New, Improved Version of the mCOP-PCR Screening System for Detection of Spinal Muscular Atrophy Gene (SMN1) Deletion

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BACKGROUND: Spinal muscular atrophy (SMA) is a frequent autosomal recessive disorder, characterized by lower motor neuron loss in the spinal cord. More than 95% of SMA patients show homozygous survival motor neuron 1 (SMN1) deletion. We previously developed a screening system for SMN1 deletion based on a modified competitive oligonucleotide priming-PCR (mCOP-PCR) technique. However, non-specific amplification products were observed with mCOP-PCR, which might lead to erroneous interpretation of the screening results. AIM: To establish an improved version of the mCOP-PCR screening system without non-specific amplification. METHODS: DNA samples were assayed using a new version of the mCOP-PCR screening system. DNA samples had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP), showing the presence or absence of SMN1 exon 7. The new mCOP-PCR method contained a targeted pre-amplification step of the region, including an SMN1-specific nucleotide, prior to the mCOP-PCR step. mCOP-PCR products were electrophoresed on agarose gels. RESULTS: No non-specific amplification products were detected in electrophoresis gels with the new mCOP-PCR screening system. CONCLUSION: An additional targeted pre-amplification step eliminated non-specific amplification from mCOP-PCR screening.

INTRODUCTION

Spinal muscular atrophy (SMA) is a frequent autosomal recessive disorder with an incidence of 1/10,000 live births [11]. The SMA locus has been mapped to chromosome 5q13 using linkage analysis [1,9], and is divided into telomeric and centromeric parts because of the presence of a large inverted duplication in the chromosome [7,8].

In 1995, the survival motor neuron gene (SMN) was reported as a candidate gene for SMA [7]. SMN exists in two nearly identical copies, SMN1 (the telomeric copy) and SMN2 (the centromeric copy) [7]. SMN1 is present in all healthy individuals, but absent from more than 95% of SMA patients and deleteriously mutated in the remaining patients [7]. The gene product, SMN, may be an essential protein in distinct cellular functions including heterogeneous nuclear ribonucleoprotein biogenesis (a ubiquitous function) and axonal transport (a motor neuron-specific function) [10]. Thus, SMN1 is now considered as an SMA-causing gene.

On the other hand, SMN2 was previously considered to be dispensable because approximately 5% of normal individuals do not carry the gene [7]. However, SMN2 also expresses SMN [7], albeit at considerably lower levels than SMN1. Thus, SMA is caused by low SMN levels rather than the complete absence of SMN [2,12]. It was shown that a high copy number of SMN2 can partially compensate for the lack of SMN1 [3,15]. Therefore, SMN2 is also now considered to be an SMA-modifying gene.

Complete loss of both SMN genes may cause embryonic lethality, therefore all SMA patients with homozygous SMN1 deletions carry at least one copy of the SMN2 gene [5,13]. SMN2 is highly homologous to SMN1, with only five nucleotide differences between them [7]. However, high similarity with SMN2 hampers detection of homozygous SMN1 deletions by conventional PCR methods.
To address this problem, we developed a screening system for homozygous SMN1 deletion based on a competitive oligonucleotide priming-PCR (COP-PCR) technique, which separately amplifies SMN1 exon 7 and SMN2 exon 7 [6]. We previously used the term “COP-PCR” for this method, in spite of some modifications added to the original COP-PCR method [4]. We have decided now to use the term “modified competitive oligonucleotide priming-PCR (mCOP-PCR)” in this study, and hereafter, to refer to the method.

Although mCOP-PCR separately amplified SMN1 exon 7 and SMN2 exon 7, we were also aware that non-specific amplification products of unexpected sizes often appeared in mCOP-PCR. It might lead to erroneous interpretation of screening results. In this study, we established a new, improved mCOP-PCR screening system, without non-specific amplification, to avoid any ambiguity of the results.

MATERIAL AND METHODS

Patient and control samples

Two DNA samples were assayed from each of three individuals (two controls and one SMA patient). One DNA sample was extracted from fresh whole blood by the SepaGene agglutination partition method (EIDIA, Tokyo, Japan). The other sample was extracted from a dried blood spot on filter paper by the method of Kato et al. [6]. The individuals had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP), showing the presence or absence of SMN1 exon 7. Prior to analysis, informed consent was obtained from study participants. The study was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

Outline of the new mCOP-PCR screening system

The new mCOP-PCR screening system included the following steps: (1) preparation of DNA samples; (2) targeted pre-amplification of sequence containing SMN1/SMN2 exon 7 by conventional PCR to secure the quality and quantity of each DNA sample; and (3) gene-specific amplification of SMN1/SMN2 exon 7 using mCOP-PCR. PCR products were electrophoresed on 4% agarose gels. The primer locations and sequences used in each PCR step are shown in Figure 1.

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 (equivalent to 100 ng DNA from fresh blood or 200–300 ng DNA from a dried blood spot) was directly added to PCR mixture (total volume, 28 μl) containing 1× PCR buffer, 2 mM MgCl2, 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 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 56°C for 1 min, and extension at 72°C for 1 min (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA); (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. Afterwards, an aliquot of pre-amplified product was electrophoresed on a 4% agarose gel in 1× TBE buffer, and visualized by Midori-Green staining (Nippon Genetics, Tokyo, Japan).
Gene-specific amplification of SMN1 and SMN2 exon 7 by mCOP-PCR

mCOP-PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific). After 100-fold dilution of pre-amplified product, 2 µl diluted solution was added to PCR mixture (total volume, 28 µl) containing 1× PCR buffer, 2 mM MgCl₂, 0.1 mM of each dNTP, 0.3 µM of common forward primer (R111), 3 µM of gene-specific reverse primer (SMN1-COP or SMN2-COP), and 1.0 U FastStart Taq DNA polymerase. The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 20 cycles of denaturation at 94°C for 1 min, annealing at 35°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. Afterwards, an aliquot of each PCR product was electrophoresed on a 4% agarose gel in 1× TBE buffer, and visualized by Midori-Green staining.

RESULTS

We performed the old and new versions of mCOP-PCR analysis using 100 ng fresh blood DNA or 200–300 ng dried blood spot DNA. The old version included 30 or 40 cycles of mCOP-PCR (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA) without targeted pre-amplification, while the new version included 30 or 40 cycles of conventional PCR for targeted pre-amplification (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA) followed by 20 cycles of mCOP-PCR.

We succeeded in gene-specific amplification of SMN1 exon 7 and/or SMN2 exon 7 from all three individuals using fresh blood DNA and dried blood spot DNA, with or without the targeted pre-amplification step (Figure 2B). The target amplification products were easily identified by their size (or band location in agarose gels after electrophoresis). PCR product sizes of SMN1 exon 7 and SMN2 exon 7 were 168 bp and 169 bp, respectively. The results of mCOP-PCR analysis were fully compatible with genotyping analysis data obtained by PCR-RFLP.

Without the targeted pre-amplification step, non-specific amplification products were observed in fresh blood DNA, with many detected in dried blood spot DNA samples. Indeed, the number and intensity of non-specific amplification products were so high in dried blood spot DNA samples that the reliability of any potential results would be hampered. In contrast, when mCOP-PCR was performed with targeted pre-amplification, these non-specific amplification products were absent in both fresh blood and dried blood spot DNA samples.

DISCUSSION

Here, we compared a new version of mCOP-PCR with a targeted pre-amplification step to the old version without a targeted pre-amplification step. The old version of mCOP-PCR screening system has been described previously [6]. In comparison, the new version simply incorporates a targeted pre-amplification step. Targeted pre-amplification was so efficient that non-specific amplification was eliminated. Consequently, interpretation of mCOP-PCR results becomes simplified and straightforward.
Frequent appearance of non-specific amplification products in the old mCOP-PCR screening system may be related to DNA quality. This is supported by the fact that non-specific amplification products were more abundant in dried blood spot DNA samples than fresh blood DNA samples. The quality of DNA extracted from dried blood spots on filter paper is poorer than DNA extracted from fresh blood, and this disparity becomes larger with a longer storage period. Addition of a targeted pre-amplification step prior to mCOP-PCR may overcome the poor quality of dried blood spot DNA.

The new mCOP-PCR screening system takes longer time compared with the old version (~ 4 hours vs. ~ 3 hours). However, the results obtained with the new version are unambiguous. In addition, the primers used in the targeted pre-amplification step, R111 and X7-Dra, are also used for PCR-RFLP analysis [14]. Accordingly, the pre-amplified products can be used directly for PCR-RFLP. The results of mCOP-PCR can be checked by PCR-RFLP, although an overnight incubation at 37°C is needed.

In conclusion, we have established an improved version of the mCOP-PCR screening system without non-specific amplification. An additional targeted pre-amplification step prior to mCOP-PCR succeeded in eliminating non-specific amplification in the mCOP-PCR screening system. Finally, it should be noted that although our screening system described here is very useful for the detection of SMN1-deletion, it is not for the detection of an intragenic mutation in SMN1. The absence of SMN1 deletion does not neglect the possibility of SMA caused by an intragenic mutation in SMN1.

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