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Spinal Muscular Atrophy: New Screening System with Real-Time mCOP-PCR and PCR-RFLP for *SMN1* Deletion

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BACKGROUND: Spinal Muscular Atrophy (SMA) is a common autosomal recessive neuromuscular disorder characterized by degeneration or loss of lower motor neurons. More than 95% of SMA patients show homozygous deletion for the survival motor neuron 1 (*SMN1*) gene. For the screening of *SMN1* deletion, it is necessary to differentiate *SMN1* from its highly homologous gene, *SMN2*. We developed a modified competitive oligonucleotide priming-PCR (mCOP-PCR) method using dried blood spot (DBS)-DNA, in which *SMN1* and *SMN2*-specific PCR products are detected with gel-electrophoresis. Next, we added a targeted pre-amplification step prior to the mCOP-PCR step, to avoid unexpected, non-specific amplification. The pre-amplification step enabled us to combine mCOP-PCR and real-time PCR. In this study, we combined real-time mCOP-PCR and PCR-restriction fragment length polymorphism (PCR-RFLP) to develop a new screening system for detection of *SMN1* deletion. METHODS: DBS samples of the subjects were stored at room temperature for a period of less than one year. Each subject had already been genotyped by the first PCR-RFLP using fresh blood DNA. *SMN1/SMN2* exon 7 was collectively amplified using conventional PCR (targeted pre-amplification), the products of which were then used as a template in the real-time PCR with mCOP-primer sets. To confirm the results, the pre-amplified products were subject to the second PCR-RFLP. RESULTS: The real-time mCOP-PCR separately amplified *SMN1* and *SMN2* exon7, and clearly demonstrated *SMN1* deletion in an SMA patient. The results of the real-time mCOP-PCR using DBS-DNA were completely consistent with those of the first and second PCR-RFLP analysis. CONCLUSION: In our new system for detection of *SMN1* deletion, real-time mCOP-PCR rapidly proved the presence or absence of *SMN1* and *SMN2*, and the results were easily tested by PCR-RFLP. This solid genotyping system will be useful for SMA screening.

INTRODUCTION

Spinal Muscular Atrophy (SMA) is a common autosomal recessive neuromuscular disorder with a prevalence of 1 in 10,000 newborns [8]. It demonstrates loss of spinal motor neurons due to muscle weakness and progressive loss of motor function. SMA is caused by the Survival Motor Neuron (*SMN*) gene that was genetically mapped to chromosome 5q11 [2, 7]. *SMN* exists in two nearly identical copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [6].

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SMN1, which produces the SMN protein (SMN), is present in all healthy individuals. However, more than 95% of SMA patients show homozygous *SMN1* deletion, while the remaining patients harbor some deleterious mutations in *SMN1* [6].

Although *SMN2*, a gene highly homologous to *SMN1*, was previously considered to be dispensable because 5% of healthy individuals carry the gene, it has been shown that *SMN2* produces small amount of SMN and that it modifies the SMA phenotype [3]. A higher copy number of *SMN2* can partially compensate for the lack of *SMN1* [4]. Therefore, *SMN1* is a responsible gene for SMA, and *SMN2* functions as an SMA-modifying gene.

For the screening of *SMN1* deletion, it is necessary to differentiate *SMN1* from *SMN2*. To differentiate *SMN1* from *SMN2*, van der Steege et al. developed a PCR-restriction fragment length polymorphism (PCR-RFLP) with a mismatched-primer [10]. This method is very solid, and it has been widely used in many countries.

In 2014, we developed a new technology using dried blood spot (DBS)-DNA, a modified competitive oligonucleotide priming-PCR (mCOP-PCR) method [5]. In this method, *SMN1* and *SMN2*-specific PCR products are detected with gel-electrophoresis. Then, we added a targeted pre-amplification step prior to the mCOP-PCR step, to overcome the poor quality and quantity problems of DBS-DNA [1] and to avoid unexpected, non-specific amplification [9]. We also noticed that pre-amplification products with a primer set of PCR-RFLP can be directly subjected to an enzyme digestion [9].

In this study, we developed a more improved screening system for homozygous *SMN1* deletion. Here, we combined two technologies, (1) real-time mCOP-PCR and (2) PCR-RFLP. Pre-amplification with a primer set for PCR-RFLP enabled us to easily confirm the mCOP-PCR results by PCR-RFLP, as the pre-amplification product was directly digested by the restriction enzyme.

MATERIAL AND METHODS

DNA sample preparation

DNA samples were extracted from three individuals (two controls and one SMA patient) from a dried blood spot (DBS) on filter paper by the method of Kato *et al*. [5]. Each individual had been genotyped by PCR-RFLP using fresh blood DNA. Prior to analyses, informed consent was obtained from the patients' families. The study was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

Targeted pre-amplification

Targeted pre-amplification of the sequence containing *SMN1/2* exon 7 was performed by conventional PCR using GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Foster City, CA, USA). Two µl of template solution (200~300 ng DNA from DBS) was directly added to PCR mixture (total volume, 28 µl) containing 1× PCR buffer [final concentration], 2 mM $MgCl_2$ [final concentration], 0.1 mM of each dNTP, 0.3 µM of each primer (R111 and X7-Dra), and 1.0 U FastStart Taq DNA polymerase (Roche Applied Science, Mannheim, Germany). The primer sequences are shown in Table I and Figure 1. The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min; (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. Following this, an aliquot of pre-amplified product was electrophoresed on a 4% agarose gel in 1× TBE buffer, and visualized by Midori-Green Advance staining (Nippon Genetics, Tokyo, Japan).

Table I. Sequences of forward and reverse primers used in this study

Steps	Forward primers	Reverse primers
Pre-amplification/ PCR-RFLP	R111: AGACTATCAACTTAATTTCTGATCA	X7-Dra: CCTTCCTTCTTTTTGATTTTGTTT
mCOP-PCR	R111: AGACTATCAACTTAATTTCTGATCA	SMN1-COP: TGTCTGAAACC SMN2-COP: TTGTCTAAAACC

For pre-amplification and PCR-RFLP, R111 and X7-Dra were used. For mCOP-PCR, R111 was used in combination with either SMN1-COP or SMN2-COP.

Real-time mCOP-PCR

The real-time mCOP-PCR was performed using the Light Cycler® 96 Real-time PCR system (Roche Molecular Systems, Inc). After 100-fold dilution of pre-amplified product, 4 µl diluted solution was added to the PCR mixture (final volume, 30 µl) containing 1 × PCR buffer [final concentration], 2 mM $MgCl_2$ [final concentration], 0.1 mM of each dNTP, 0.3 µM of common forward primer (R111), 0.3 µM of gene-specific

reverse primer (SMN1-COP or SMN2-COP), 1.0 U FastStart Taq DNA polymerase and 1.5 µl of 20× EvaGreen® Dye (Biotium, Hayward, CA, USA). The primer sequences are shown in Table 1 and Figure 1. The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 30 or 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 1 min; (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. The cycle number was 30 for *SMN1*, and 40 for *SMN2*.

PCR-RFLP

DraI site was introduced into *SMN2* product during the targeted pre-amplification step (Figure 1). The pre-amplified *SMN2* product was digested by overnight incubation with *DraI*. More specifically, 8 µl of pre-amplified products was added to the enzyme solution (final volume, 20 µl) containing 1 × buffer M [final concentration] and 1 µl of *DraI* (15 U/µl) (Takara Biomedicals, Shiga, Japan), and the mixture was incubated at 37°C overnight. Then, an aliquot of digested pre-amplified product was electrophoresed on a 4% agarose gel in 1×TBE buffer, and visualized by Midori-Green Advance staining (Nippon Genetics). After *DraI* digestion, the pre-amplified *SMN2* product (187 bp) generated two fragments of 163 bp and 24 bp, while the pre-amplified *SMN1* product (187 bp) did not undergo *DraI* digestion and remained the same size as the non-digested one.

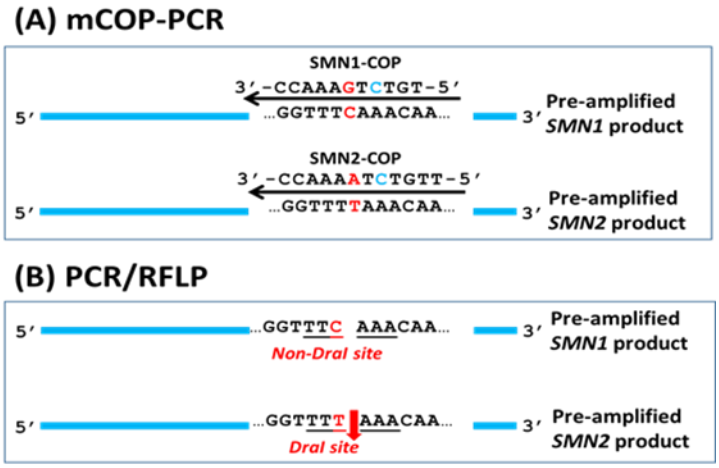

Figure 1.
Schematic representation of the pre-amplified PCR product of *SMN1* and *SMN2*
(A) mCOP-PCR: Annealing site of the gene-specific mCOP-PCR primers of *SMN1* or *SMN2*. An additional mismatched nucleotide between mCOP-PCR primers and pre-amplified product is shown in blue. Matched nucleotides to the gene-specific nucleotides are shown in red. (B) PCR-RFLP: A DraI site is introduced in the sequence of *SMN2* exon 7 after pre-amplification.

RESULTS

Real-time mCOP-PCR

For *SMN1* amplification in a real-time mCOP-PCR (Figure 2A), the quantification cycle (Cq) values for *SMN1* positive samples [*SMN1* (+) / *SMN2* (+) *and SMN1* (+) / *SMN2* (-)] were less than 23 (Figure 2A). However, the Cq value for *SMN1* negative sample [*SMN1* (-) / *SMN2* (+)] could not be obtained, because no apparent elevation of amplification curve was observed prior to 30 cycles. Thus, the presence or absence of *SMN1* was unambiguously detected in spite of the presence of *SMN2*.

For *SMN2* amplification (Figure 2A), the quantification cycle (Cq) values for *SMN2* positive samples [*SMN1* (+) / *SMN2* (+) *and SMN1* (-) / *SMN2* (+)] were less than 35 (Figure 2A). However, the Cq value for *SMN2* negative sample [*SMN1* (+) / *SMN2* (-)] could not be obtained, because no apparent elevation of amplification curve was observed prior to 40 cycles. Thus, the presence or absence of *SMN2* was unambiguously detected in spite of the presence of *SMN2*.

The results of real-time mCOP-PCR were completely consistent with the first PCR-RFLP, which had been performed in an earlier experiment.

PCR-RFLP

For the purpose of confirming the results of real-time mCOP-PCR, the pre-amplified product of each DNA sample was digested by overnight incubation with *DraI* (the second PCR-RFLP). The pre-amplified product derived from *SMN2* had a restriction enzyme site of *DraI* (TTT AAA) and was digested by *DraI*, while the pre-amplified product derived from *SMN1* did not have a *DraI* site and was not digested by *DraI* (Figure 1B).

As shown in Figure 2B, the first DNA sample with a genotype of [*SMN1* (+) / *SMN2* (+)] presented a non-digested band and a digested band, indicating the presence of both *SMN1* and *SMN2*. The second DNA sample with a genotype of [*SMN1* (+) / *SMN2* (-)] presented only a non-digested band but no digested band, indicating the presence of *SMN1* and the absence of *SMN2*. The third DNA sample with a genotype of [*SMN1* (-) / *SMN2* (+)] presented only a digested band but no non-digested band, indicating the presence of *SMN2* and the

absence of *SMN1*. The findings of the second PCR-RFLP were completely consistent with the results of real-time mCOP-PCR.

DISCUSSION

Combination of real-time mCOP-PCR and PCR-RFLP

In this study, we developed a new screening system for homozygous *SMN1* deletion. Our new system consists of real-time mCOP-PCR and PCR-RFLP. According to our previous observation [1], real-time mCOP-PCR is a very rapid, but at the same time, it is well enough equipped to distinguish *SMN1* and *SMN2*, as long as the test samples are preserved from contamination of other samples' DNA.

However, it should be considered that low-level contamination can preclude us from misdiagnosis, because our real-time mCOP-PCR method is very sensitive. According to our experience, PCR-RFLP is time-consuming and less sensitive compared to real-time mCOP-PCR, but low-level contamination may not affect the diagnosis. Thus, we developed the idea that PCR-PFLP could check the ambiguous results of real-time mCOP-PCR.

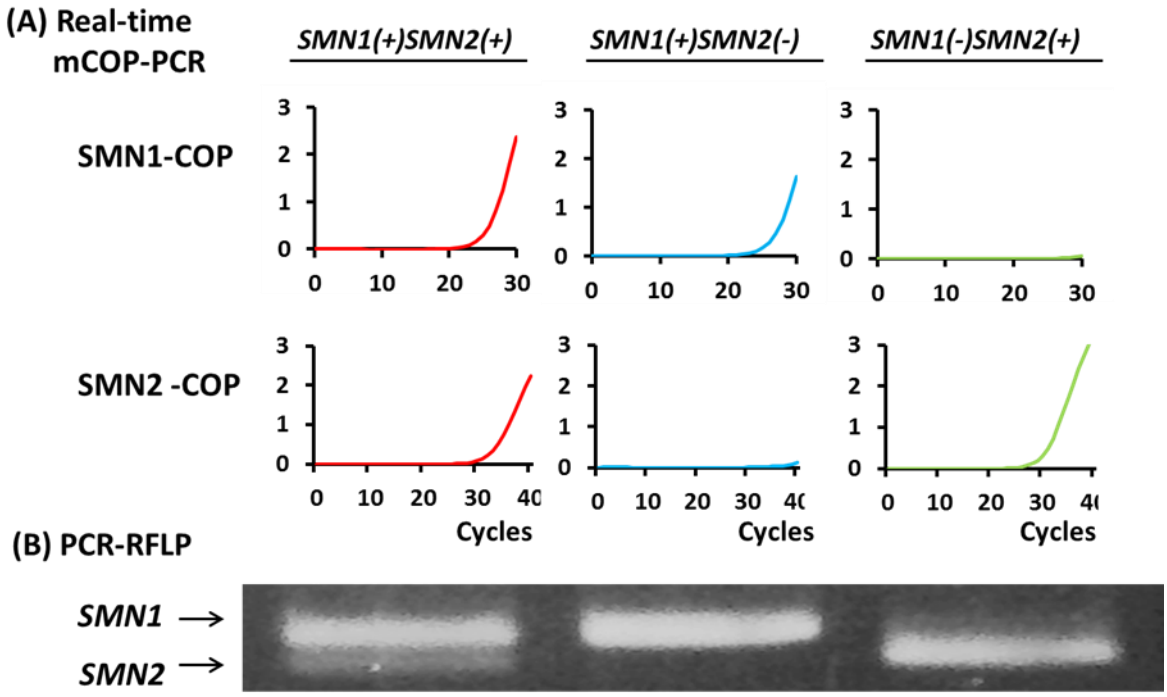

Figure 2. Real-time mCOP-PCR and PCR-RFLP analyses of pre-amplified products.
(A) Real-time mCOP-PCR analysis. The samples with [*SMN1 (+) / SMN2 (+)]* showed amplification with SMN1-COP and SMN2-COP. The sample with [*SMN1 (+) / SMN2 (-]* showed amplification with SMN1-COP, but no amplification with SMN2-COP. The sample with [*SMN1* (-) / *SMN2* (+] showed amplification with SMN2-COP, but no amplification with SMN1-COP. (B) PCR-RFLP analysis. Two bands of *SMN1* exon 7 and *SMN2* exon 7 were present in the sample of *[SMN1 (+) / SMN2 (+)]*. However, the band of *SMN2* exon 7 was absent in the sample with *[SMN1 (+) / SMN2 (-)]*, whereas the band of *SMN1* exon 7 was absent in the sample with *[SMN1 (-) / SMN2 (+)]*.

Pre-amplification with a primer set for PCR-RFLP

When DBS is used as a DNA source, a pre-amplification step is essential in real-time mCOP-PCR, because it overcomes the poor quality and quantity problems of DBS-DNA [1] and prevents unexpected non-specific PCR amplification [9]. Here, we arrived at the second idea: pre-amplification with a primer set for PCR-RFLP, which enabled us to check the results of real-time mCOP-PCR easily, as direct digestion of the pre-amplification product completes PCR-RFLP (the second PCR-RFLP).

Each participant in this study had been genotyped by PCR-RFLP using fresh blood DNA (the first PCR-RFLP). Our data showed that the results of two PCR-RFLP experiments, one with DBS and another with fresh blood, were fully consistent.

Gene-specific COP-primers with an additional mismatched nucleotide

In this study, the pre-amplified product was generated with a reverse primer, X7-Dra. X7-Dra was a mismatched primer which introduced a *DraI* site into the sequence of *SMN2* exon 7. Thus, the pre-amplified product contained a mismatched nucleotide with the genuine sequence of *SMN1* and *SMN2*. Consequently, the gene-specific mCOP-PCR primers, SMN1-COP and SMN2-COP, contained an additional mismatched nucleotide to the sequence of pre-amplified product.

As shown in Figure 1, SMN1-COP primer contained a mismatched nucleotide (C) with the sequence of pre-amplified *SMN1* product, therefore it contained two mismatched nucleotides (G, C) with the sequence of pre-amplified *SMN2* product. Similarly, SMN2-COP primer contained a mismatched nucleotide (C) with the sequence of pre-amplified *SMN2* product, thus containing two mismatched nucleotides (A, C) with the sequence of pre-amplified *SMN1* product.

Surprisingly, our experimental results showed that SMN1-COP and SMN2-COP did not lose gene-specificity in spite of the presence of a mismatched nucleotide. However, two or more additional mismatched nucleotides and/or the positions of the mismatched nucleotides in the COP-primers may affect the gene-specificity.

Conclusion

In our new screening system for detection of *SMN1* deletion, real-time mCOP-PCR rapidly proved the presence or absence of *SMN1* and *SMN2*, and the results were easily tested by PCR-RFLP. This solid genotyping system will be useful for SMA screening.

DECLARATION OF INTEREST

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

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