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 exon 7, 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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SMA SCREENING WITH MCOP-PCR USING DRIED BLOOD SPOTS

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. 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 MgCl2 [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

<table>
<thead>
<tr>
<th>Steps</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tbody>
<tr>
<td>Pre-amplification/</td>
<td>R111: AGACTATCAACTTAAATTTCGATCA</td>
<td>X7-Dra: CCTTCCCTCTTGGATTTGT</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td></td>
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</tr>
<tr>
<td>mCOP-PCR</td>
<td>R111: AGACTATCAACTTAAATTTCGATCA</td>
<td>SMN1-COP: TGATGAAACC</td>
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<tr>
<td></td>
<td></td>
<td>SMN2-COP: TTGCTGAAACC</td>
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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 MgCl2 [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 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 Bimomedicals, 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.

**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-RFLP 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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REFERENCES