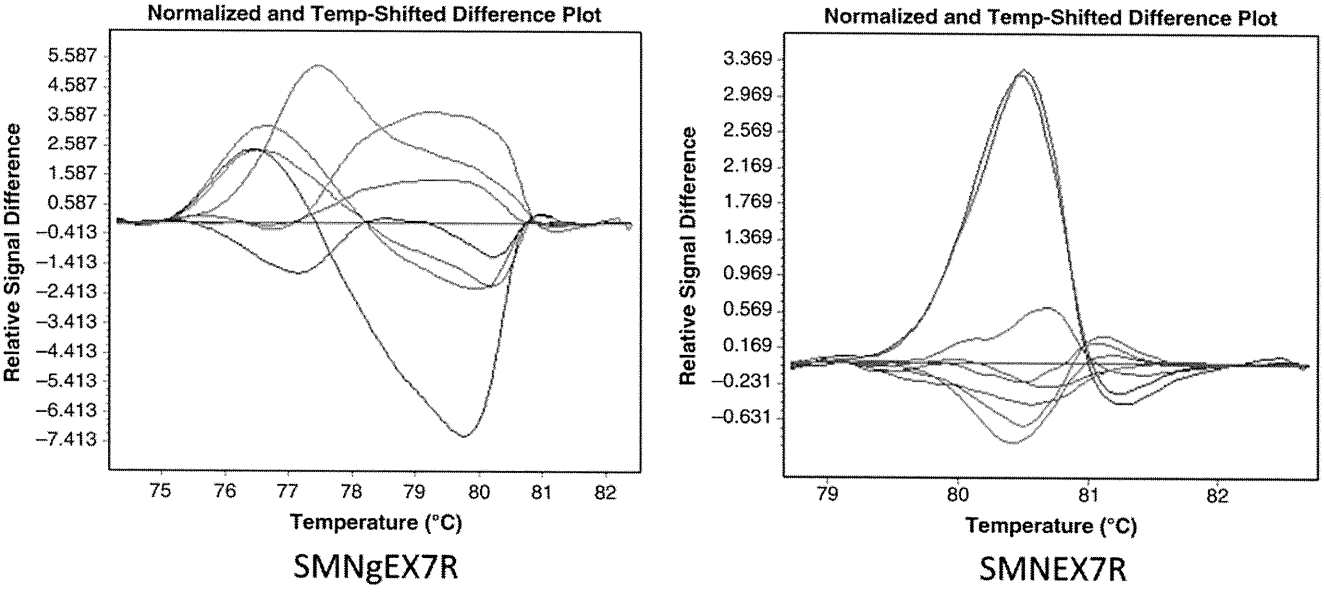


FIG. 7. *SMN* mutation hunting in exon 3 by HRMA: samples who have an intragenic mutation (red), and samples who do not have any mutation (blue).

possibly be overcome by combining other methods using a reference gene with a known copy number ratio for comparison to determine copy number variations (Harada *et al.*, 2002; Tran *et al.*, 2008). Further, copy number variants of the *SMN2* gene are known to have implications on the prognosis and severity of the SMA phenotype. Unfortunately, for samples lacking the *SMN1* gene, the method proposed in the present study is currently unable to distinguish between the different copy numbers of *SMN2*.

After our initial series of deletion testing using symmetric PCR-HRMA, we next evaluated whether this method is proficient in detecting intragenic mutations in *SMN1* exon 3. This region of *SMN1* is unique given its inherent complexity toward attempts at amplification posed by a polyadenine tract. This polyadenine tract stretches for 13 nucleotide (nt), and extends from 11 nt from the 3′ end of SMN exon 3 into the 5′ region of SMN intron 3 (Gunadi *et al.*, 2008). A previous study investigating the stability of mononucleotide PCR amplification found that mononucleotide sequences 11 ≤nt were not correctly amplified. The errors introduced during amplification of mononucleotide sequences longer than 11 nt were primarily contractions of repeat motifs (Clarke *et al.*, 2001). To circumvent this issue, we performed HRMA by using two primer sets. In assessments using primer set D (Fig. 2), we encountered difficulties in distinguishing SMA patients who had c.275 G>C mutations, from normal controls (Fig. 7). We suspect that HRMA with primer set D was unsuccessful due to errors introduced during amplification of the polyadenine tract. Heteroduplex formation of amplicons with repeat contractions or expansions, and those without errors canceled out any observable differences on the difference plots between patient and control samples. When we used using primer set E, samples from patients who had a c.275 G>C mutation could clearly be distinguished from controls (Fig. 7).

The *SMN1* deletion tests, along with screening assessments for intragenic *SMN1* mutations, proved once again the existence of unresolved issues associated with HRMA. However, one of the most striking outcomes of this study was the simple reminder of how important it is to reconsider basic protocol when initial attempts at a novel method of analyses end in failure. Where a past study had dismissed symmetric PCR-HRMA as a potent addition to the existing arsenal of screening/detection methods for pathogenic variants, we show that a simple modification to the primers adopted in analyses can result in monumental differences. Here, primer design is crucial, because the results of HRMA can also be influenced by factors, such as the presence of a polyadenine tract, and can lead to the introduction of errors during the prescreening amplification process.

In recent years, multiplex ligation-dependent probe amplification (MLPA) has also emerged as an additional tool for detecting copy number differences in the *SMN1* and *SMN2* genes (Scarciolla *et al.*, 2006). MLPA provides the unique ability to hybridize several probes specific for the target region and control sequences. However, the HRMA method proposed in this article offers several advantages over MLPA. The first and foremost advantage of HRMA is its rapid turnaround time of ~90 min. Conversely, MLPA requires ~24 h to obtain the same results. In terms of convenience, HRMA is also superior in the sense that the method requires only a single device and PCR to generate results. On the other hand, MLPA is a multistep method that entails DNA naturation and hybridization, followed by PCR, and finally the loading of samples onto a genetic analyzer for analysis. Another noteworthy advantage of HRMA is its low cost per sample analysis. The cost to analyze a sample via HRMA is a fraction of the cost using MLPA.

In the present study we demonstrate that symmetric PCR-HRMA is a simple but powerful tool for diagnosing SMA, and that even seemingly modest modifications to existing screening protocol, such as a simple change in primer sets, can culminate monumental benefits.

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Disclosure Statement

No competing financial interests exist.

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Long-Term Stabilization of Respiratory Conditions in Patients with Spinal Muscular Atrophy Type 2 by Continuous Positive Airway Pressure: a Report of Two Cases

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ABSTRACT

Spinal muscular atrophy (SMA) type 2 is a motor neuron disease that leads to severe congenital muscle atrophy. The majority of adult patients are at risk of death due to respiratory failure. Here, we report on two patients with SMA type 2 who repeatedly developed bronchitis and pneumonia. The patient in Case 1 was a 48-year-old female lacking exon 7 of the survival motor neuron gene (SMN) 1. The patient in Case 2 was a 37-year-old female lacking exons 7 and 8 in SMN 1 and exon 5 in the neuronal apoptosis inhibitory protein (NAIP) gene.

We applied continuous positive airway pressure (CPAP) in both cases because their data on polysomnography showed obstructive sleep apnea (OSA). CPAP treated their respiratory symptoms as well as those due to OSA. Moreover, CPAP stabilized the respiratory condition of Case 1 for seven years and seven months and that of Case 2 for five years and four months. These findings suggest that CPAP alone can achieve long-term improvement in the respiratory condition in patients with SMA type2.

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CPAP stabilizes respiratory condition in SMA type 2

INTRODUCTION

Spinal muscular atrophy (SMA) is a congenital motor neuron disease that shows an autosomal recessive hereditary pattern (1, 2). It was previously reported that the genes responsible for SMA were the survival motor neuron gene (SMN) 1(1) and the neuronal apoptosis inhibitory protein (NAIP) gene (2). Patients with SMA usually develop severe deformity of the thorax with severe scoliosis due to muscle atrophy from infancy (3). Therefore, these patients develop severely restrictive ventilatory defect complicated by repetitive episodes of bronchitis and pneumonia (4-6).

However, noninvasive mechanical ventilation (NIV) was reported to control the respiratory problems of patients with neuro-muscular disorder containing SMA (7). NIV has two modes: continuous positive airway pressure (CPAP) and biphasic positive airway pressure (BiPAP) (8). CPAP sends the patient airway a constant level of positive pressure during breathing. CPAP is mainly used to treat the obstructive sleep apnea (OSA). OSA is reported to be associated with hypertension, cardio-vascular disorder, stroke and chronic bronchitis (9-11). BiPAP provides the patient airway at a higher level of pressure during inspiration, and a lower pressure during expiration. BiPAP is applied to OSA that cannot be adequately managed by CPAP and is utilized for sleep-associated disorders involving central sleep apnea, heart failure, COPD and respiratory failure due to neuromuscular disorders (8,9). Generally, BiPAP is applied to assist respiratory function in patients with of neuromuscular diseases such as SMA (7, 8, 12). However, whether CPAP is effective for respiratory disorders in patients with SMA has remained unknown until now.

We applied CPAP to our patients with SMA to treat OSA and found an unexpected overall effect on their respiratory disorder. Here, we examine the usefulness of CPAP for respiratory problems in patients with SMA type2.

CASE 1

The patient was a 48-year-old right-handed woman who was diagnosed with SMA type 2 at two years of age. She lacked SMN 1 gene exon 7, and demonstrated the neuronal apoptosis inhibitory protein (NAIP) gene (Figure 1a) and three copies of SMN 2 exon 7. In addition, she was reported to have a hybrid SMN involving the fusion of SMN 2 intron 7 and SMN 1 exon 8 (13). She could not hold her back upright in a chair without support, and could not raise her limbs upwards or raise her head to an upright position. Her spinal column showed severe scoliosis and kyphosis due to muscle atrophy since infancy (Figure 2a). Examination demonstrated an alert and well- oriented woman with normal speech and eye movements. Her tongue showed slight fasciculation and moderate atrophy (Figure 3). She could not open her mouth completely. The muscles of all extremities showed severe atrophy. Motor strength was 1/5 in all extremities; deep-tendon reflexes were 0/4 distally and 0/4 proximally. There was no Babinski's sign. Sensory perceptions were normal. Her mother had recognized the physical weakness of the patient at three to four months of age; she was unable to raise her lower limbs or brace her feet. She could not roll over even at six months of age. She showed poor muscular development and was diagnosed with SMA at two years of age. She could sit by herself until seven years of age, but she could never stand by herself. Her older brother also had SMA type 2. He died of respiratory failure after developing severe pneumonia and chest emphysema at 32 years of age.

The patient had shown a gradual increase in the frequency of coughing fits along with episodes of bronchitis and pneumonia since 30 years of age. She also had to struggle with severe respiratory problems every morning, which was the worst time of the day. After awakening, she could not cough up the phlegm by herself abundantly accumulated sour

phlegm by herself, and needed the assistance of someone to repeatedly push against her abdomen to enable her to expel the phlegm. The phlegm was abundantly, and then the taste was sour.

In addition, when she tried to ingest tablets, beans, hijiki seaweed or rice crackers, she often felt as if these were caught in her throat.

Following an episode of severe pneumonia, the patient was referred to our hospital in May 2001 at 38 years of age. In June 2001, she started to inhale a bronchodilator (β -agonist: salbutamol sulfate) for continuous wheezing. She developed bronchitis with high fever five times during 2001, six times during 2002 and three times between January and September in 2003. In December 2002, she was hospitalized with severe pneumonia. In April 2003, gastroesophageal reflux disease (GERD) was suspected as the cause of her cough, but she refused to be examined by esophagogastroduodenoscopy at that time. Ranitidine hydrochloride and dried aluminum hydroxide gel・magnesium hydroxide were prescribed, but respiratory symptoms were not improved.

In October 2003, we analyzed the cycle of her respiratory symptoms in detail and found that the condition was worst in the morning, and then gradually recovered during the day. In addition, medical examination by interview disclosed that she was frequently awakened by coughing during the night, snored while sleeping and showed fatigue and sleepiness during the day. Polysomnography demonstrated obstructive sleep apnea (OSA). The apnea and hypopnea index (AHI) was 11.9 (supine: 7.9, non-supine: 14.0). The minimal arterial oxygen saturation (SaO_2) was 69%. In September 2003, arterial blood gas analysis performed during the daytime showed normal values. Pulmonary function tests demonstrated severe restrictive and mild obstructive ventilatory impairment (forced vital capacity (FVC): 0.93 L; forced expiratory volume in 1 second ($FEV_{1.0}$): 0.84 L; 50%FVC/25%FVC: 3.6). CPAP was initiated because of an extremely low minimal SaO_2 during sleep. The positive pressure was set to 5.0 cmH_2O. The morning after the initiation of CPAP, she immediately showed recovery from severe respiratory symptoms including coughing, wheezing, and sputum production. Between October 2003 and December 2004, she developed upper respiratory infection twice due to a cold. Between January 2005 and October 2010, she developed upper respiratory infection only once per year. CPAP has stabilized her respiratory condition for seven years seven months to date. In addition, she became able to naturally swallow tablets, beans, hijiki seaweed and rice crackers, and her swallowing ability normalized. The patient reported that sputum often stayed in her throat before she used CPAP, and the sputum affected her ability to swallow foods, but she has not felt sputum in her throat since the initiation of CPAP, and has become able to swallow foods naturally.

In July 2008, she underwent esophagogastroduodenoscopy because she showed severe anemia (red cell count $353x10^4/\mu l$, Hb 7.7 g/dl Ht 25.7%) and appetite loss. The procedure demonstrated stenosis at the lower esophagus similar to esophageal achalasia (Figure 4). The cause of her anemia was hemorrhoids.

CASE 2

The patient was a 37-year-old right-handed woman who was diagnosed with SMA type 2 at one year of age. She lacked SMN 1 gene exons 7 and 8 and the NAIP gene exon 5 (Figure 1b), but she had three copies of SMN 2 exon 7.

She could not hold her back upright in a chair without support, and could not raise her limbs upwards or hold her head in an upright position. Her spinal column showed severe scoliosis and kyphosis (Figure 2b). Examination demonstrated an alert, and well-oriented

woman with normal speech and eye movements. Her tongue showed fasciculation and moderate atrophy on the left side. She could not open her mouth completely. The muscles of all extremities showed severe atrophy. Motor strength was 0/5 in all extremities, although motor strength was 1/5 in fingers; deep-tendon reflexes were 0/4 distally and 0/4 proximally. There was no Babinski's sign. Sensory perceptions were normal. Muscle strength in her limbs was weak from birth. She could raise her head, and roll over at six months of age, but she could not roll over at 10 months of age. She was diagnosed with SMA at one year of age. After the diagnosis, atrophy and decrease in muscle strength gradually progressed. She underwent tracheal incision during an episode of pneumonia at 15 years of age. The situation recovered thereafter the tracheal cannula was removed. She developed pneumonia twice at 25 years of age, and was admitted to the hospital due to pneumothorax twice at 29 years of age.

She reported that she felt choked by food and that her breath stopped at night, and she developed bronchitis more than three times per year before CPAP was initiated. She consulted a doctor specializing in sleep disorder. Portable polysomnography demonstrated AHI at 26.4, and then minimal SaO_2 was 74%. Therefore, she received a diagnosis of OSA. However, based on her overall condition, it was speculated that she would have had considerable difficulty in recovering from sleep apnea. It was also thought that apnea might become fatal. Therefore, in January 2006, CPAP was initiated. The patient has not felt choked by food, and has been able to sleep soundly since the initiation of CPAP. In addition, she has had only one episode of bronchitis after the start of CPAP up to October 2010. CPAP has stabilized her respiratory condition for five years and four months to date.

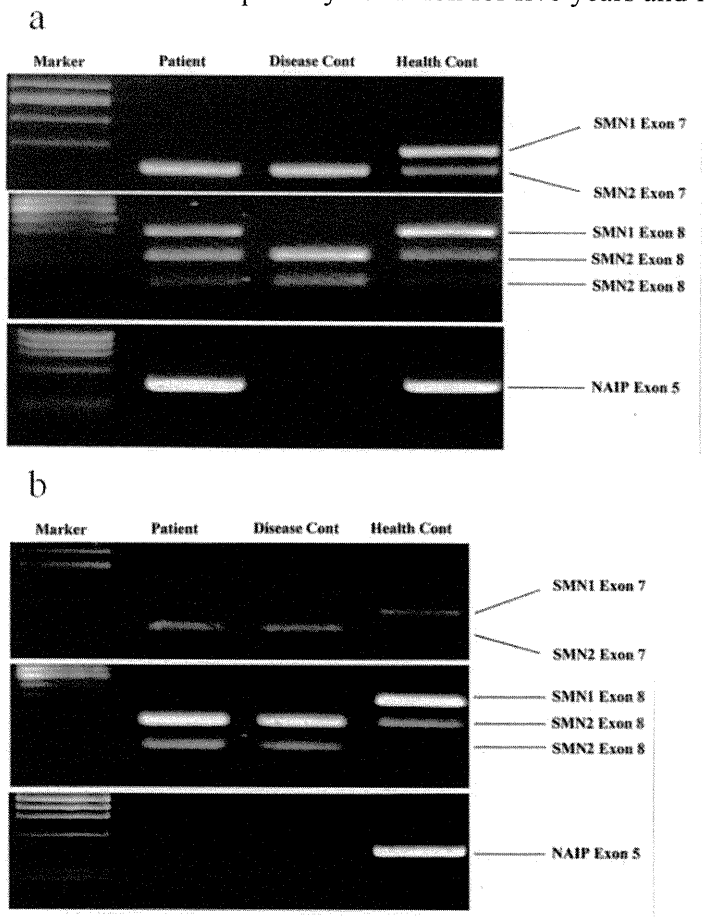

Figure 1. Detection of SMN1, SMN2 and NAIP gene deletion by polymerase chain reaction (PCR). (a) Data from Case 1. (b) The data from Case 2. The marker is ϕ X174 Hae III digest.

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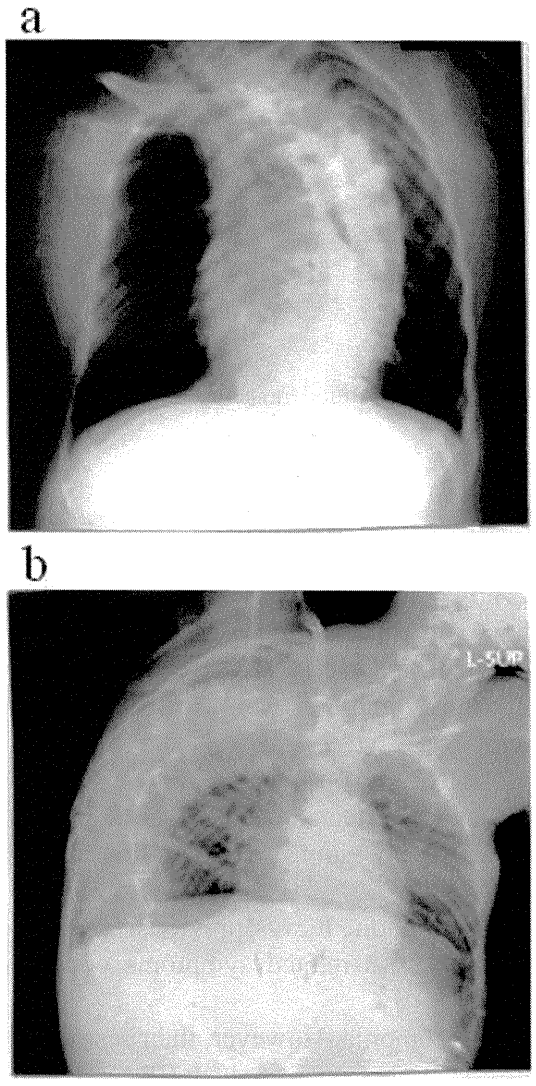

Figure 2. Deformed thorax on chest X-ray. (a) Chest X-ray of Case 1. (b) Chest X-ray of Case 2.

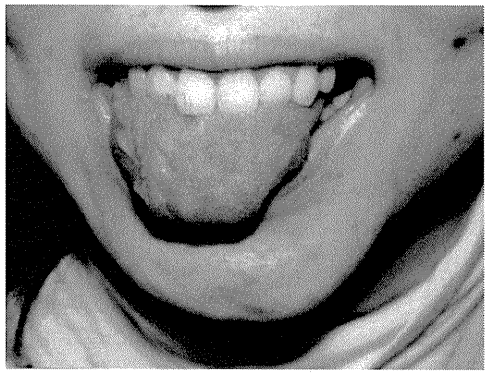

Figure 3. The tongue of Case 1.

E102

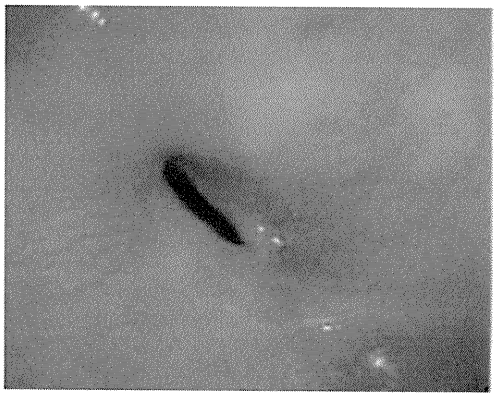

Figure 4. Stenosis at the lower esophagus of Case 1 on esophagogastroduodenoscopy.

DISCUSSION

When we diagnose patients as having SMA, we must consider their symptoms, course and genetic abnormality. Our patients started to show muscle atrophy of the extremities before one year of age, and then they could not stand and walk without support. Symptoms and course were characteristic for SMA type 2 (4-6). In addition, Case 1 was lacked the SMN 1 exon 7, and Case 2 was lacked the SMN 1 exons 7 and 8 as well as the NAIP gene exon 5. Therefore, we diagnosed our patients as having SMA type2.

Coincidently, each features three copies of SMN 2 exon 7. However, it remains unknown whether the number of the copies of SMN2 exon 7 affects the course of SMA (14). Furthermore, Case 1 had a hybrid SMN (13). However, the impact of that finding also remains unknown.

Patients with SMA demonstrate OSA as a complication. It was previously reported that when patients with SMA demonstrate OSA, their OSA-related symptoms could be treatable by CPAP (15-17).

Our patients had chronic bronchitis and repeated pneumonia. However, their respiratory state had not yet progressed to respiratory failure. Therefore, we applied CPAP for the treatment of OSA. As a result, CPAP not only improved the sleep apnea, but also suppressed the occurrence of chronic bronchitis and pneumonia. Moreover, CPAP achieved long-term stabilization of their respiratory condition.

Here, we discuss the effect of CPAP on chronic bronchitis and pneumonia of the patients with SMA type 2. First, Case 1 demonstrated chronic bronchitis and repeated pneumonia. In addition, she had expectorated sour sputum every morning before CPAP was initiated. It is highly possible that sour sputum was mainly composed of gastric juice. Therefore, we can speculate that gastric juices had further damaged the lung due to gastroesophageal reflux. In addition, she had severe scoliosis and kyphosis as complications. It was reported that the deformed spine causes deterioration of reflux esophagitis (18, 19). However CPAP can inhibit OSA, and therefore, lessen the reflux esophagitis (20, 21).

Thus, we speculate that CPAP was able to prevent the reflux of gastric juice to bronchi, and then stabilized the respiratory condition of Case 1. In addition, the patient had a swallowing disorder before CPAP, but the symptom disappeared after CPAP was initiated. She had complained that she constantly felt residual sputum and that the sputum mixed with the bolus of food, preventing her from fully swallowing the food before CPAP. Hence, it is highly possible that the swallowing disorder of the patient was related to chronic bronchitis. That is, CPAP may have improved the swallowing disorder because CPAP successfully suppressed the chronic bronchitis.

In Case 2 as well as in Case 1, the repeated occurrence of pneumonia ceased and both the chronic bronchitis and the swallowing disorder were improved after the initiation of CPAP. The patient had a deformed spine and sleep apnea, although she did not have symptoms of reflux esophagitis. Therefore, Case 2 had milder symptoms of chronic bronchitis than Case 1.

However, the deformed spine and sleep apnea may increase the severity and frequency of gastric reflux to the bronchi in Case 2 because these diseases can aggravate reflux esophagitis (18-21). That is, we speculate that the aggravated reflux might contribute to chronic bronchitis and pneumonia as one of the silent symptoms in Case 2. In addition, it is possible that the improvement of swallowing disorder in Case 2 was associated with the same mechanism as in Case 1.

One of the serious respiratory disorders caused by neuromuscular disorders was reported to be atelectasis (22, 23, 24). CPAP is also effective in preventing the atelectasis (25, 26). The effect may be also associated with the stabilization of the respiratory condition in our patients after CPAP.

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Key words
脊髄性筋萎縮症
SMN1 遺伝子
SMN 蛋白
スプライシング
低分子量リボ核蛋白

小児医学最近の進歩

脊髄性筋萎縮症とSMN蛋白と低分子量リボ核蛋白合成

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要旨 脊髄性筋萎縮症（SMA）は，*SMN1* 遺伝子の欠失や変異によって引き起こされる運動ニューロン病である．*SMN1* 遺伝子産物であるSMN蛋白は，ヒトのさまざまな臓器で発現していることが知られているが，機能については十分にはわかっていない．最近，SMN蛋白は，pre-mRNAスプライシングに必要な低分子量リボ核蛋白の合成・輸送過程に関与していることが明らかにされた．また，SMN蛋白が，低分子量リボ核蛋白の合成以外にも，さまざまな生体機能に関与していることも明らかにされつつある．これらの知見から，SMAはヒトのすべての細胞活動にかかわる病気であり，運動ニューロンに限局する病気ではないと考えられるようになった．

はじめに

脊髄性筋萎縮症（spinal muscular atrophy：SMA）は，脊髄前角細胞（運動ニューロン）の変性・脱落に伴い，体幹・四肢近位部優位の筋緊張低下・萎縮を生じる遺伝性疾患である．従来，SMAは，発症年齢と獲得運動能力の観点から，1型，2型，3型に分類されてきた[1]．1型（Werdnig-Hoffmann病）は生後6カ月までに発症する重症型である．座位保持の能力を獲得することなく，2歳までに人工呼吸管理あるいは死亡の転帰をとることが多い．2型（中間型）は生後18カ月までに発症する中等症型である．自力での座位保持は可能であるが，自力で立って歩行する能力を獲得するには至らない．3型（Kugelberg-Welander病）は18カ月以降に発症する軽症型である．自力で立って歩行することが可能な時期がある．早期新生児期から呼吸困難などの症状で発症する最重症型を0型とすることがある[2)3)]．また，逆に，成人期以降に歩行困難などの症状で発症する最軽症型を4型とすることがある[4)5)]．SMAは単一疾患でなく，複数の疾患単位を包含した概念である．ただし，本稿で扱うSMAは，もっとも頻度の高い，常染色体劣性遺伝形式をとる，5番染色体長腕に関連した5q-SMAに限ることにする（そこで，以後，5q-SMAのことをSMAとのみ記す）．

1995年に，*SMN1* 遺伝子がSMAの責任遺伝子であることが明らかになり，その遺伝子産物であるSMN蛋白の研究が始まった[6]．翌年，SMN蛋白がヘテロ核内リボ核蛋白U（hnRNP U）やフィブリラリンと結合することが示されて以来，SMN蛋白とRNA結合蛋白との相互

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作用のことが精力的に研究された[7]．1998年には，SMN蛋白がpre-mRNAスプライシングにかかわっていることが示された[8]．その後，SMN蛋白が，低分子量リボ核蛋白（snRNP）の合成過程で働き，pre-mRNAスプライシングに重要な役割を果たしていることが明らかにされていった[9]．本稿では，snRNP合成におけるSMN蛋白の役割を解説し，SMAの分子病態に関する新しい考え方を紹介したい．

Ⅰ SMN蛋白の研究の歴史

1．*SMN1*遺伝子，*SMN2*遺伝子，SMN蛋白

1995年に，Lefebvreらによって，染色体5q13領域からSMA発症に関連する遺伝子としてsurvival motor neuron（*SMN*）遺伝子がクローニングされた[6]．染色体5q13領域には大規模な重複が生じていて，テロメア側とセントロメア側に2つの相同遺伝子が並んでいる．そのため，*SMN*遺伝子でも，テロメア側とセントロメア側に2つの相同遺伝子が存在する．テロメア側のものは*SMN1*遺伝子，セントロメア側のものは*SMN2*遺伝子とよばれている．

現在，*SMN1*遺伝子はSMAの責任遺伝子，*SMN2*遺伝子はSMAの修飾因子であると考えられている[10]．*SMN1*遺伝子が責任遺伝子であるとする根拠は，90%以上のSMA患者で*SMN1*遺伝子の欠失が認められることや，およそ5%のSMA患者で*SMN1*遺伝子内の微小変異が認められていることである．また，*SMN2*遺伝子が修飾因子であるとする根拠として，*SMN2*遺伝子のコピー数が多いほど軽症型になる傾向が認められていることが挙げられる．

*SMN1*遺伝子は9つのエクソン（1，2a，2b，3，4，5，6，7，8）で構成され，その産物であるSMN蛋白（全長型）は38 kDa，294個のアミノ酸からなる[11][12]．SMN蛋白（全長型）は，その大部分が*SMN1*遺伝子に由来するものであるが，*SMN2*遺伝子からも少量産生されていることが知られている[12]．

2．SMN蛋白とRNA代謝

1995年に*SMN1*遺伝子がクローニングされた時点で，その遺伝子産物であるSMN蛋白の機能・役割についてはまったく何もわかっていなかった．SMN蛋白はどの既知の蛋白とも似ていなかったので，SMN蛋白の機能・役割について類推することも困難であった．ただ，SMAとの関連から，「SMN蛋白は運動ニューロンに特異的に発現し，機能している蛋白であろう」と予想されていた．

*SMN1*遺伝子が同定された翌年（1996年）に，LiuとDreyfussは，HeLa細胞を使って，SMN蛋白がhnRNP Uやフィブリラリンのようなな RNA結合蛋白と複合体を形成することを明らかにした[7]．また，彼らは，HeLa細胞の核では，SMN蛋白が凝集して濃く染色され，周囲から区別できる小斑点を形成することも見出した．この小斑点は，ジェム（Gem）[*1]とよばれ，新しい核内サブドメインと認識されるようになった[7]．

このように，LiuとDreyfussは「SMN蛋白は運動ニューロンに特異的に発現し，機能している蛋白であろう」という当初の予想を覆し，「SMN蛋白は運動ニューロン以外の細胞でも発現し，RNA代謝に関連する蛋白である」ということを示したのである．

1998年に，Pellizzoni，Dreyfussらは，SMN蛋白がpre-mRNAスプライシングにかかわっていることを示した[8]．その後，Dreyfussらのグループやその他のグループが精力的に研究を進め，SMN蛋白はsnRNP合成に関与すること

[*1]：ジェム（Gem）という名称は，Gemini of coiled bodies，すなわち「コイル体の双子星」というところからきている．「コイル体」とは，従来「カハール体」とよばれていた核内サブドメインであり，本稿では以後「カハール体」という名称の方を用いる．

で，pre-mRNA スプライシングに重要な役割を果たしている蛋白であることを明らかにした[9]．

3．SMN 蛋白の空間的・時間的発現様式

やがて，SMN 蛋白はヒトのさまざまな臓器，組織，細胞に広く存在する蛋白，いわゆるユビキタスな蛋白であることも明らかになった．そして，ユビキタスな発現様式をもつことから，*SMN1* 遺伝子はハウスキーピング遺伝子[*2]ではないか，SMN 蛋白はハウスキーピング蛋白[*2]ではないかと考えられるようになった[13]．

ただし，SMN 蛋白の発現レベルは臓器，組織によって異なる．SMN 蛋白は，脳，脊髄，腎臓，肝臓では高度に発現しているのに対して，心臓や骨格筋では中等度にしか発現していない．とりわけ SMN 蛋白が脊髄で高度に発現していることは，脊髄における SMN 蛋白の役割の重要性を示唆するものである．SMA1 型患者の脊髄の SMN 蛋白の発現量は，同年齢の標準の 1/100 であったという報告もある[14]．このことは，SMA マウスモデルでも確認されている．脊髄の SMN 蛋白の発現量の低下が，脊髄前角細胞の変性・脱落と関連しているのは確かである．

SMN 蛋白の発現レベルは，ヒトのライフステージによっても異なることも興味深い．ヒト組織（骨格筋，心筋，脳）を用いたイムノブロット解析によれば，SMN 蛋白は胎児期には非常に高度に発現していたのに対し，出生後は急速に発現が減少していた[15]．このことは，マウスでも確認されている[16]．胎児期は，出生後と比較して，Smn 蛋白（Smn 蛋白はマウスの蛋白で，ヒトの SMN 蛋白に相当するものである）の必要性あるいは要求度が高いことが予想される（Ⅱ3．参照）．

4．SMN 蛋白の多種多様な機能と役割

現在までに，SMN 蛋白はさまざまなパートナー蛋白と結合して複合体を作っていることが証明され，さまざまな細胞活動に関与していることが明らかにされてきた[17]．SMN 蛋白が関与している細胞活動として，遺伝子転写，pre-mRNA スプライシング，snRNP の合成，RNA 代謝，アポトーシス，ストレス反応，軸索における RNA 輸送などが挙げられる．しかし，SMA の発症に，SMN 蛋白の発現低下がどのようにかかわっているのかはいまだ明らかではない．

その一方，①*SMN1* 遺伝子と *SMN2* 遺伝子の両方を欠失している SMA 患者は見つかっていない，すなわち SMN 蛋白が完全に欠如している SMA 患者は見つかっていないという臨床観察[17]，②マウスモデル[*3]の実験において，*Smn* 遺伝子を破壊するだけでヒト *SMN2* 遺伝子を導入しなければ，胎生早期に致死となるという実験事実[18]があり，これらのことは「SMN 蛋白が完全に欠如すればヒトは誕生できない」ことを示している．すなわち，SMN 蛋白は，運動ニューロンの生存にかかわる機能のみならず，胎生期の細胞の生存あるいは増殖・分化にかかわるような重大な機能をも有していることが示唆される．

Ⅱ 低分子量リボ核蛋白（snRNP）の合成

1．pre-mRNA スプライシングと snRNP と SMN 蛋白

ヒトでは，どの蛋白も同じ過程を経て合成さ

[*2]：ハウスキーピング遺伝子とは，多くの組織や細胞中に共通して一定量発現する遺伝子のことであり，常に発現され，細胞の維持，増殖に不可欠な遺伝子を指す．ハウスキーピング蛋白とは，ハウスキーピング遺伝子産物であり，細胞の維持，増殖に不可欠な蛋白である．

[*3]：SMA マウスモデルでは，*Smn* 遺伝子が破壊され，代わりにヒト *SMN2* 遺伝子が導入されている．このような遺伝子型は（$Smn^{-/-}$, $SMN2^{+/+}$）と記される．マウスは，本来，ヒト *SMN1* 遺伝子に相当する *Smn* 遺伝子をもっているが，ヒト *SMN2* 遺伝子に相当する遺伝子をもたない．

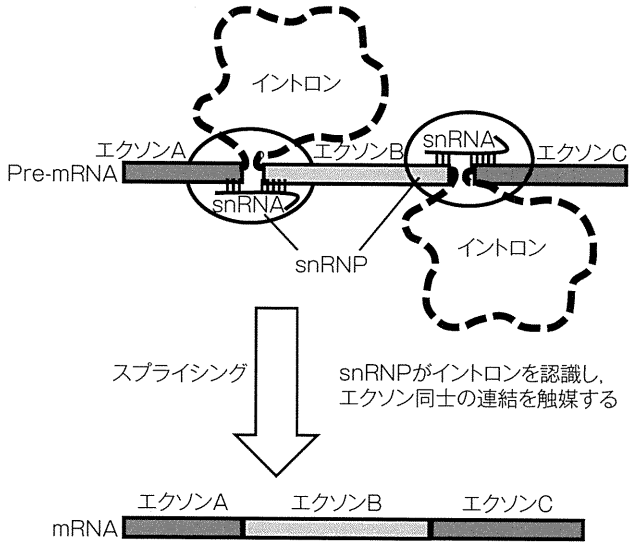

図1　snRNP とスプライシング

れる．すなわち，ある蛋白の設計図をコードしている DNA は pre-mRNA に転写され，pre-mRNA はさらにスプライシングなどの加工処理を受けて mRNA にまで成熟し，その mRNA の情報に基づいて目的の蛋白が合成される．

pre-mRNA のスプライシングは，イントロン配列を除去し，エクソン同士を連結する過程である．この過程を担当する装置がスプライソソームである．スプライソソームは，snRNP といくつかの蛋白，RNA 因子から構成されている．snRNP がイントロンを認識し，エクソン同士の連結を触媒する機能をもっている(**図1**)．

snRNP は，低分子量リボ核酸（snRNA），Sm コア蛋白，その他の蛋白の複合体である．Sm コア蛋白は，7 つの Sm 蛋白サブユニット SmB，SmD1，SmD2，SmD3，SmE，SmF，SmG がリング状に並んでいる蛋白複合体で，snRNA 内の特定の部位（Sm 部位）に結合することが知られている[*4]．

この 15 年の間に，SMN 蛋白が snRNP 合成にかかわる重要な因子であることが明らかになった．現在，SMN 蛋白は，SMN/Gemin 複合体として，Sm コア蛋白形成と snRNP の核内輸送に深く関与していることが示されている．**図2**に，Dreyfuss らによるモデル図を示す．以下に，最近の知見に基づいて[17)19)]，多くの snRNP に共通する合成過程（あるいは成熟過程）のあらましを述べる．

2．snRNP の合成

図3に snRNP の合成の概要を示す．

a．snRNA の核外輸送（核内，細胞質での過程）

核内で遺伝子からポリメラーゼⅡによって転写された snRNA は，さまざまに加工される．まず，7-メチルグアノシン（m^7G）キャップが snRNA の 5' 端に付加され，次に，そのキャップ部分に核外輸送に関連する蛋白が結合し（輸送因子結合），核膜孔を通って核外（細胞質）へ輸送される．

m^7G キャップを付加された snRNA に直接的，間接的に結合する蛋白は，キャップ結合複合体（CBC），PHAX（アダプター蛋白），XPO1/CRM1（核外輸送受容体），Ran（核外輸送受容

[*4]：snRNA，snRNP は U-snRNA，U-snRNP と表記されている報告も多い．snRNA，snRNP がウリジンに富んでいるからである．