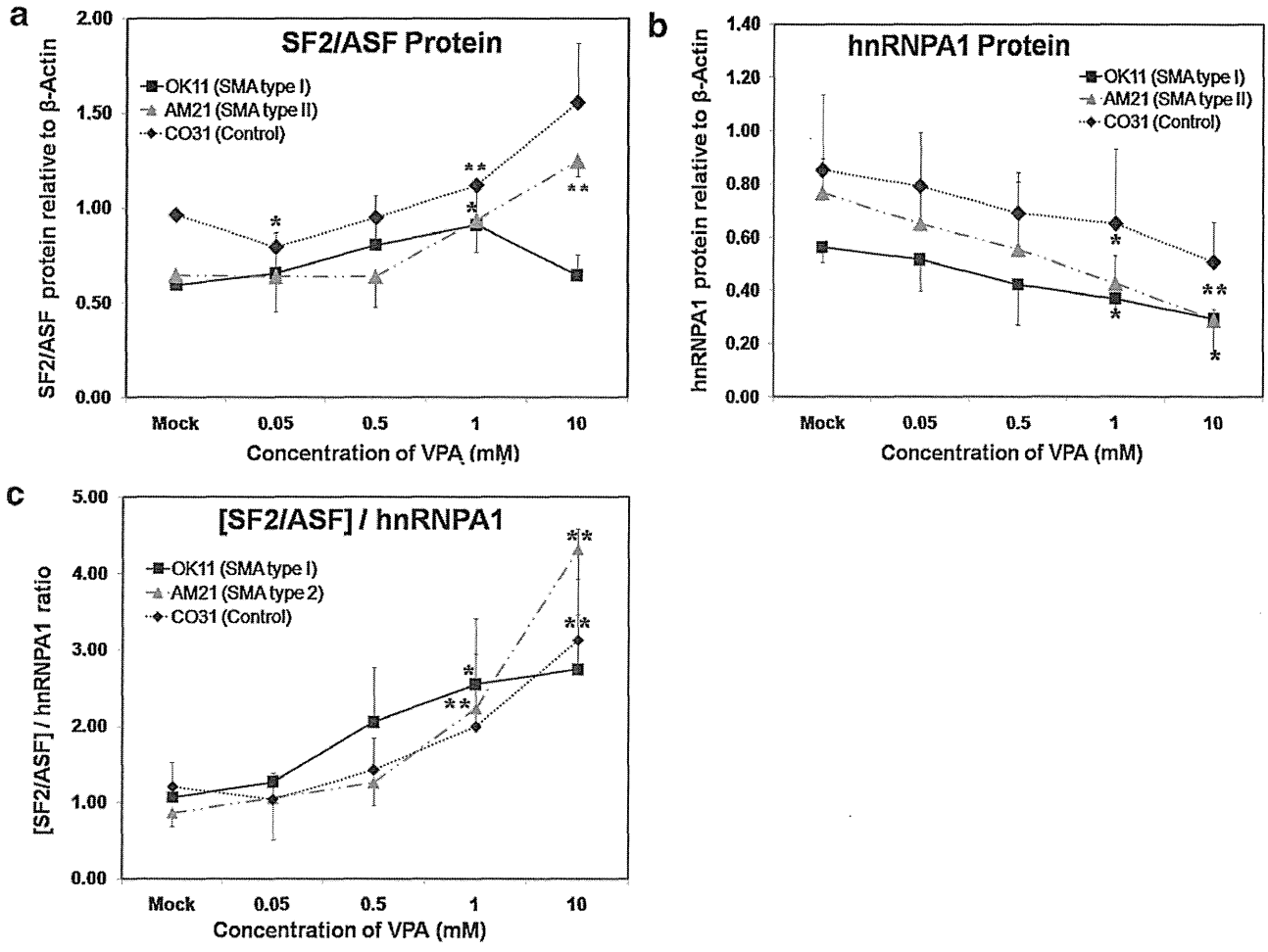

Fig. 4. Quantitative analysis of SF2/ASF protein. (a) Upregulation of SF2/ASF protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry revealed increasing SF2/ASF protein levels with increasing concentration of VPA. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock). (b) Downregulation of hnRNPA1 protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry indicated decreasing hnRNPA1 protein levels with increasing VPA concentration. Asterisks indicate a significant decrease compared with the mock status in each cell line. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock). (c) The calculated [SF2/ASF]/hnRNPA1 ratios showed modulation of splicing factor expression induced by VPA. The FL/Δ7 ratio was not affected by the increase in the [SF2/ASF] / hnRNPA1 ratio, suggesting that *SMN2* gene splicing was not affected by modulation of SF2/ASF and hnRNPA1 levels. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock).

an important role in correcting the splicing pattern [33]. This notion was supported by previous studies showing that Htra2-β1 overexpression can promote the inclusion of *SMN2* exon 7 [39,48,49].

We examined two crucial splicing factors: SF2/ASF, related to *SMN1* exon 7 inclusion, and hnRNPA1, related to *SMN2* exon 7 exclusion. In our study, VPA increased SF2/ASF and decreased hnRNPA1 at the protein level (Fig. 4a and b). It should be noted here that VPA does not always activate gene expression, but sometimes inactivates gene expression indirectly. For example, HDAC inhibitors such as trichostatin A and VPA suppress the expression of steroidogenic gene *CYP11A1* and reduce CY11A1 levels in adrenocortical cell lines [50].

According to Kashima et al., *SMN2* exon 7 inclusion was enhanced by treatment with small interfering RNAs against hnRNPA1 [19]. Thus, it can be expected that decreased hnRNPA1 expression prevents the skipping of *SMN2* exon 7, correcting the splicing of *SMN2* exon 7. In contrast, our experiment shows that VPA did not change the FL/Δ7 ratio in any cell line, indicating that the decrease in hnRNPA1 caused by VPA did not contribute to a splicing correction of *SMN2* exon 7. This may be explained by at least three possibilities. The first explanation is that the decrease in hnRNPA1 was insufficient for splicing correction. The second is that additional effects of VPA may weaken the influence of decreased hnRNPA1 expression in correcting the splicing. As an example of the latter case, if VPA could

increase or retain the amount of hnRNPA2 which is related and potentially redundant with hnRNPA1 [51], it may attenuate the influence of decreased hnRNPA1 expression in splicing correction. The third is the possibility that in our cell lines, VPA could not increase Htra2-β1. If VPA could increase the amount of Htra2-β1, the ratio of FL/Δ7 would have been corrected. Further analysis of the effects of VPA on splicing factors, including Htra2-β1, is required.

4.3. Clinical application of VPA in SMA treatment

Brichta et al. showed the effect of VPA treatment in 20 SMA patients [40]. There was an increase in *SMN* transcript and SMN protein levels in blood cells in 7 patients (1 of 5 in type I; 4 of 11 in type II and 2 of 4 in type III). According to these authors, there are two types of SMA patients: responders and nonresponders to the VPA treatment, mantaining serum VPA levels of 0.48–0.70 mM [40]. Responders showed a 1.6–3.4-fold elevation of FL-*SMN* transcripts while nonresponders showed only minor changes in FL-*SMN* transcript levels. For nonresponders, a higher dose of VPA may be required to obtain a sufficient increase in FL-*SMN* transcripts or SMN protein. However, Brichta et al. did not demonstrate an effect of VPA on the improvement of clinical symptoms including muscle strength.

Weihl et al. presented evidence for improvement in muscle strength in seven adult patients with SMA type III/IV patients after VPA treatment [52]. These authors collected quantitative data of muscle strength determined by hand-held dynamometry [53]. They also reported the patient's subjective benefits from VPA treatment, such as feeling stronger, ease of breathing, ease of rising from chairs, ability to dress themselves, ability to comb hair and pick grapes and walking endurance in a marching band.

Tsai et al. reported that age may play a role in the improvement of muscle strength in six SMA patients (two SMA type II and four SMA type III) with VPA treatment [41]. The therapeutic effects of VPA in SMA patients were inversely related to age. These authors assessed the muscle strength of SMA patients using the Medical Research Council score (from 0 to 5) [41]. Two SMA children showed some improvement in muscle power, and two SMA adolescents showed a slight increase. In contrast, two SMA adult patients showed no response to VPA.

Swoboda et al. [54] reported results similar to those of Tsai et al. and found that the Modified Hammersmith Functional Motor Scale showed some improvement in gross motor function in 27 SMA type II children upon VPA treatment. However, no children $\geqslant$5 years of age showed a six-point improvement after 1 year. In 8 of 16 children (50%) under 5 years of age, the improvement was significantly more pronounced.

In light of these data, the question arises as to whether VPA can have a negative effect on the motor neuron function. Rak et al. found that VPA can increase *Smn* expression and lead to reduced growth cone size and decreased excitability in axon terminals of the motor neurons of SMA mice ($Smn^{-/-}$; *SMN2*) [55]. These authors reported that the potential positive effects of VPA are counteracted by its negative effects of on neuronal excitability and axon growth. Similarly, Van der Berg et al. reported that VPA reduced neuronal excitation through sodium channels [56]. Recently, Swoboda et al. have demonstrated no benefit from six months treatment with VPA and L-carnitine in a young non-ambulatory cohort of subjects with SMA [57]. However, their patients may have included responders and non responders to VPA. We should rather emphasize the importance of estimating prior to the treatment whether the SMA patients are responders or non responders to VPA. If we can identify the responders for VPA treatment before clinical trial, feasible outcome would be gained.

We can say that it is worthwhile treating SMA patients with VPA, especially who are VPA-responders, because it is a safe, widely-used and long-term drug used in epileptic children. Based on the studies using cells derived from SMA patients, including ours, the responsiveness to VPA of *SMN2* expression may vary from patient to patient. Some patients require a small dose of VPA to activate *SMN2* expression and improve clinical symptoms, but others require a much larger dose. Thus, dose-related side effects should always be considered. In fact, children need a higher dose of VPA per kilogram than adults to obtain an equivalent serum concentration [58]. Regular monitoring of serum VPA levels is necessary to avoid any harmful side effects.

5. Conclusion

We demonstrated here that VPA increases FL-*SMN2* transcript and SMN protein levels, suggesting that VPA is a candidate drug for SMA. Because splicing correction of *SMN2* exon 7 was not observed, the increase in FL-*SMN2* transcript and SMN protein levels may be explained mainly by the activation of *SMN2* transcription.

In addition, we also demonstrated that VPA modulates the expression of the splicing factors SF2/ASF and hnRNPA1 in our cell lines: VPA increased SF2/ASF protein levels and decreased hnRNPA1 protein levels. Based on our data, *SMN2* exon 7 splicing may be regulated not only by SF2/ASF and hnRNPA1 but also by other factors as well. However, the number of SMA cell lines in our study was very limited: we could not neglect the possibility that these findings were specific to our SMA cell lines. Thus, it is necessary to study

the VPA-effects on expression of the splicing factors using a larger number of cell lines.

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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

Satoru Morikawa,[1,2] Indra Sari Kusuma Harahap,[1] Richard Hideki Kaszynski,[3] Tomoto Yamamoto,[1,2] Dian Kesuma Pramudya,[1] Huyen Thi Van Pham,[1,2] Tri Budi Hartomo,[1] Myeong Jin Lee,[1] Ichiro Morioka,[2] Noriyuki Nishimura,[1,2] Naoki Yokoyama,[2] Yasuhiro Ueno,[3] Masafumi Matsuo,[2] and Hisahide Nishio[1,2]

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),

Departments of [1]Epidemiology, [2]Pediatrics, and [3]Legal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan.

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*

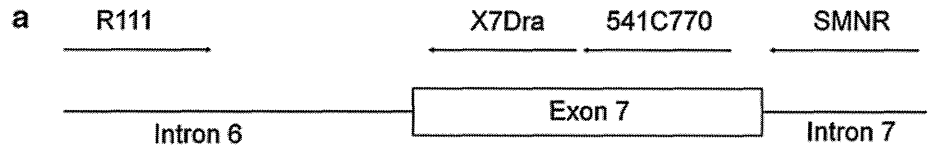
In applying HRMA to the detection of intragenic *SMN1* exon 3 mutations, designing optimal primer sets—which took into consideration the unique polyadenine tract in intron 3—was a crucial factor. Considering this unique feature of intron 3, two different primer sets, D (gEX3F and gInt3R) and E (gEX3F 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

a



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°C	Poor
	SMNR	5'-GAT TCA CTT TCA TAA TGC TGG-3'			
B	R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	183bp	56°C	Poor
	X7Dra	5'-CCT TCT TTT TGA TTT TGT TT-3'			
C	R111	5'-AGA CTA TCA ACT TAA TTT CT-3'	202bp	56°C	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.

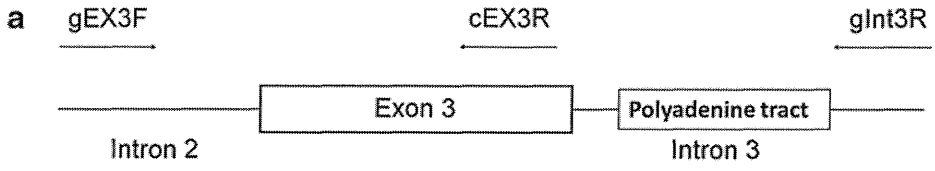
samples lacking *SMN1*, along with control samples lacking *SMN2*, had a higher melting temperature due to homoduplex formations of the amplicons. As shown in Figure 3, under the primer set A protocol, it was nearly impossible to distinguish patient samples lacking *SMN1* from control samples lacking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

a



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°C	Poor
	gInt3R	5'-TGT ATA CTG TTT AAC AGT TT-3'			
E	gEX3F	5'-GTG TGC ATG TAT ATA TCT TT-3'	224bp	50°C	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.

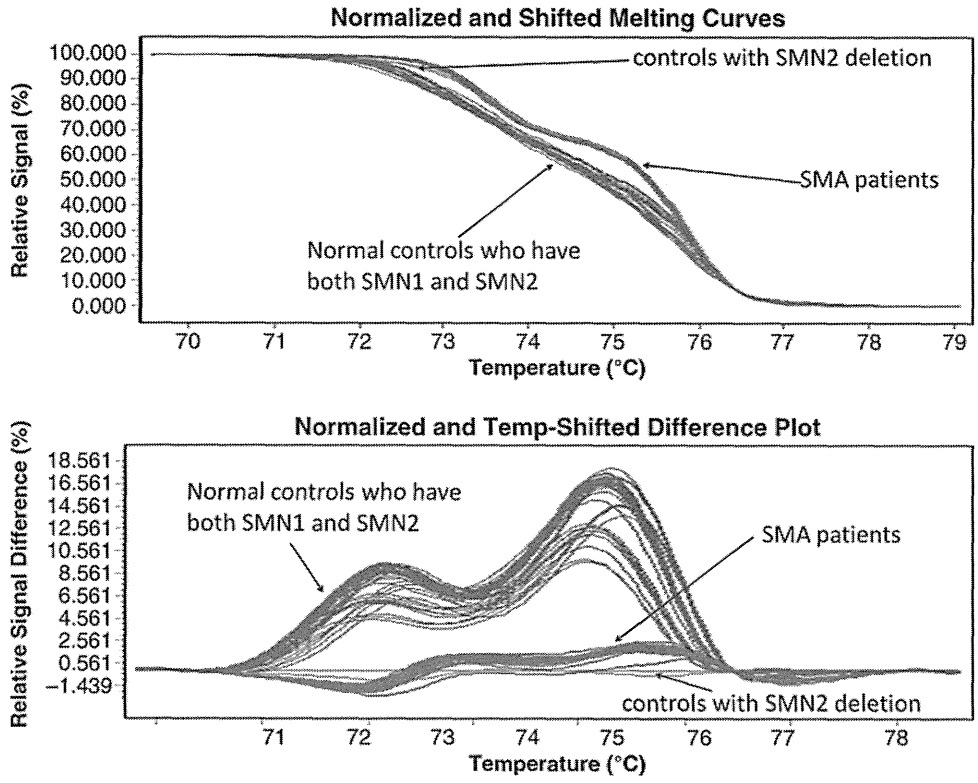


FIG. 3. Results of HRMA by using primer set A. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

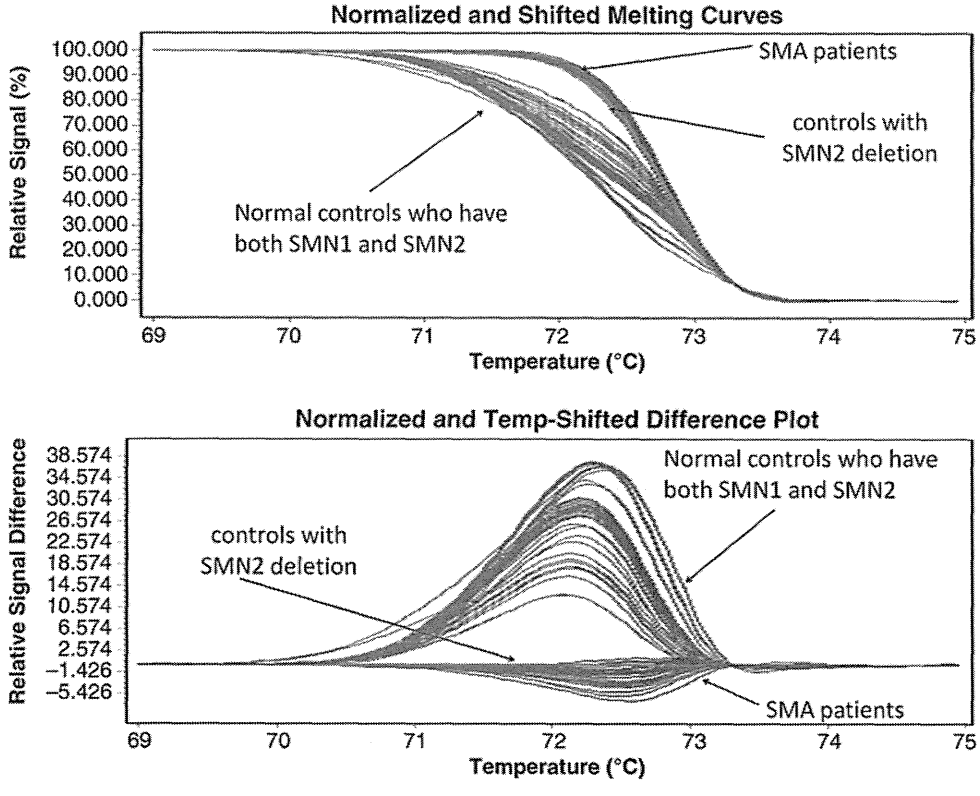


FIG. 4. Results of HRMA by using primer set B. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

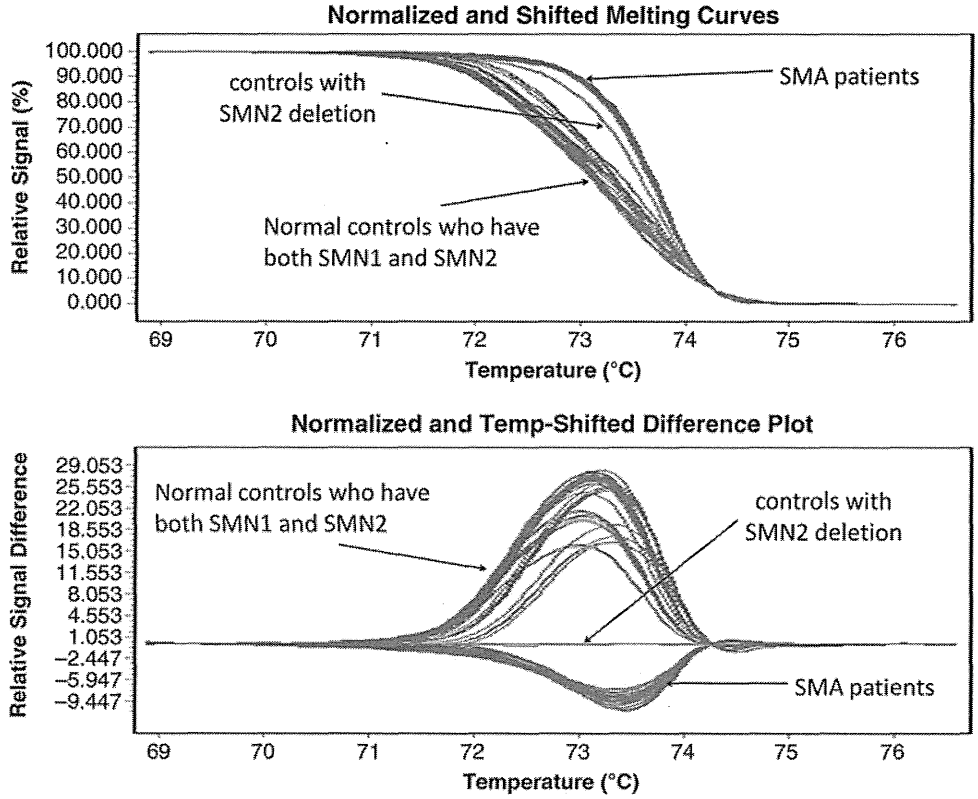


FIG. 5. Results of HRMA by using primer set C. Normal individuals retaining both *SMN1* and *SMN2* (blue), SMA patients lacking *SMN1* (red), and individuals lacking *SMN2* (green).

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.

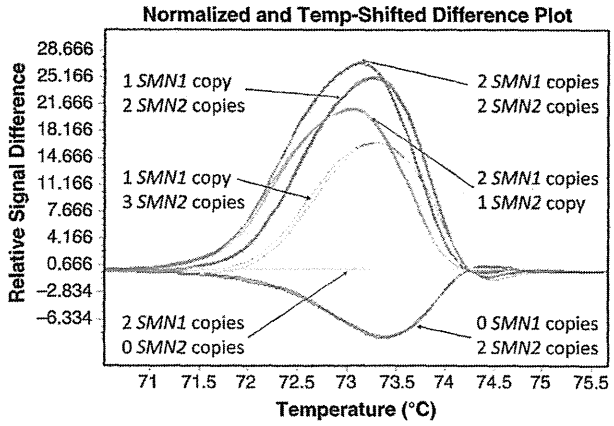


FIG. 6. Copy number analysis of HRMA by using primer set C.

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

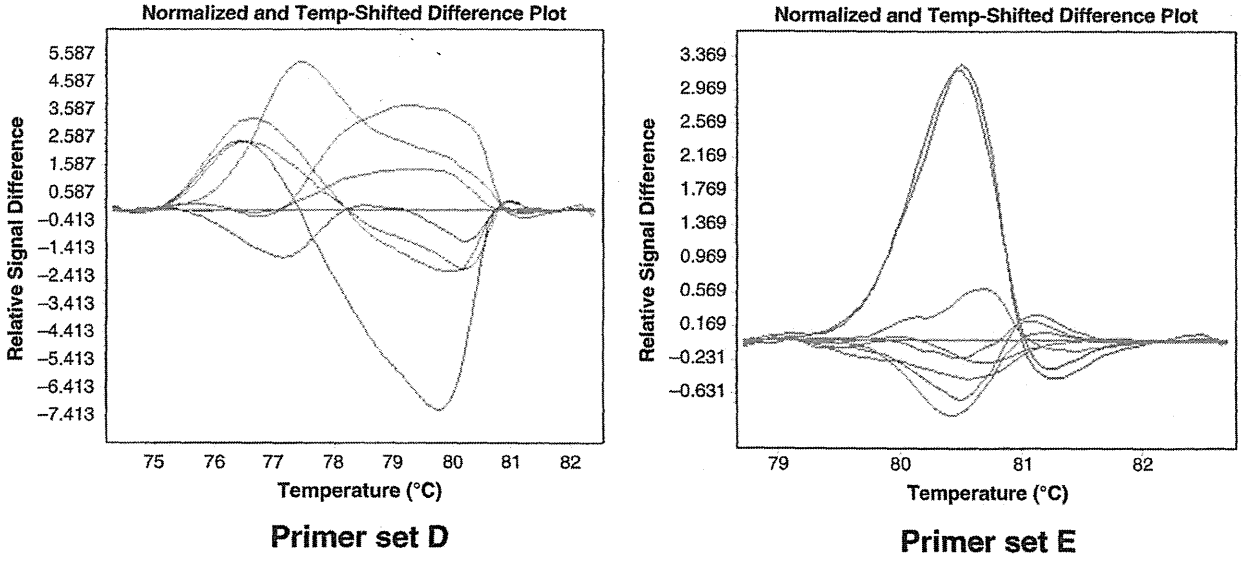


FIG. 7. *SMN* mutation hunting in exon 3 by HRMA: samples who have an intragenic mutation (red), and samples who do not have any mutation (blue).

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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Address correspondence to:
Satoru Morikawa, M.D.
Department of Community Medicine
and Social Healthcare Science
Kobe University Graduate School of Medicine
7-5-1 Kusunoki-cho, Chuo-ku
Kobe 650-0017
Japan

E-mail: msatoru@med.kobe-u.ac.jp

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ORIGINAL ARTICLES

AU1 ▶

Spinal Muscular Atrophy Patient Detection and Carrier Screening Using Dried Blood Spots on Filter Paper

Nur Imma Fatimah Harahap,[1,2] Indra Sari Kusuma Harahap,[1,3] Richard Hideki Kaszynski,[4] Dian Kesuma Pramudya Nurputra,[1] Tri Budi Hartomo,[1] Huyen Thi Van Pham,[1] Tomoto Yamamoto,[1,5] Satoru Morikawa,[1,5] Noriyuki Nishimura,[1,5] Imam Rusdi,[3] Retno Widiastuti,[2] and Hisahide Nishio[1,5]

Aim: Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder. It is caused by mutations in the *SMN1*, and its clinical severity is modified by copy number variations of the *SMN2*. According to previous studies, deletion of *SMN1* exon 7 is the most frequently observed in patients with SMA. Therefore, molecular analyses exploiting this genetic lesion could be beneficial in the diagnosis of SMA. Unfortunately, in many geographical regions, physicians do not have the latest molecular screening technologies at their immediate disposal. Thus, to overcome this issue, we developed an SMA-diagnosing system using dried blood spots (DBS) placed on filter paper to facilitate remote diagnosis. *Methods:* In this study, we validate the applicability of DBS on Flinders Technology Associates (FTA) filter paper for detecting *SMN1* exon 7 deletions and copy number variations of *SMN1* and *SMN2*. To detect exon 7 deletions in *SMN1*, polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis was conducted by using DNA extracted from the DBS on FTA filter paper that had been stored at room temperature for a period of up to 4 years. To determine the copy numbers of *SMN1* and *SMN2*, we carried out SYBR green-based real-time PCR by using the same blood specimens. *Results:* The results obtained from the DBS on FTA filter paper were in complete concordance with those analyses using fresh blood specimens. This indicates that DBS on filter papers is a reliable method for SMA patient detection and carrier screenings. *Conclusion:* The SMA-diagnosing system, combined with the mailing of DBS on filter paper, will be beneficial for patients suffering from neuromuscular disorders in areas with limited or no access to diagnostic facilities with molecular capabilities.

Introduction

SPINAL MUSCULAR ATROPHY (SMA) is an autosomal recessive disease characterized by degeneration of motor neurons in the spinal cord, resulting in atrophy of skeletal muscles. SMA is clinically divided into four subtypes based on age at onset and the type of motor function achieved: SMA type I (Werdnig-Hoffman disease, severe type), SMA type II (intermediate type), SMA type III (Kugelberg-Welander disease, mild type), and SMA type IV (adult onset type or very mild type) (Lunn and Wang, 2008).

The gene responsible for SMA is the survival motor neuron (*SMN*) gene. This gene has two nearly identical copies, *SMN1* and *SMN2* (Lefebvre *et al.*, 1995). It has been shown that SMA is caused by a homozygous disruption of *SMN1*, and further, the clinical severity of the disease is modified by copy number variations of *SMN2* (Feldkötter *et al.*, 2002). To a limited degree, the presence of *SMN2* copies can compensate for the deletion of *SMN1*, and it has been shown that in the absence of *SMN1*, increased copy numbers of *SMN2* can improve the clinical severity of the disease (Harada *et al.*, 2002). Additionally, the presence of the neuronal apoptosis inhibitory protein (*NAIP*) gene is also associated with the clinical severity (Glotov *et al.*, 2001).

Most cases of SMA present with the complete absence of *SMN1* (Parsons *et al.*, 1998). Patients with an SMA-like presentation should be tested for the presence of a homozygous deletion of *SMN1* by using methods that are 98.4%–99% sensitive and 98.3%–100% specific (Beck *et al.*, 2001; Mailman *et al.*, 2002). Therefore, the detection of *SMN1* deletions is highly beneficial in diagnosing SMA. Additionally, copy number analysis of *SMN1* may also be valuable for carrier testing, because all carriers have only 1 copy of *SMN1*.

When discussing the topic of molecular testing, it is essential to note that the means to conduct molecular analysis is not universally available in all geographical regions of the world.

[1]Division of Genetic Epidemiology, Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan.
[2]Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Indonesia.
[3]Department of Neurology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia.
Departements of [4]Legal Medicine and [5]Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan.

With this in mind, if a simple method of collection, storage, and shipment of samples could be established, then all patients can gain access to the benefits of molecular analysis regardless of geographical or industrial limitations. This is true not only for SMA but also for other inherited disorders as well (Aggarwal *et al.*, 1992). In this study, we proposed a novel SMA-diagnosing system using dried blood spot (DBS) on filter paper, which effectively facilitates the simplified collection, storage, and shipment of patient samples to centralized testing facilities.

Patients and Methods

Patients

ID-1 (woman, SMA type 1). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 1 month old. She did not obtain head control or sit by 3 years.

ID-2 (woman, SMA type II). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 6 months old. She obtained head control at 4 months old, but she could not sit without aid. She underwent tracheostomy at 2 years of age.

ID-3 (woman, SMA type III). She was referred to Kobe University Hospital for the evaluation of muscle weakness affecting dominantly lower limbs at 12 years old. Her gait disturbance had been noticed since she was 3 years old.

ID-4 (woman, SMA type I). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 1 year 5 months old. She did not obtain head control or sit without aid.

ID-5 (man, SMA type III). He was referred to Kobe University Hospital for the evaluation of muscle weakness affecting dominantly lower limbs at 5 years of age.

Before the molecular analysis, informed consent was obtained from the parents of the patients. This study was approved by the Ethical Committee in Kobe University.

Extraction of DNA from Flinders Technology Associate elute microcard™

For the collection of blood samples from SMA suspicious patients, we adopted the use of FTA® Elute Cards (Whatman® Inc, Schleicher & Schuell, Clifton, NJ, art no. WB 120410). Storage periods of the Flinders Technology Associate (FTA) Elute Cards varied from 1 to 4 years. All FTA Elute Cards were stored at room temperature. To extract DNA from the DBS, 5 circles were punched out from the center of a blood spot by using a
F1 ▶ Φ3 mm hole puncher, as shown in Figure 1. The punched circles were put into a sterile 1.5 mL microfuge tube and washed by adding 500 µL of sterile water and 3 cycles of pulse vortexing. Excess water used for washing was removed from the tube by using a sterile pipette. After this step, the circles obtained were further centrifuged for 5 s, and excess water was removed again by pipetting. After washing, these punched circles were completely immersed in 100 µL of TE buffer, used as an eluant followed by heating at 95°C for 30 min. At the end of the incubation period, the tubes were pulse vortexed ~60 times. The eluant was stored at −20°C until being used as a DNA source for further examination (Whatman, Inc., Clifton, NJ).

Deletion test

Polymerase chain reaction (PCR) was performed according to the methods described by van der Steege *et al.* (1995). For each reaction, 300–500 ng of DNA was used. The oligonucleotide primers for exon 7 in the *SMN1* and *SMN2* were R111 (Lefebvre *et al.*, 1995) and ×7-Dra (van der Steege *et al.*, 1995), and those applied for use on exon 8 of both genes were 541C950 (Lefebvre *et al.*, 1995) and 541C1120 (Lefebvre *et al.*, 1995). To discriminate between *SMN1* and *SMN2* products, PCR amplicons were completely digested with *Dra*I (Takara Biomedical, Tokyo, Japan) for exon 7 and *Dde*I (Takara Biomedicals) for exon 8. The restriction enzymes *Dra*I and *Dde*I only cleave the amplified fragments from *SMN2* exon 7 and 8, respectively. PCR amplification of the *NAIP*-specific sequence, exon 5, was performed according to the method reported by Roy *et al.* (1995).

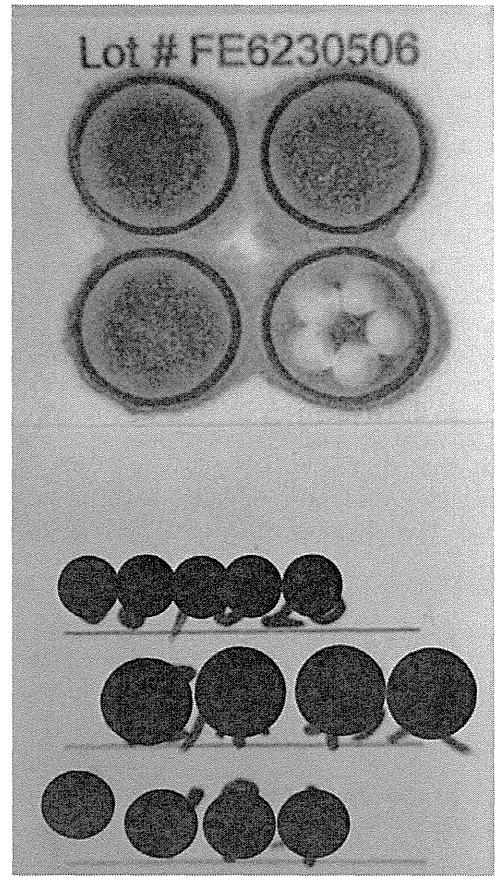

FIG. 1. Image of FTA Elute microcard™ after being punched. On filter paper, five holes in the center of one well can be observed. To avoid cross contamination between samples, a micropunch was carried out thrice on a clean piece of filter paper followed by wiping of the holepuncher with 95% ethanol. This was done between all FTA samples. FTA, Flinders Technology Associates.

Sequencing analysis

To identify and confirm the presence of hybrid genes, direct sequencing analysis was performed by using a BigDye Terminator V3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a genetic analyzer (ABI Prism 310; Applied Biosystems), with DNA Sequencing Analysis Software (Applied Biosystems). PCR amplicons were used as templates along with the forward primer of intron 6 and reverse primer of exon 8 for sequencing reactions.

Copy number analysis

For determination of *SMN1* and *SMN2* copy numbers, we used a quantification method utilizing real-time PCR and a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). *SMN1* exon 7 was amplified with a primer set of telSMNex7forw (5′-TTT ATT TTC CTT ACA GGG TTT C-3′) (Feldkötter *et al.*, 2002) and telSMNint7rev (5′-GTG AAA GTA TGT TTC TTC CAC GTA-3′) (Feldkötter *et al.*, 2002). *SMN2* exon 7 was amplified with a primer set of cenSMNex7forw (5′-TTT ATT TTC CTT ACA GGG TTT TA-3′) (Feldkötter *et al.*, 2002) and cenSMNint7rev (5′-GTG AAA GTA TGT TTC TTC CAC GCA-3′) (Feldkötter *et al.*, 2002). The *CFTR* was used as a reference gene for the relative quantification of *SMN1* and *SMN2*. *CFTR* was amplified with a primer set of CF621F (5′-AGT CAC CAA AGC AGT ACA GC-3′) (McAndrew *et al.*, 1997) and CF621R (5′-GGG CCT GTG CAA GGA ATG TTA-3′) (McAndrew *et al.*, 1997). The details of the experimental procedure we applied have been described elsewhere (Tran *et al.*, 2008).

Statistical analysis

To evaluate the performance of the FTA Elute Microcard method described in this study, sensitivity and specificity were calculated by using standard statistical analyses.

Results

DNA concentration or recovery of DNA from the DBS on FTA elute microcard

To assess the performance of FTA Elute microcards (Whatman Inc, Schleicher & Schuell, art no. WB 120410), we selected cards based on their storage time (Table 1). The storage time of the individual cards varied from 1 to 4 years.

TABLE 1. DNA CONCENTRATION FROM DRIED BLOOD SPOT ON FLINDERS TECHNOLOGY ASSOCIATES ELUTE MICROCARD™

Age of dried blood on FTA elute Microcard™	*Personal ID*	*DNA concentration (mg/L TE Buffer)*	*OD 260: OD280*
1 year	16	255.9	1.68
	17	151.4	1.75
	9	200.1	1.62
	19	244.8	1.61
2 year	3	66.5	1.76
	8	73.6	1.86
	5	215.3	1.67
	6	321.3	1.61
	7	239.9	1.67
	4	270.5	1.57
	12	180.2	1.64
	13	223.6	1.66
3 year	1	143.8	1.56
	14	314.8	1.61
	10	180.4	1.61
	11	200.7	1.67
	18	226.4	1.75
	20	268.0	1.77
4 year	2	187.6	1.81
	15	123.2	1.62

Sample was prepared from five punched circles that had been (Φ3 mm) incubated in 100 μL of TE buffer.

Concentration of DNA extracted from the cards ranged between 66.5 and 321.3 mg/L (mean 204.4 mg/L). DNA samples had OD 260: OD 280 values of 1.56 and 1.86, respectively. The concentration and purity of the isolated DNA was sufficient for subsequent PCR analysis.

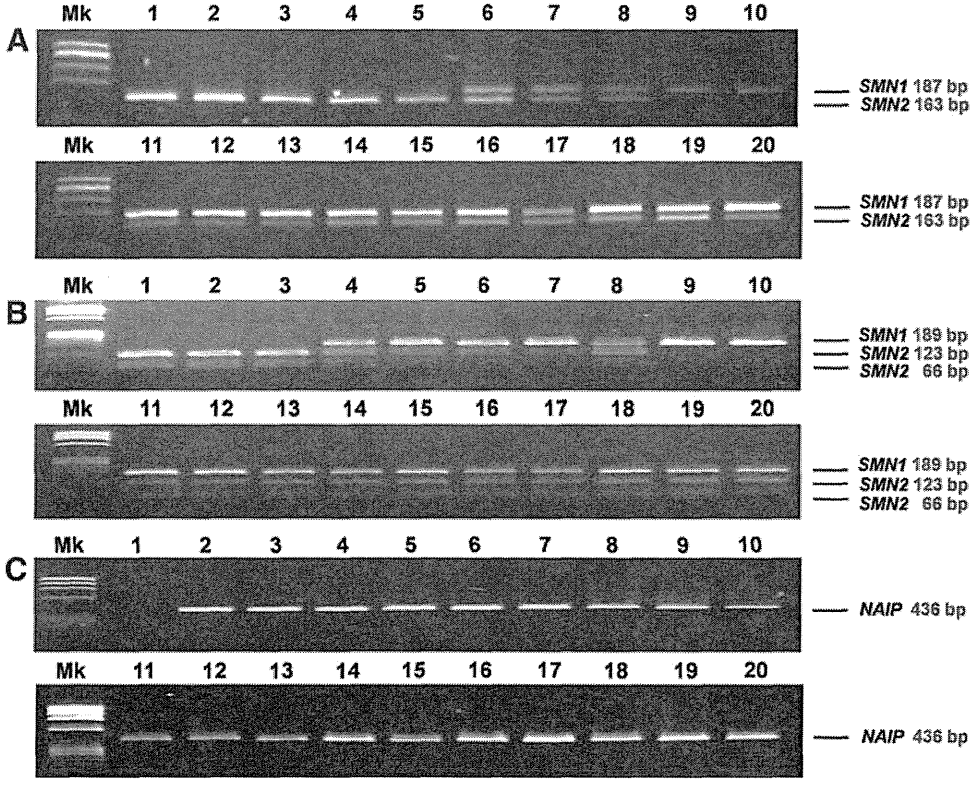

FIG. 2. **(A)** *SMN1* exon 7 deletion test. About 5/20 samples showed a homozygous deletion of *SMN1* exon 7. About 2/20 samples showed a homozygous deletion of *SMN2* exon 7. **(B)** *SMN1* exon 8 deletion test. About 3/20 samples showed a homozygous deletion of *SMN1* exon 8. 2/20 samples showed a hybrid *SMN* gene. Another 2/20 samples showed a homozygous deletion of *SMN2* exon 8. **(C)** *NAIP* exon 5 deletion test. About 1/12 samples showed a homozygous deletion *NAIP* exon 5. Lane numbers in this figure are identical to personal ID of the samples in Tables 1 and 2.

TABLE 2. SURVIVAL MOTOR NEURON-DELETION TEST AND *SMN1* AND *SMN2* COPY NUMBER ANALYSIS USING DRIED BLOOD ON FLINDERS TECHNOLOGY ASSOCIATES ELUTE MICROCARD

			Deletion test					*Copy number* SMN1				*Copy number* SMN2			
			SMN *Exon 7*		SMN *Exon 8*			*Freshly prepared sample*		*Card DNA*		*Freshly prepared sample*		*Card DNA*	
	Personal ID	*Clinical status*	SMN1	SMN2	SMN1	SMN2	*NAIP Exon 5*	*CNR*	*Copy number*	*CNR*	*Copy number*	*CNR*	*Copy number*	*CNR*	*Copy number*
Patient	1	SMA type I	Del	Nondel	Del	Nondel	Del	–	0	–	0	1.96	2	1.91	2
	2	SMA type II	Del	Nondel	Del	Nondel	Nondel	–	0	–	0	3.00	3	2.96	3
	3	SMA type III	Del	Nondel	Del	Nondel	Nondel	–	0	–	0	3.32	3	3.25	3
	4	SMA type I	Del	Nondel	Nondel	Nondel	Nondel	–	0	–	0	2.67	3	2.87	3
	5	SMA type III	Del	Nondel	Nondel	Nondel	Nondel	–	0	–	0	3.65	4	3.72	4
Carrier	6	Father of Patient no 5	Nondel	Nondel	Nondel	Nondel	Nondel	0.95	1	0.93	1	1.75	2	2.38	2
	7	Mother of Patient no 5	Nondel	Nondel	Nondel	Nondel	Nondel	1.05	1	0.66	1	2.65	3	2.58	3
	8	Mother of Patient no 3	Nondel	Nondel	Nondel	Nondel	Nondel	0.95	1	1.00	1	2.97	3	3.13	3
Control	9	Healthy	Nondel	Del	Nondel	Del	Nondel	1.99	2	1.91	2	–	0	–	0
	10	Healthy	Nondel	Del	Nondel	Del	Nondel	1.96	2	1.91	2	–	0	–	0
	11	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.84	2	1.55	2	0.93	1	1.07	1
	12	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.30	2	2.30	2	1.00	1	1.14	1
	13	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.20	2	2.32	2	1.15	1	1.20	1
	14	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.87	2	2.23	2	1.80	2	2.20	2
	15	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.88	2	1.90	2	1.97	2	1.74	2
	16	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.97	2	2.04	2	2.05	2	2.16	2
	17	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.95	2	1.90	2	2.00	2	2.19	2
	18	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.28	2	2.22	2	2.22	2	1.52	2
	19	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.02	2	1.96	2	2.84	3	3.19	3
	20	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.96	3	2.71	3	1.91	2	2.26	2

These data obtained from analyses using DNA extracted from DBS on the FTA Elute microcards were compatible with those obtained from analyses using DNA prepared from EDTA-blood. (The data of *SMN*-deletion test using DNA prepared from EDTA-blood not shown).

[a]The deletion test using card blood was completely compatible with the freshly prepared sample.

DBS, dried blood spot; FTA, Flinders Technology Associates; CNR, calibrator-normalized ratio; del, deletion; nondel, nondeletion.

◀ AU3

TABLE 3. SENSITIVITY AND SPECIFICITY ANALYSIS OF DRIED BLOOD ON FLINDERS TECHNOLOGY ASSOCIATES ELUTE MICROCARD

		Freshly prepared DNA		
		(+)	*(−)*	*Total*
Card blood	*SMN1* Exon 7 deletion test (+)	5	0	5
	SMN1 Exon 7 deletion test (−)	0	15	15
	Total	5	15	20

Sensitivity test = 5/(5+0) × 100% = 100%.
Specificity test = 15/(15+0) × 100% = 100%.
From Fisher analysis, exact Fisher *p* was 0.72. It indicated a nonsignificant difference between the result from DBS on filter paper and EDTA-blood.

Deletion test

To determine the presence and/or absence of *SMN* exon 7, we performed a PCR-enzyme digestion analysis of these exons by using DNA samples extracted from the FTA Elute microcards. After complete digestion by *DraI*, nondigested PCR products from *SMN1* exon 7 (187 bp) could not be observed in five samples (lanes 1–5), although these samples clearly show the presence of digested PCR products from *SMN2* exon 7 (163 bp) (Fig. 2). In 13 samples (lanes 6–8, 11–20), nondigested PCR products from *SMN1* exon 7 were present along with the digested PCR products from *SMN2* exon 7. In two samples (lanes 9 and 10), the presence of nondigested PCR products from *SMN1* exon 7 *SMN1* were confirmed, but digested PCR products from *SMN2* exon 7 could not be observed. These data obtained from analyses using DNA extracted from DBS on the FTA Elute microcards were compatible with those obtained from analyses using DNA prepared from EDTA-blood (Table 2). In the current study, we compared and evaluated the results of the *SMN1* exon 7 deletion test by using DNA extracted from DBS on the FTA Elute Microcard with test results obtained from DNA prepared from EDTA-blood. Both sensitivity and specificity displayed outstanding results at 100% (Table 3). Data obtained from analyses using DNA prepared from EDTA-blood are not shown in this article.

Similar—but not identical—results were obtained from the analysis conducted on *SMN* exon 8. After complete digestion by *DdeI*, three samples (lanes 1–3) did not have nondigested PCR products from *SMN1* exon 8 (189 bp), although the presence of two digested PCR products from *SMN2* exon 8 (123 bp, 66 bp) could be detected (Fig. 2). Fifteen samples (lanes 4–8, 11–20) showed the presence of nondigested PCR products from *SMN1* exon 8 and two digested PCR products from *SMN2* exon 8. Two samples (lanes 9 and 10) showed the presence of a nondigested PCR product from *SMN1* exon 8 but did not show digested PCR products from *SMN2* exon 8. These two samples were taken from healthy controls. The results obtained using DNA extracted from DBS on the FTA Elute microcards were compatible with those obtained from DNA prepared from EDTA-blood.

Interestingly, two samples (lanes 4 and 5) were absent for *SMN1* exon 7, whereas the presence of *SMN1* exon 8 could be readily confirmed. These samples were taken from patients with the so-called hybrid *SMN* genes (Figs. 2 and 3B). To confirm the initial results suggesting the presence of a hybrid *SMN* gene in DNA samples extracted from DBS on the FTA Elute microcard, we performed a sequence analysis of the PCR product that spanned intron 6, exon 7, intron 7, and exon 8. In the sequence between intron 6 and intron 7, three *SMN2*

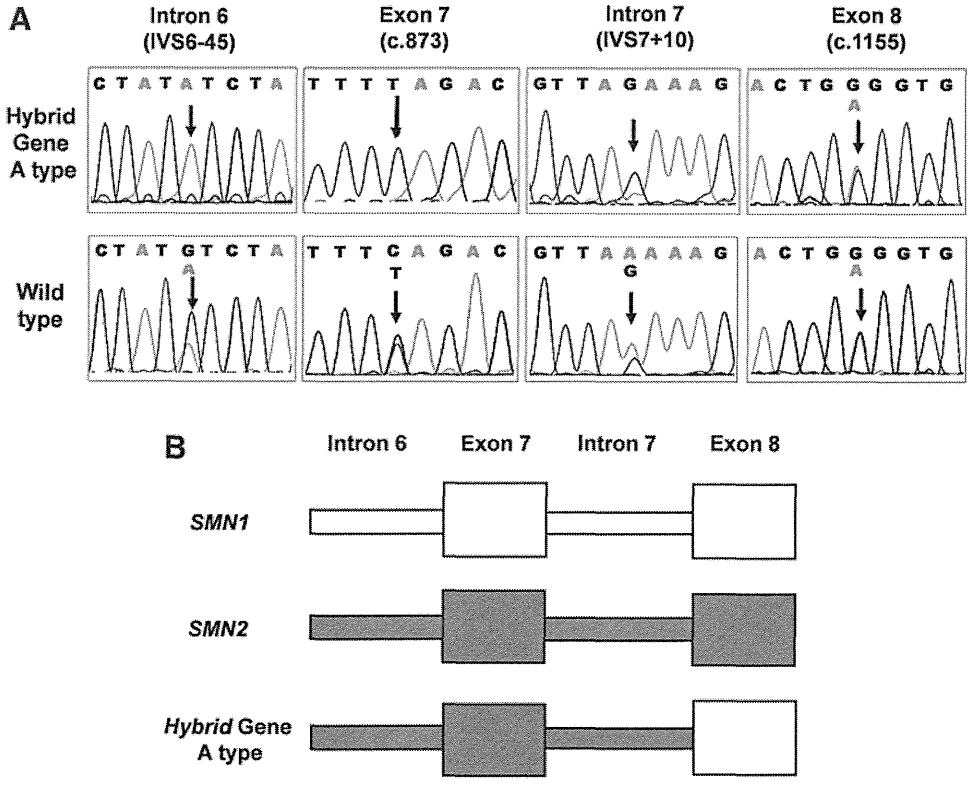

FIG. 3. **(A)** Direct DNA sequence analysis of the *SMN* gene: wild-type (lower) and hybrid SMN gene (Personal ID no.4) (upper). In the hybrid *SMN* gene, we found the following: (1) Intron 6 (−45 bp/exon7)—*SMN2* showed nucleotide **a**, as same nucleotide as *SMN2*. (2) Exon 7 (nucleotide 873)—*SMN2* showed nucleotide **T**, as same nucleotide as *SMN2*. (3) Intron 7 (+100 bp/exon7 and +214 bp/exon7)—*SMN2* showed nucleotide **g**, as same nucleotide as *SMN2*. (4) Exon 8 (nucleotide 1155)—*SMN2* showed nucleotide **G** and **A**, as same nucleotide as *SMN1* and *SMN2*. **(B)** Data sequencing analysis showed that the nucleotide sequences of intron 6, exon 7, and intron 7 were identical to those of *SMN2*, whereas the sequence of exon 8 exhibited both *SMN2* and *SMN1*. Thus, we can conclude that this patient has A type hybrid *SMN* (Nishio *et al.*, 1999).

specific nucleotides were present. In stark contrast, in exon 8, both *SMN1* and *SMN2* specific nucleotides were present in the sequence (Fig. 3A).

In addition, we performed PCR analysis on *NAIP* exon 5. Only 1 out of 20 samples showed the absence of *NAIP* exon 5 (lane 1) (Fig. 2). The sample was obtained from a patient with type I SMA. This result was compatible with a previous result obtained using DNA prepared from EDTA-blood (data not shown).

Copy number analysis

Copy number variations in *SMN1* and *SMN2* were determined by a relative quantification method based on the calibrator-normalized ratios (CNR) (Tran *et al.*, 2008). When DNA samples showed CNR values of 0.6–1.4, 1.5–2.4, 2.5–3.4, and 3.5–4.4, it was considered that they carried 1, 2, 3, and 4 copies of the gene, respectively. Copy numbers determined from CNR values obtained for the DBS samples were consistent with those determined for EDTA-blood samples.

In this study, *SMN1* copy number variations in patients with SMA (ID 1–5) was not a major issue, because they had been determined through previous deletion studies that there was a deletion of *SMN1*. For these samples, we only conducted copy number analyses for *SMN2*. In these patients, *SMN2* copy number varied from two to four copies; two copies in Type I, three copies in Type I, II, and III, and four copies in Type III. The results displayed a tendency that *SMN2* copies had a more profound effect on the phenotype (Table 2).

In the present study, 3 out of 15 non-SMA samples (ID 6–8) carried a single copy of *SMN1*. These samples belong to the parents of patients with SMA. Consequently, the parents were also diagnosed as with carrier status of SMA at the molecular genetic level, as well as at the clinical level.

We also examined *SMN1* and *SMN2* copy number variations in 12 controls (ID 9–20). The samples were obtained from healthy persons. With the exception of one sample (ID 20), all control samples had two copies of *SMN1* (Table 2). Sample ID 20, on the other hand, had three copies of *SMN1*. The presence of *SMN2* in control samples varied from 0 to 3 copies.

Discussion

We demonstrated the applicability of DBS on filter paper for molecular diagnostic analysis of SMA. In this study, a simple boiling method was applied for DNA extraction from DBS affixed on five Φ3 mm-circles of filter paper. This amount corresponds to $\sim$50 µL of whole blood, and the method has been adapted from a method previously described (Kogan *et al.*, 1987; Jinks *et al.*, 1989). The total amount of DNA extracted from five Φ3 mm-circles was between 6.65 and 32.13 µg. The OD260/OD280 ratio of the DNA extracted from DBS was 1.56–1.86 (Table 1), which retained enough purity for subsequent PCR analysis. Without exception, in all samples, we successfully amplified the target genes: *SMN1*, *SMN2*, and *NAIP*. To determine the hybrid *SMN* gene, we amplified a 1011 bp-fragment encompassing intron 6 to exon 8 in the *SMN1* or *SMN2* genes from DBS. Similarly, Chaisomchit *et al.* (2005) amplified a 1039 bp-fragment of the β-globin gene from DBS on filter paper stored for 11 years, thus demonstrating the remarkable stability of DNA in DBS affixed on filter paper.

By conducting PCR using DNA derived from DBS on filter paper, we were successful in demonstrating the existence or absence of *SMN1*, *SMN2*, and *NAIP*. PCR-enzyme digestion experiments in this study were performed according to the method described by van der Steege *et al.* (1995). To determine the copy numbers of the *SMN* genes, we conducted a set of quantitative real-time PCR studies according to previously described methods (Tran *et al.*, 2008). All data obtained from DBS on filter paper experiments were compatible with those obtained using EDTA-blood samples.

Majumdar *et al.* (2005) used multiplex-PCR to detect zero copies of *SMN1* in patients with SMA and a single copy of *SMN1* in carriers. Their results, combined with the results reported in this study, collectively indicate that the molecular diagnosis of SMA using EDTA-blood samples can be replaced by DBS on filter paper. Wijnen *et al.* (2008) sums up the potential advantages of genotyping with DBS as follows: (1) no phlebotomist is necessary, (2) genotyping results are known when the patient visits the clinician, and the clinician can take these results into account when he/she prescribes (other) drugs, (3) transport is easy, and there is a reduction in transport costs, because only the cost of an envelope and filter paper are incurred, and (4) DNA extraction from DBS is rapid, simple, and expensive DNA isolation kits are unnecessary. Accurate diagnosis based on molecular analysis can be simply obtained by mailing the filter paper with a DBS affixed to a central diagnostic center.

In conclusion, the evolution of molecular diagnostic studies have been ground breaking rather than incremental; however, the consideration of how these novel techniques can be utilized to exploit their full range of effectiveness has not been granted ample attention. In this study, we showed that DNA samples isolated from DBS serve as a satisfactory source for subsequent SMA molecular diagnosis even after 4 years of storage at room temperature. An SMA-diagnosing system, combined with the mailing of DBS on filter paper, will be beneficial for patients suffering from neuromuscular disorders in areas with limited or no access to diagnostic facilities with molecular capabilities.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Indra Sari Kusuma Harahap, M.D., Ph.D.
Division of Genetic Epidemiology
Department of Community Medicine
and Social Healthcare Science
Kobe University Graduate School of Medicine
7-5-1 Kusunoki-cho
Chuo-ku
Kobe 650-0017
Japan

E-mail: iin_999@yahoo.com