that *SMN2* splicing in Patient A was similar to that of the disease controls.

3.3. Identification of GCC polymorphism in the SMN2 promoter

According to Monani et al., a ~200-bp element lying between −441 and −228 (numbering is based on the article of Monani et al. [14]) in *SMN* promoters drives strong expression. Thus, we explored the *SMN2* promoter of Patient A. Direct sequence analysis of the element showed overlapping nucleotide peaks (Fig. 2), suggesting an insertion or deletion of some nucleotides. Sequencing analysis of subcloned fragments identified two different alleles with and without a GCC insertion at position c.-320_-321. However, no other mutations were detected in this region.

The Ag1CA multicopy microsatellite marker sequence is present in the promoter region downstream of the GCC insertion at c.-320_-321. Therefore, the GCC location may be different from allele to allele. We did not identify the real size of the Ag1CA multicopy marker sequence or the real position of the GCC insertion in Patient A. The GCC insertion at c.-320_-321 does not represent the real location in Patient A. In this study, based on the report by Monani et al. [14], we named this GCC insertion polymorphism in the *SMN1/SMN2* promoter as "the GCC insertion at c.-320_-321").

3.4. Frequency of GCC insertion in control individuals and SMA patients

We performed DHPLC screening for the GCC insertion polymorphism in 50 control individuals and 50 SMA patients (Patient A was not included in the 50 SMA patients) (Fig. 3). All control individuals carried *SMN1* and *SMN2*, and all SMA patients lacked *SMN1* but retained *SMN2*.

The GCC insertion polymorphism was found in 12 out of 50 control individuals (24%), while it was not found in any SMA patients. Using long-range PCR, we also confirmed that the GCC insertion polymorphism was present in the *SMN1* promoter in a healthy control. However, the GCC insertion polymorphism was absent in all SMA patients (Table 1). This observation suggested that the GCC insertion polymorphism propagates mainly in *SMN1* genes.

3.5. Effects of GCC insertion on transcription efficiency in luciferase transcription assay

To clarify the effect of the GCC insertion polymorphism on transcription activity, we performed transient transfection experiments with pGL2BTK, pGCCGCC, and pGCC (Fig. 4). The sequences of inserted fragments, GCCGCC and GCC, are presented in Fig. 1. It should be noted that the cyclic AMP-response element (CRE)-like site, TGACGACA, is present at 84 bp upstream of the polymorphism position.

Neuroblastoma cell lines have been used because of their closely resembled characteristic of neurons. In the assay with neuroblastoma cell lines, BE(2)-C cells, the baseline activity of pGCCGCC was lower than that of pGCC. Furthermore, the response to dibutyryl cAMP (a cyclic nucleotide derivative which mimics the action of endogenous cAMP), forskolin (a reagent elevating cAMP via activation of the adenylyl cyclase), or both reagents was slightly lower in pGCCGCC than in pGCC. These results suggested that the presence of the GCC insertion polymorphism slightly decreased transcription efficiency of the *SMN2* promoter in neuronal cells. Thus, the GCC insertion polymorphism in the *SMN2* promoter of Patient A may not increase, but decrease transcription efficiency. This finding was

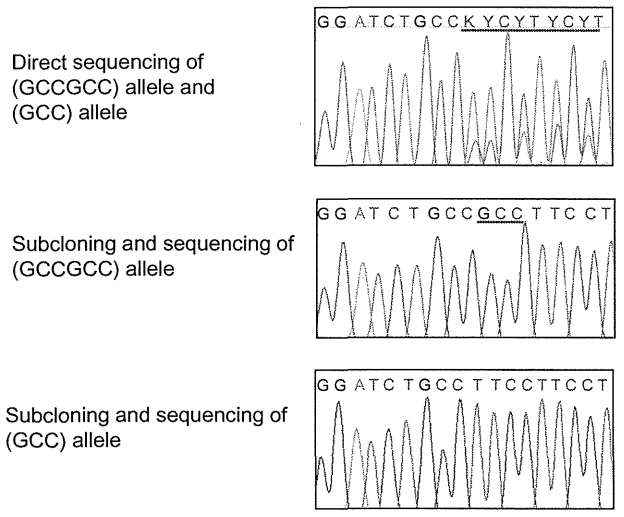

Fig. 2. Sequencing of the *SMN2* promoter region of Patient A. Direct sequencing analysis showed overlapping nucleotide peaks suggesting the presence of two different alleles (top). Subcloning sequencing analysis separately identified (GCCGCC) allele and (GCC) allele (middle and bottom).

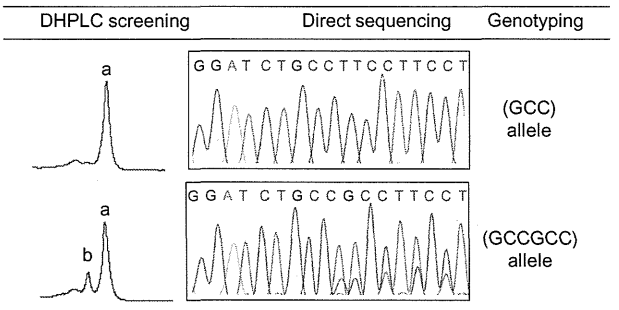

Fig. 3. DHPLC screening for the GCC insertion polymorphism. DHPLC analysis for the (GCC) allele showed a single peak (a-peak), but the presence of (GCCGCC) allele generated an additional peak (b-peak) to the a-peak.

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Table 1
Frequency of GCC insertion polymorphism in control individuals and *SMN1*-deleted SMA patients.

	GCC insertion polymorphism		
	Present	Absent	
SMN1-deleted SMA patients*	0	50	50
Healthy control individuals	12	38	50
	12	88	100

* Patient A was excluded from the *SMN1*-deleted SMA patients.

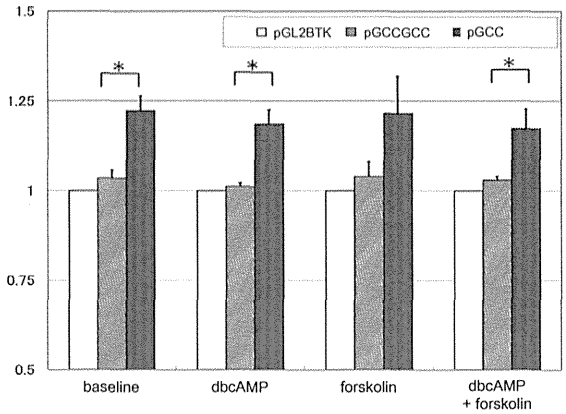

Fig. 4. Effect of GCC insertion polymorphisms on transcriptional activity. The effect of the GCC insertion polymorphism on transcription activity after transient transfection with pGL2BTK, pGCCGCC and pGCC into a neuroblastoma cell line, BE(2)-C cells. According to our data, the presence of the GCC insertion polymorphism slightly decreased transcription efficiency of the *SMN2* promoter in neuronal cells, with or without dibutyryl cAMP (dbcAMP) and/or forskolin treatment. The asterisk (*) shows the significant difference ($p < 0.01$).

compatible with a decreased amount of total *SMN2* transcript in white blood cells of Patient A.

4. Discussion

4.1. SMN2 copy number analysis

In this study, we presented a *SMN1*-deleted SMA patient with two copies of *SMN2*. He showed a milder phenotype than we had expected based on a low *SMN2* copy number. Patient A was able to sit without support, and also able to take a few steps sideways when supported. The clinical phenotype of the patient was judged to be SMA type 2/3, or type 2.9 according to the classification of Dubowitz [23].

Feldkötter et al. [24] reported that 138 out of 153 patients carrying two copies of *SMN2* were type 1 patients (90%). Swoboda et al. [25] reported that 21 out of 22 patients carrying two copies of *SMN2* were ventilator dependent or unable to sit unsupported (95%). These results are fully compatible with our previous observation of 37 out of 39 patients carrying two copies of *SMN2* being diagnosed as SMA type 1 [26].

Even though the *SMN2* copy number is a modifying factor of the SMA phenotype, there are some exceptional cases. Prior et al. and Vezain et al. reported that some unrelated SMA patients with 1–2 copies showed much milder clinical phenotypes than expected [20,21]. They identified a single base substitution, c.859G>C, in *SMN2* exon 7, leading to inclusion of the exon in *SMN2* mRNA. Patient A may also belong to such a patient group with a discrepancy between clinical phenotype and *SMN2* copy number. However, Patient A did not carry a c.859G > C substitution.

Recently, Oprea et al. reported the possibility of a female-specific modifying factor [27]. They suggested that high plastin 3 (*PLS3*) expression may be a female-specific modifying factor, which was observed in non-symptomatic females lacking *SMN1* (discussed further below). However, Patient A was a male, not a female.

It is, therefore, reasonable to think that unknown modifiers, other than an increase of *SMN2* copy number, the presence of an *SMN2* mutation facilitating exon 7 inclusion or a female-specific modifying factor, ameliorated the phenotype of this patient.

We found a GCC insertion polymorphism at c.-320_-321 in the *SMN2* promoter of Patient A. This trinucleotide insertion has already been identified in *SMN1*, but not in *SMN2*. Monani et al. [13] found more than 10 nucleotide differences between the *SMN* promoter regions of PAC 125D9 and PAC 125D15. Among them, a GCC insertion polymorphism was found in PAC 125D9 containing *SMN1*, but not in PAC 125D15 containing *SMN2* [13]. However, Boda et al. reported that the promoter sequences of *SMN1* and *SMN2* were identical [12]. They suggested that the different nucleotides in the sequence of PAC 125D9, including the GCC insertion, could be accounted for by errors in the sequencing of this clone.

Our study demonstrates that the GCC insertion polymorphism is, without doubt, present in the human population. The GCC insertion polymorphism was found in one fourth of control individuals, but was absent in all SMA patients, except for Patient A. In addition, strictly speaking, the possibility exists that in Patient A the upstream region, including the *SMN1* promoter, was connected to the downstream region, including *SMN2* exons, during gene conversion switching. These findings suggest that the GCC insertion polymorphism is perpetuated in *SMN1* genes.

4.2. Effect of the GCC insertion polymorphism on transcriptional activity

Next, we examined the possibility of the GCC insertion polymorphism in *SMN2* as a modifying factor of the SMA phenotype. Comparison between patient A (two copies of *SMN2*) and other SMA type 2 patients

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(three copies of *SMN2*) revealed a lower total *SMN* transcript level in peripheral blood cells of patient A. This finding suggests that the *SMN2* transcript level in patient A is mainly influenced by *SMN2* gene dosage. However, we are unable to examine *SMN2* transcription in motor neurons.

Monani et al. [14] showed that a ~200-bp fragment lying between −441 and −228 (numbering is based on the article of Monani et al. [14]) drives strong expression in a motor neuron cell line. The ~200-bp fragment contains a CRE-like site (TGACGACA), which interacts with the cyclic-AMP responsive element binding protein (CREB). Because the GCC insertion polymorphism resides in this fragment, we suggest that it may exert a negative (suppressing) or positive (enhancing) effect on transcriptional efficiency.

To examine the effect of the polymorphism on transcriptional efficiency, we constructed plasmids containing the ~200-bp fragment, and performed a luciferase reporter gene analysis using a neuroblastoma cell line (as an alternative cell line of a motor neuron cell line). Under baseline conditions and stimulated conditions with dbcAMP, forskolin, or both reagents, luciferase activity of the plasmid construct with the GCC insertion was lower compared with that without the GCC insertion.

These data suggest that the presence of the GCC insertion polymorphism slightly decreases transcription efficiency of the *SMN2* promoter in neuronal cells. Thus, the GCC insertion polymorphism in the *SMN2* promoter of Patient A might partially decrease transcription efficiency.

However, we must mention some limitations of this study. First, we do not know whether the *SMN2* promoter of Patient A carries any polymorphisms other than the GCC insertion polymorphism. The existence or not of other polymorphisms should be determined for a more accurate evaluation of the promoter function. Furthermore, the promoter region that we used to study *SMN2* transcription efficiency was not large enough to evaluate the combined effect of promoter-binding proteins, although the *SMN2* transcript levels of Patient A were significantly lower than those of other individuals tested in this study.

4.3. Modifying genes outside SMN genes

Our data show that FL-*SMN* transcript levels in Patient A were similar to those in five other SMA patients with three copies of *SMN2*. The amount of FL-*SMN* transcript is regulated by two processes, transcriptional activity and splicing of exon 7; however, transcription was not up-regulated in this patient. Patient A, therefore, suggests positive effects of splicing-related factors on the increase in FL-*SMN* transcript levels and that the modifier gene in Patient A may be related to the splicing machinery. However, the phenotype of our patient was much milder compared with the phenotypes of patients with typical SMA type 2. This indicates that the phenotype was positively modified by factors other than FL-*SMN* transcript levels (and levels of FL-*SMN* protein).

It is certain that modifying factors other than *SMN* genes are closely related to the SMA phenotype. Recently, Oprea et al. reported the possibility of a female-specific modifying factor [27]. They suggested that high plastin 3 (*PLS3*) expression, observed in non-symptomatic females lacking *SMN1*, may be such a factor (discussed further below). However, Patient A was a male, not a female.

The presence of asymptomatic females with no *SMN1* copies strongly suggests that non-*SMN* genes may be related to the pathogenesis or anti-pathogenesis of SMA. In this context, it is also necessary to clarify modifying factors other than *SMN* genes to establish an effective treatment for SMA.

4.4. Conclusion

In this study, we found a GCC insertion polymorphism at c.-320_-321 in the *SMN2* promoter of Patient A, an *SMN1*-deleted SMA patient with a milder phenotype than expected considering the low *SMN2* copy number. Transcription assays demonstrated that the polymorphism had a negative effect on transcription efficiency. This finding was compatible with a decreased amount of total *SMN2* transcript in white blood cells from Patient A. Thus, this GCC insertion polymorphism in the *SMN2* promoter may not be associated with the mild phenotype of the patient. Furthermore, our experiment in this study show that *SMN* gene and milder clinical phenotype of SMA may not always been correlated. We suggest non *SMN* gene factor maybe worked in this patient. Future studies are necessary to identify the modifying genes outside *SMN* gene that ameliorated the clinical phenotype of the patient.

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SMA Screening System Using Dried Blood Spots on Filter Paper: Application of COP-PCR to the *SMN1* Deletion Test

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Keywords: spinal muscular atrophy, *SMN1*, *SMN2*, COP-PCR, dried blood spot

BACKGROUND: Spinal muscular atrophy (SMA) is a common neuromuscular disorder caused by mutations in *SMN1*. More than 95% of SMA patients carry homozygous *SMN1* deletions. Thus, the *SMN1* deletion test should be performed initially as part of the diagnostic process. However, *SMN2*, a highly homologous gene, hampers detection of *SMN1* deletion. To differentiate between *SMN1* and *SMN2*, many analysis methods have been developed yet they are not all available worldwide. AIM: To establish a simple but accurate *SMN1*-deletion detection system that can be used worldwide. METHODS: Fifty DNA samples (29 SMA patients and 21 controls) from dried blood spots (DBS) on filter paper were assayed. All participants had previously been screened for SMA by PCR-restriction fragment length polymorphism (PCR-RFLP) using DNA extracted from freshly collected blood. DNA was extracted from DBS that had been stored at room temperature (20–25°C) for between 1 and 8 years. Competitive oligonucleotide priming-PCR (COP-PCR) was performed to distinguish *SMN1* and *SMN2* exon7. RESULTS: DNA yield from an 11-mm diameter DBS circle was 21,171 ± 7,485 ng (mean ± SD), with an 260/280 OD ratio from 1.49 to 2.1(mean ± SD; 1.67 ±0.13). Nucleotide sequencing confirmed gene-specific amplification of *SMN1* and *SMN2* by COP-PCR. *SMN1* and *SMN2* COP-PCR results are completely consistent with those obtained by PCR-RFLP. CONCLUSION: We have combined DNA extraction from DBS on filter paper with COP-PCR that specifically detects *SMN1* and *SMN2*, establishing a new *SMN1*-deletion detection system with practical application worldwide.

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INTRODUCTION

Spinal muscular atrophy (SMA) causes general muscle weakness because of a loss of lower motor neurons in the spinal cord. SMA is a common autosomal recessive disorder, with an incidence of 1/10,000 live births [24]. SMA is classified into three subtypes that depends on age of disease onset and achievement of motor milestones [19]: type 1 (severe form; onset age of 0–6 months, unable to sit unaided), type 2 (intermediate form; onset age of <18 months, unable to stand or walk unaided), and type 3 (mild form; onset age of >18 months, able to stand or walk unaided). Additionally, two other forms have been reported, SMA type 0 is the most severe and has a prenatal onset, while SMA type 4 is mildest and manifests after 20 years of age [12].

The gene for SMA, survival motor neuron 1 (*SMN1*), was cloned in 1995. *SMN1* encodes the SMN protein, which plays a critical role in cellular metabolism [22]. More than 95% of SMA patients have homozygous *SMN1* deletions [13], while the rest (< 5%) may carry intragenic *SMN1* mutations or be non-*SMN1*-related SMA (non-5q-SMA) [13]. Thus, at the beginning of diagnosis, *SMN1* deletion tests should be performed.

All SMA patients with homozygous *SMN1* deletions carry at least one copy of the *SMN2* gene, as complete loss of both *SMN* genes causes embryonic lethality [9, 27]. *SMN2* is highly homologous to *SMN1*, with only five nucleotide differences between them [11], and *SMN2* also produces a small amount of SMN protein [14]. The presence of *SMN2* may compensate for loss of *SMN1* to some degree, and higher copy numbers of *SMN2* are correlated with milder SMA phenotypes [3,7,15,29,31]. However, with regard to diagnosis or screening by conventional PCR methods, *SMN2* hampers detection of homozygous *SMN1* deletion.

To address this problem, various unique PCR methods have been established, for example, PCR and single-strand conformation polymorphism (PCR-SSCP) [13], PCR and restriction fragment length polymorphism (PCR-RFLP) [30], radio-isotope competitive PCR [15], PCR and denaturing high-performance liquid chromatography (DHPLC) [28], real-time PCR [4], multiplex ligation probe amplification (MLPA) [1], tetra-primer PCR [2],and high-resolution melting analysis[18]. However, not one of these methods can be readily introduced into all laboratories, some require expensive equipment or special reagents (e.g. radioisotopes), while others require much effort and are time-consuming procedures. Thus, further research is needed for the innovation of new simpler methods for distinguishing between *SMN1* and *SMN2*. We previously found that competitive oligonucleotide priming-PCR (COP-PCR) can separately amplify *SMN1* and *SMN2* [21], with each of the genes specifically amplified as usual by PCR.

In addition, when considering molecular analysis of any disease (including SMA), it should be noted that the capability to perform molecular analyses are not universally available in all geographical regions of the world. If simple method of collection, storage, and transfer of samples can be established, all patients will gain access to the benefits of molecular analysis regardless of geographical or industrial limitations [8]. We have previously reported that dried blood spots (DBS) on filter paper, which can be sent by mail, are a good DNA source for SMA diagnosis [8].

In this study, we combined DNA extraction from DBS on filter paper and COP-PCR analysis to establish a simple but accurate *SMN1*-deletion detection system that has practical utility in any area of the world. Here, we report on the yield and purity of DNA extracted from DBS, storage year effect on PCR amplification, and specificity of COP-PCR amplification.

MATERIAL AND METHODS

Patient and control samples

Fifty individuals were assayed, 28 SMA patients and 22 controls. All individuals had been previously analyzed by PCR-RFLP using DNA extracted from freshly collected blood [30]. Prior to analysis, informed consent was obtained from study participants. The study was approved by the Ethical Committee of Kobe University Graduate School of Medicine.

DNA extraction from DBS on filter paper

Blood samples were collected from SMA patients and controls using filter paper FTA® Elute Cards (Thermo Fisher Scientific, Waltham, MA, USA). Storage periods of blood samples on FTA® Elute Cards varied from 1 to 8 years. All FTA® Elute Cards were stored in the dark at room temperature (20–25°C). Genomic DNA was extracted from DBS according to our previous report [8]. Here, the protocol is briefly described. To extract DNA from DBS on cards, a hole punch was used to punch out seven 3-mm diameter circles from an 11-mm diameter DBS, equivalent to 50 μL of whole blood. The seven circles were placed into a sterile tube, washed in 500 μL distilled water, and vortexed three times. Washing water was completely removed from the circles, and 75 μL Tris-EDTA Buffer, pH 8.0 (TE buffer) added to the tube. The tube was heated at 95°C for 30 min, and then TE buffer containing DNA eluted and used as a DNA template for PCR or stored at −20°C until use. The

concentration and absorbance ratio of the DNA solution were measured at 260/280 nm (260/280 OD ratio) using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific).

COP-PCR for *SMN1/SMN2* exon 7

To separately amplify exon 7 from *SMN1* and *SMN2*, we performed COP-PCR using a thermal cycler (Bio-Rad iCycler Thermal Cycler, Bio-Rad Laboratories, Inc., Foster City, CA, USA). The PCR mixture total volume was 30 μL, consisted of distilled water, genomic DNA template (50–500 ng), 10 pmol/μL of common forward primer (R111), 100 pmol/μL of gene-specific reverse primer (SMN1-COP or SMN2-COP), 10 mM dNTP, 10 ×buffer with $MgCl_2$, and Taq DNA polymerase (Fast Start Taq DNA polymerase, Roche Applied Science, Mannheim, Germany). Primer sequences are shown in Fig. 1. After an initial denaturing step of 94°C for 7-min, 30 cycles were performed consisting of 1-min denaturing at 94°C, 1-min annealing at 35°C, and 1-min extension at 72°C, followed by 7-min at 72°C. An aliquot of each COP-PCR product was electrophoresed on a 4% agarose gel with 1×TBE buffer, and visualized by ethidium bromide staining.

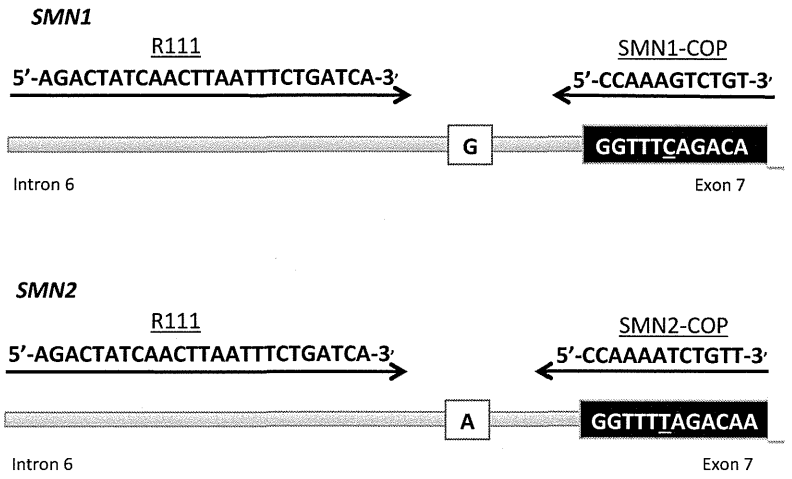

Fig. 1. Sequences, positions and directions of primers (R111, SMN1-COP and SMN2-COP). Nucleotides G and A in intron 6, and C and T in exon 7 indicate gene-specific differences between *SMN1* and *SMN2*. The expected sizes of *SMN1*- and *SMN2*-specific products are 168 bp and 169 bp, respectively.

Sequencing of COP-PCR products

Amplified COP-PCR products of *SMN1/SMN2* exon 7 were purified and used for cycle sequencing reactions with R111 primer. Cycle sequencing products were directly sequenced using a dye terminator cycle-sequencing kit (Life Technologies Corporation, Carlsbad, CA, USA). Reaction products were electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation).

RESULTS

Extracted DNA yield and storage period of DBS on filter paper

The DNA yield in an 11-mm diameter DBS circle was 21,171 ± 7,485 ng (mean ± SD), with 260/280 OD ratio from 1.49 to 2.1 (mean ± SD; 1.67 ± 0.13) (Table I).

Table I. DNA yield and 260/280 OD ratio

	DNA samples with good amplification (n=46)	DNA samples with no amplification (n=4)	All DNA samples (n=50)
DNA yield (ng) (11-mmØDBS circle)	21,564± 7,268	16,650 ± 8,403	21,171 ± 7,485
260/280 OD ratio	1.68 ± 0.13	1.60 ± 0.06	1.67 ± 0.13

There were four DNA samples (three controls and one SMA patient) with no COP-PCR amplification of either gene. Non-amplification of both genes is considered a false result, as the combination of *SMN1* and *SMN2* deletion causes embryonic lethality [9,27]. To determine if these four samples were of sufficient quality for PCR, we performed a conventional PCR experiment using a primer set for the *CFTR* gene [15], which resulted in failure of *CFTR* fragment amplification (data not shown). Thus, we concluded that these four samples had deteriorated and were not suitable for PCR amplification, and therefore omitted them from further analysis.

These four DNA samples had been stored more than 4 years before analysis (Table II). Nevertheless, it should be noted that the DNA concentrations and 260/280 OD ratios of these samples were similar to those showing good amplification with SMN1-COP or SMN2-COP primers (Table I).

Table II. Storage period of DBS on filter paper

Years	0 - 1	2	3	4	5	6	7	8
Samples with amplification	8	6	7	4	9	7	2	3
Samples with non-amplification	0	0	0	1	0	0	1	2

COP-PCR amplification patterns

COP-PCR allowed us to separate *SMN1* exon 7 from *SMN2* exon 7 (Fig. 2). The COP-PCR diagnostic screening results are summarized in Fig. 3.

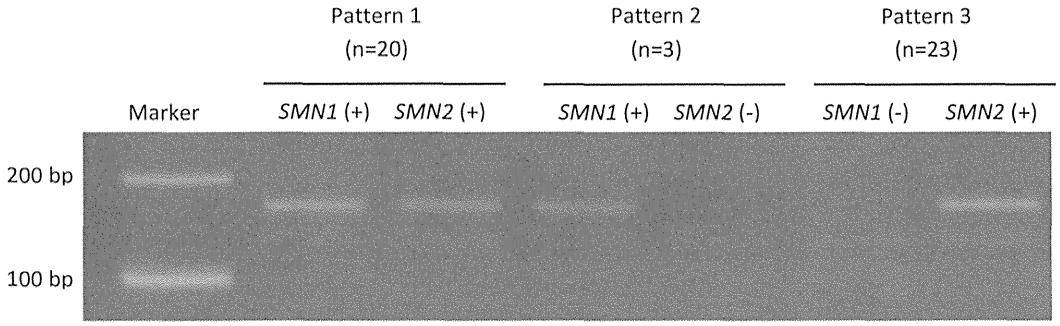

Fig. 2 COP-PCR amplification patterns. COP-PCR clearly distinguishes three genotypes: *SMN1*(+)/*SMN2*(+) (Pattern 1; *SMN1* and *SMN2* retention), *SMN1* (+)/*SMN2* (−) (Pattern 2; *SMN1* retention and *SMN2* deletion), and *SMN1*(−)/*SMN2* (+) (Pattern 3; *SMN1* deletion and *SMN2* retention).

In 18 controls and two patients with intragenic *SMN1*mutations, COP-PCR using either R111 and SMN1-COP or R111 and SMN2-COP produced amplification products (Pattern 1 in Figs. 2 and 3). In three controls, COP-PCR using R111 and SMN1-COP produced an amplification product, while R111 and SMN2-COP produced no amplification product (Pattern 2 in Figs. 2 and 3). In 23 patients with homozygous *SMN1* deletions, COP-PCR using R111 and SMN1-COP produced no amplification product, while R111 and SMN2-COP produced an amplification product (Pattern 3 in Figs. 2 and 3).

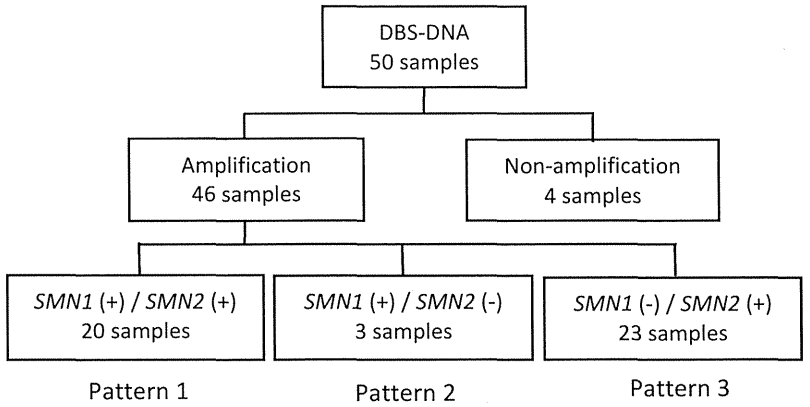

Fig. 3 Summary of the COP-PCR data. Pattern 1 group: 18 control individuals and 2 SMA patients with intragenic *SMN1* mutations. Pattern 2 group: 3 control individuals. Pattern 3 group: 23 SMA patients with homozygous deletions.

To confirm gene-specific amplification by COP-PCR, we performed nucleotide sequence analysis of COP-PCR products. According to a previous report [17], both *SMN1* and *SMN2* have gene-specific nucleotides in intron 6, with G and A nucleotides at position -44 from exon 7 of *SMN1* and *SMN2*, respectively. As shown in Fig. 4, the COP-PCR product using R111 and SMN1-COP had a G nucleotide at position -44 from exon 7, while R111 and SMN2-COP had an A nucleotide. These findings show that COP-PCR can separately amplify *SMN1* and *SMN2* exon 7 in a gene-specific way.

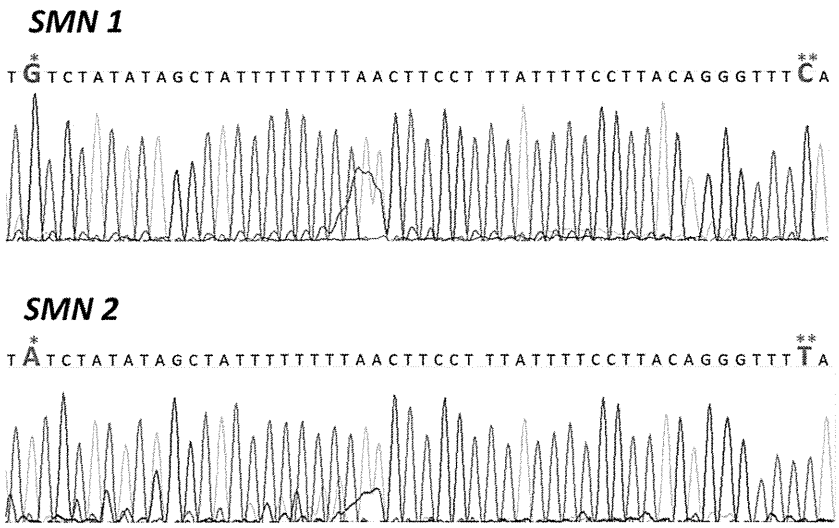

Fig. 4 COP-PCR specificity confirmed by sequencing. Nucleotide sequencing analysis confirmed *SMN1*- and *SMN2*-specific amplification. Nucleotide differences in intron 6 (*) and exon 7 (**) prove differential amplification of *SMN1* and *SMN2* by COP-PCR.

COP-PCR with DBS-DNA vs. PCR-RFLP with DNA from freshly collected blood

COP-PCR results using DBS-DNA completely matched those of PCR-RFLP using DNA from freshly collected blood (Table III). COP-PCR results using R111 and SMN1-COP also completely matched those of PCR-RFLP (Table III A). Sensitivity and specificity of COP-PCR using DBS-DNA for *SMN1* and *SMN2* exon 7 were 100%. These results suggested that COP-PCR using DBS-DNA is useful for diagnostic screening of SMA patients.

Table III. Comparison between COP-PCR and PCR-RFLP

(A)

		PCR-RFLP (freshly collected blood)		Total
		SMN1 Exon 7 Deletion	*SMN1* Exon 7 Non-deletion	
COP-PCR (dried blood)	*SMN1* Exon 7 Deletion	23	0	23
	SMN1 Exon 7 Non-deletion	0	23	23
Total		23	23	46
				sensitivity 1.0, specificity 1.0

(B)

		PCR-RFLP (freshly collected blood)		Total
		SMN2 Exon 7 Deletion	*SMN2* Exon 7 Non-deletion	
COP-PCR (dried blood)	*SMN2* Exon 7 Deletion	3	0	3
	SMN2 Exon 7 Non-deletion	0	43	43
Total		3	43	46
				sensitivity 1.0, specificity 1.0

DISCUSSION

DBS on filter paper

In this study, we combined DNA extraction from DBS on filter paper with COP-PCR analysis of *SMN1* and *SMN2*, establishing a simple but accurate *SMN1*-deletion detection system that has practical worldwide utility.

We used DBS-DNA as the PCR template, and obtained sufficient DNA for diagnosis of SMA. DBS on filter paper are practical and convenient with regard to handling (involves only spotting and drying blood on filter paper), transport (can be transferred by mail), storage (can be maintained at room temperature, and it is not necessary to store in a refrigerator), and cost (filter paper can be bought at minimal cost).

DBS have been used as biological specimens for more than 50 years, since Guthrie and Susi developed the phenylketonuria screening method to measure phenylalanine levels from DBS on filter paper [6]. Many technologies using DBS have already been developed and applied for medical purposes, including measurement of metabolites (e.g. adrenoleukodystrophy [20]), hormones (e.g. thyroid-stimulating hormone [11]), enzyme activities (e.g. glucose-6-phosphate dehydrogenase [26]), and drugs (e.g. antimalarial drugs [23]).

DBS are now recognized as a good source of DNA, and the combination of PCR with DNA from DBS enables genetic analysis [10, 16]. Molecular screening using DNA from DBS has already been reported for Duchenne/Becker muscular dystrophy and cystic fibrosis [25]. Our findings also support the use of DBS on filter paper as a good source of DNA for disease diagnosis. However, there are limitations to using DBS samples, and in particular, long-term storage of DBS cards may be associated with no PCR amplification, as in our study.

COP-PCR

We show here that COP-PCR can separately amplify the *SMN* genes, *SMN1* and *SMN2*. COP-PCR is a type of allele-specific amplification, in which two oligonucleotide primers compete for DNA annealing. Competitive primers are shorter than usual PCR primers (which are typically18–25 mer) and identical except for a nucleotide change that is located in the middle of the primer [5]. Amplification with the better-matched primer is favored 100-fold over the mismatched primer [5, 32]. Nucleotide sequencing confirmed that COP-PCR specifically amplified *SMN1* and *SMN2* exon 7. Comparison between COP-PCR and PCR-RFLP demonstrated a complete compatibility of both methods, proving the accuracy of COP-PCR. In addition, *SMN1*-deletion detection by COP-PCR is much faster than that by PCR-RFLP, because COP-PCR has no enzyme-digestion step. As to the required time from the start of PCR to the end of gel electrophoresis separating gene-specific products, COP-PCR takes only 3 hours, while PCR-RFLP takes more than 8 hours.

Conclusion

Here, we have combined DNA extraction from DBS on filter paper with COP-PCR that specifically detects *SMN1* and *SMN2*, and established a new *SMN1*-deletion detection system that is practical for use even in remote areas of the world. Moreover, we also demonstrate that DBS on filter paper are a good source of DNA for disease diagnosis.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

AUTHORS' CONTRIBUTION

NOZOMU KATO and NIHAYATUS SA'ADAH contributed equally to this work.

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A Rapid, Accurate and Simple Screening Method for Spinal Muscular Atrophy: High-Resolution Melting Analysis Using Dried Blood Spots on Filter Paper

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Running title: SMA screening with HRMA using dried blood spots

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Declaration of Interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Summary

Background: Spinal muscular atrophy (SMA) is a common neuromuscular disorder caused by mutation of the survival of the motor neuron 1 (*SMN1*) gene. More than 95% of SMA patients carry a homozygous deletion of *SMN1*. SMA can be screened for by polymerase chain reaction and high-resolution melting analysis (PCR-HRMA) using DNA extracted from dried blood spots (DBSs) stored on filter paper. However, there are two major problems with this approach. One is frequent poor quality/quantity of DNA extracted from DBSs on filter paper. The other is the difficulty in designing primer sets or probes to separate allele-specific melting curves. In this study, we addressed these problems and established a rapid, accurate and simple screening system for SMA with PCR-HRMA using DNA extracted from DBSs on filter paper.

Methods: Seventy individuals were assayed in this study, 42 SMA patients and 28 controls, all of whom had been previously screened for SMA by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) using DNA extracted from freshly collected blood. In this study, the DNA of each individual was extracted from dried blood that had been spotted onto cards and stored at room temperature (20–25°C) for between 1 and 8 years. PCR amplification of 30 or 45 cycles was performed using 50 ng of DNA and was immediately followed by HRMA. *SMN1 and SMN2* products co-amplified using a previously designed primer set (R111 and 541C770) contain two single nucleotide differences.

Results: The absorbance ratio at 260/280 of DNA extracted from DBSs ranged from 1.49 to 2.1 (mean ± SD; 1.66 ± 0.12), suggesting high-purity DNA. Thirty cycles of PCR amplification were insufficient to amplify the target alleles; PCR with 45 cycles was, however, successful. PCR-HRMA using the R111/541C770 primer set enabled separation of the normalized melting curves of the samples with no *SMN1* from those with *SMN1* and *SMN2*.

Conclusions: DBSs on filter paper can be a good source of DNA for the diagnosis of diseases and PCR-HRMA using DNA extracted from DBSs is an alternative method to detect *SMN1* deletion. These findings suggest that the SMA screening system using

PCR-HRMA with DBSs on filter paper is practicable in a large population study over a long time period.

Keywords: Spinal muscular atrophy, *SMN1*, *SMN2*, high-resolution melting analysis, dried blood spot on filter paper

Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by the loss of lower motor neurons in the spinal cord. SMA is one of the most prevalent autosomal recessive disorders with an incidence of 1/6,000–10,000 births [1]. Mutations in the survival of the motor neuron 1 gene (*SMN1*) located on chromosome 5q13 are the most common cause of SMA [2]. *SMN1* encodes the SMN protein, which plays a critical role in cellular metabolism.

More than 95% of SMA patients are homozygous for *SMN1* deletion [1]. The remaining SMA patients carry subtle intragenic mutations in *SMN1* [1]. In every SMA patient with a homozygous deletion of *SMN1*, at least one copy of the *SMN2* gene is retained. *SMN2* is a highly homologous gene to *SMN1* [2], and produces small amounts of SMN [3]. There are only five nucleotide differences between *SMN1* and *SMN2* [1].

SMA is recognized as an incurable disease, but there are now promising therapeutic candidates from recent clinical trials [1]. Researchers and clinicians have just started to investigate the possibilities of mass screening for SMA according to ELSI (Ethical, Legal and Social Implications) and mutation-screening techniques [1].

The most efficient strategy for SMA screening is to detect homozygous *SMN1* deletion in the neonatal period. However, the presence of *SMN2* hampers the detection of homozygous *SMN1* deletion using conventional PCR. To differentially amplify *SMN1* and *SMN2*, various unique PCR methods have been established, including single-strand conformation polymorphism (SSCP) of PCR products [2], restriction fragment length polymorphism (RFLP) of PCR products [4], competitive PCR [5], denaturing high-performance liquid chromatography (DHPLC) of PCR products [6], real-time PCR [7], multiplex ligation probe amplification (MLPA) [8] and tetra-primer PCR [9].

High-resolution melting analysis of PCR products (PCR-HRMA) using dried blood spots (DBSs) is considered to be a promising method for SMA screening in a large population [1]. However, there are two major problems with this approach. One is poor quality and/or quantity of the DNA extracted from DBSs (DBS-DNA) [10]. The

other is difficulty in designing primer sets or probes to separate allele-specific melting curves [11-14].

We overcame these problems, and established a rapid, accurate and simple screening system for SMA with PCR-HRMA using DBS-DNA on filter paper. Our method can be applied to mass screening in a large population. In this study, we also evaluated the diagnostic accuracy of this method (the primary outcome measure) and the life length of DBSs with PCR availability (the secondary outcome measure).

Materials and Methods

Subjects

Seventy individuals were assayed in this study, 42 SMA patients and 28 controls. All the individuals had been previously analyzed by PCR-RFLP using DNA extracted from freshly collected blood [4]. Forty one patients were homozygous for deletion of *SMN1* allele, while one patient was compound heterozygous for deletion of one *SMN1* allele and intragenic mutation in the retained *SMN1* allele. Prior to analysis, informed consent was obtained from the study participants. The study was approved by the Ethical Committee of Kobe University Graduate School of Medicine.

DNA extraction from dried blood spots on filter paper

Blood samples were collected from SMA patients and controls using FTA® Elute Cards (Thermo Fisher Scientific, Waltham, MA, USA). Storage periods of blood samples on the FTA® Elute Cards varied from 1 to 8 years. All FTA® Elute Cards were stored in the dark at room temperature (20–25°C). To extract DNA from the dried blood spot on the card, seven 3-mm-diameter circles were punched out from the 11-mm-diameter dried blood spot using a hole-punch. The detailed procedures to extract DNA from the punched circles are described in our previous paper [15]. The extracted DNA was stored in TE buffer at −20°C until use. The concentration and absorbance ratio of the DNA solution at 260/280 were measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific).

PCR-HRMA to test for SMN1 deletion

The PCR-HRMA equipment in this study was LightCycler®480 System II (Roche Diagnostics GmbH, Mannheim, Germany) provided with LightCycler®480 Gene Scanning Software (Roche Diagnostic).

PCR-HRMA was carried out in 10 mL final volume containing 1 μL of template DNA (50 ng) and 5 μL of Light-Cycler®480 High Resolution Melting Master (Roche Diagnostics). The concentrations of each primer and $MgCl_2$ in the reagent mixture are 0.3 mM and 3 mM, respectively. The Lefebvre's primers, R111 [2] and 541C770 [2],

were used in this study (Figure 1). These primers amplified *SMN1* and *SMN2*-specific fragments. The amplified fragments contain two single nucleotide differences, one in intron 6 (G in *SMN1*/A in *SMN2*) and one in exon 7 (C in *SMN1*/T in *SMN2*).

The PCR program consists of an initial denaturation–activation step at 95°C for 10 min, followed by a 30- or 45-cycle program (denaturation at 95°C for 10 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 30 sec).

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 the rate of 25 data acquisition per °C. The melting curves obtained in the program were first normalized, and then shifted along the temperature, and finally a difference plot was generated.

Results

Concentration and absorbance ratio of extracted DNA

The average amount of DNA extracted from an 11-mm-diameter DBS, equivalent to 50 μl of whole blood, was 19,980 ± 7,517 ng (mean ± SD). The absorbance ratio at 260/280 ranged from 1.49 to 2.1 (1.66 ± 0.12), suggesting good purity of DNA. PCR with 30 cycles using 50 ng of DNA failed to amplify the target alleles; however, PCR with 45 cycles and 50 ng of DNA was successful (Figure 2). Conversely, PCR with 30 cycles using 50 ng of DNA extracted from freshly collected blood using a commercially available kit (Sanko Junyaku, Tokyo, Japan) was successful in amplifying the target sequence (data not shown). The difference in amplification efficiency between the "DBSs on filter paper stored for a long time" and "freshly collected blood" may reflect DNA quality.

Among the 70 DNA samples, only one sample from a patient with homozygous deletion of *SMN1* (* in Figure 2) could not be amplified by 45 cycle PCR using 50 ng of DNA. The DBS had been stored on filter paper for 4 years. The amount of DNA extracted from the 11-mm-diameter DBS was 31,065 ng, and the absorbance ratio at