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Original article

Genetic screening of spinal muscular atrophy using a real-time modified COP-PCR technique with dried blood-spot DNA

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Abstract

Background: Spinal muscular atrophy (SMA) is a common neuromuscular disorder caused by mutations in *SMN1*. More than 95% of SMA patients carry homozygous *SMN1* deletion. SMA is the leading genetic cause of infant death, and has been considered an incurable disease. However, a recent clinical trial with an antisense oligonucleotide drug has shown encouraging clinical efficacy. Thus, early and accurate detection of *SMN1* deletion may improve prognosis of many infantile SMA patients.

Methods: A total of 88 DNA samples (37 SMA patients, 12 carriers and 39 controls) from dried blood spots (DBS) on filter paper were analyzed. All participants had previously been screened for *SMN* genes 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 1 week to 5 years. To ensure sufficient quality and quantity of DNA samples, target sequences were pre-amplified by conventional PCR. Real-time modified competitive oligonucleotide priming-PCR (mCOP-PCR) with the pre-amplified PCR products was performed for the gene-specific amplification of *SMN1* and *SMN2* exon 7.

Results: Compared with PCR-RFLP using DNA from freshly collected blood, results from real-time mCOP-PCR using DBS-DNA for detection of *SMN1* exon 7 deletion showed a sensitivity of 1.00 (CI [0.87, 1.00])] and specificity of 1.00 (CI [0.90, 1.00]), respectively.

Conclusion: We combined DNA extraction from DBS on filter paper, pre-amplification of target DNA, and real-time mCOP-PCR to specifically detect *SMN1* and *SMN2* genes, thereby establishing a rapid, accurate, and high-throughput system for detecting *SMN1*-deletion with practical applications for newborn screening.

Keywords: Dried blood spot; Real-time mCOP-PCR; SMN1; SMN2; Spinal muscular atrophy

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1. Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disorder with an autosomal recessive inheritance trait. It is characterized by the degeneration of lower motor neurons resulting in muscle weakness and progressive loss of movement. SMA is estimated to be one of the most frequent genetic disorders, with an incidence of 1/10,000 live births [1]. SMA has been classified into three subtypes depending on the age of disease onset and the achievement of motor milestones [2]. The subtypes are: type 1 (the most severe form; age of onset 0–6 months, unable to sit unaided), type 2 (severe form; age of onset <18 months, unable to stand or walk unaided), and type 3 (mild form; age of onset >18 months, able to stand and walk unaided). Muscle weakness is associated with not only poor motor function, but also respiratory insufficiency. In particular, respiratory insufficiency in patients with SMA type 1 results in death or the requirement for permanent artificial ventilation before the age of 2 years [3]. Thus, SMA may be considered as the leading genetic cause of death in infants.

In 1995, the survival of motor neuron gene (*SMN*) on chromosome 5q 13 was identified as a candidate gene for SMA. *SMN* exists as two nearly identical copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [4]. *SMN1* is now recognized as an SMA-causing gene as it is homozygously deleted in more than 95% of SMA patients and is deleteriously mutated in the remaining patients [4]. On the other hand, *SMN2* was previously considered to be dispensable because approximately 5% of control individuals do not carry this gene. However, *SMN2* also expresses the same protein, SMN, as *SMN1* does, albeit at considerably lower levels than *SMN1*, and high copy numbers of *SMN2* may improve survival outcomes and maintenance of motor function [5–10]. Thus, *SMN2* is also now considered to be an SMA-modifying gene.

SMN1 and *SMN2* differ by only five nucleotides [4]. One of the five nucleotide differences is in the coding region at position +6 of exon 7; c.840C in *SMN1* and c.840T in *SMN2*. Although this C-to-T transition is translationally silent, it alters the mRNA splicing pattern [11]. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% exon7-lacking (Δ7) *SMN2* transcripts and 10% FL-*SMN2* transcripts [12]. However, a high copy number of *SMN2* can produce a large amount of FL-*SMN2* to at least partially compensate for the loss of *SMN1*.

SMA has been considered an incurable disease. However, improved understanding of *SMN* splicing mechanisms has spurred the development of therapeutic compounds that modify the splicing of *SMN2* exon 7 to increase SMN protein. In 2016, clinical trial results of intrathecal administration of an antisense oligonucleotide drug, nusinersen (SPINRAZA™, Biogen and Ionis Pharmaceuticals), were reported to demonstrate acceptable safety and tolerability and encouraging clinical efficacy [13]. This antisense oligonucleotide drug masks the splicing suppressor sequence in *SMN2* intron 6 and leads to inclusion of *SMN2* exon 7 into the transcript [14]. The development of a successful drug for SMA means that early diagnosis is now far more important than when SMA was thought to be incurable. A newborn screening (NBS) system for SMA may be essential for early diagnosis of the disease. Even before a specific therapy for SMA is available, an NBS test might allow patients to be enrolled in clinical trials before irreversible neuronal loss occurs and to give patients the choice of more proactive treatments [15].

However, there is *a priori* problem for genetic diagnosis of SMA by conventional molecular analyses: the presence of *SMN2* hampers easy and quick detection of homozygous *SMN1* deletion. To overcome this problem, many kinds of PCR technologies have been invented, for example, PCR and single-strand conformation polymorphism (PCR-SSCP) [4], PCR and restriction fragment length polymorphism (PCR-RFLP) [16], radio-isotope competitive PCR and RFLP [5], PCR and denaturing high-performance liquid chromatography (DHPLC) [17], real-time PCR with gene-specific primers, multiplex ligation probe amplification (MLPA) [18], tetra-primer PCR [19], and high-resolution melting analysis [20]. Regarding the NBS-related technology for *SMN1* deletion, Pyatt et al. and Taylor et al. combined dried blood spot (DBS) sampling and a real-time PCR technology with fluorescent oligonucleotide probe [21,22].

We also previously reported that modified competitive oligonucleotide priming-PCR (mCOP-PCR) technique, which was developed in our laboratory, can separately amplify *SMN1* and *SMN2* genes [23]. Then, to establish a simple but accurate *SMN1*-deletion detection system that has practical utility in any area of the world, we combined DNA extraction from DBS on filter paper (DBS-DNA) and mCOP-PCR technique using conventional PCR [24]. Using this system, we analyzed DBS-DNA samples from the filter papers delivered to our laboratory. However, we did not always obtain clear-cut results from the low-quantity DNA samples or poor-quality DNA samples extracted from DBS on filter paper. In addition, the system could not be used for the analyses of many specimens in a short time, because it needed time-consuming gel-electrophoresis after PCR.

In this study, to establish a rapid, accurate, and high-throughput *SMN1*-deletion detection system that can be applied to high throughput NBS for SMA, we developed a new system with target DNA pre-amplification followed by mCOP-PCR technique using real-time PCR. Our new system can detect *SMN1*-deletion even