# SMA Diagnosis: Detection of SMN1 Deletion with Real-Time mCOP-PCR System Using Fresh Blood DNA

Emma Tabe Eko Niba ; Rochmah, Mawaddah Ar ; Harahap, Nur Imma Fatimah ; Awano, Hiroyuki ; Morioka, Ichiro ; Iijima, Kazumoto ; Saito, Toshio ;…

(Citation)
The Kobe journal of the medical sciences,63(3):80-83

(Issue Date)
2017

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(JaLCDOI)
https://doi.org/10.24546/81010009

(URL)
https://hdl.handle.net/20.500.14094/81010009

Kobe J. Med. Sci., Vol. 63, No. 3, pp. E80-E83, 2017

# SMA Diagnosis: Detection of *SMN1* Deletion with Real-Time mCOP-PCR System Using Fresh Blood DNA

EMMA TABE EKO NIBA[1], MAWADDAH AR ROCHMAH[1], NUR IMMA FATIMAH HARAHAP[1], HIROYUKI AWANO[2], ICHIRO MORIOKA[2], KAZUMOTO IIJIMA[2], TOSHIO SAITO[3], KAYOKO SAITO[4], ATSUKO TAKEUCHI[5], POH SAN LAI[6], YOSHIHIRO BOUIKE[7], HISAHIDE NISHIO[1*] and MASAKAZU SHINOHARA[1]

[1] *Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan;*

[2] *Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan;*

[3] *Division of Child Neurology, Department of Neurology, National Hospital Organization Toneyama National Hospital, Toneyama, Japan;*

[4] *Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan;*

[5] *Kobe Pharmaceutical University, Kobe, Japan;*

[6] *Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore*

[7] *Faculty of Nutrition, Kobe Gakuin University, Kobe, Japan*

Received 27 July 2017/ Accepted 21 August 2017

**Keywords:** spinal muscular atrophy, *SMN1*, *SMN2*, mCOP-PCR, real-time PCR

**BACKGROUND: Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders. The symptoms are caused by defects of lower motor neurons in the spinal cord. More than 95% of SMA patients are homozygous for 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 using dried blood spot (DBS) on filter paper. This system is convenient for mass screening in the large population and/or first-tier diagnostic method of the patients in the remote areas. However, this system was still time-consuming and effort-taking, because it required pre-amplification procedure to avoid non-specific amplification and gel-electrophoresis to detect the presence or absence of *SMN1* deletion. When the fresh blood samples are used instead of DBS, or when the gel-electrophoresis is replaced by real-time PCR, we may have a simpler and more rapid diagnostic method for SMA. AIM: To establish a simpler and more rapid diagnostic method of *SMN1* deletion using fresh blood DNA. METHODS: DNA samples extracted from fresh blood and stored at 4 °C for 1 month. The samples were assayed using a real-time mCOP-PCR system without pre-amplification procedures. DNA samples had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP), showing the presence or absence of *SMN1* exon 7. The DNA samples were directly subjected to the mCOP-PCR step. The amplification of mCOP-PCR was monitored in a real-time PCR apparatus. RESULTS: The genotyping results of the real-time mCOP-PCR system using fresh blood DNA were completely matched with those of PCR-RFLP. In this real-time mCOP-PCR system using fresh blood-DNA, it took only four hours from extraction of DNA to detection of the presence or absence of *SMN1* deletion, while it took more than 12 hours in PCR-RFLP. CONCLUSION: Our real-time mCOP-PCR system using fresh blood DNA was rapid and accurate, suggesting it may be useful for the first-tier diagnostic method of SMA.**

## INTRODUCTION

Spinal Muscular Atrophy (SMA) is a common autosomal recessive neuromuscular disorder with an incidence of 1 in 10,000 newborns [9]. SMA patients show muscle weakness and progressive loss of motor function because of the defects in lower motor neurons. The severity of the disease varies from patient to patient [6], but infantile SMA is a leading genetic cause of infantile death [10].

In 1990, SMA locus was mapped to chromosome 5q11[1, 8]. In 1995, the survival motor neuron (*SMN*) gene was found in the SMA locus [7]. According to Lefebvre et al., *SMN* exists in two nearly identical copies, *SMN1* (telomeric copy) and *SMN2* (centromeric copy) [7]. Although *SMN1* is present in all healthy individuals, *SMN1*

Phone: +81-78-382-5540 Fax: +81-78-382-5559 E-mail: nishio@med.kobe-u.ac.jp

is absent in more than 95% of SMA patients. They are homozygous for *SMN1* deletion, and the rest may harbor some deleterious mutations in *SMN1* [7].

Contrarily, *SMN2* was previously considered to be dispensable because ~5% of normal individuals do not carry the gene [7]. But, now, *SMN2* is considered to be an SMA-modifying gene, because *SMN2* can also produce a small amount of SMN protein [2, 11], and a high copy number of *SMN2* is related to the milder phenotype of SMA [3]. It should also be noted that all SMA patients with a homozygous *SMN1* deletion carry at least one copy of the *SMN2* gene [12].

For the diagnosis of SMA, *SMN1* deletion test should come first. However, high similarity in the nucleotide sequence between *SMN1* and *SMN2* makes it difficult to detect *SMN1* deletion by conventional PCR methods. Therefore, a more advanced technique to separately amplify *SMN1* or *SMN2* has been requested.

In 2014, we developed a screening system for *SMN1* deletion using dried blood spot (DBS) on filter paper. This system was based on a modified competitive oligonucleotide priming-PCR (mCOP-PCR) technique, which separately amplified *SMN1* exon 7 and *SMN2* exon 7 [5]. But non-specific amplification products of unexpected sizes often appeared in mCOP-PCR, especially when using DBS on filter paper. To overcome this problem, we added a targeted pre-amplification step prior to the mCOP-PCR step [13].

The current form of mCOP-PCR screening system for *SMN1* deletion using DBS may be convenient for mass screening in the large population and/or first-tier diagnostic method of the patients in the remote areas. But we thought that the system was still time-consuming and effort-taking, because it required pre-amplification procedure to avoid non-specific amplification and gel-electrophoresis to detect the presence or absence of *SMN1* deletion. If the fresh blood samples could be used instead of DBS, and if the gel-electrophoresis could be replaced by real-time PCR, we would have a simpler and more rapid diagnostic method for SMA. In this study, we established a simpler and more rapid diagnostic method for *SMN1* deletion using fresh blood DNA.

## MATERIAL AND METHODS

### Patient and control samples

Twelve DNA samples from 8 controls and 4 SMA patients were assayed in this study. Each DNA sample was extracted from fresh whole blood by a DNA extraction kit, SepaGene (EIDIA, Tokyo, Japan). The samples had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) [14], 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.

### Gene-specific amplification of *SMN1* and *SMN2* exon 7 by mCOP-PCR

*SMN1*/*SMN2* specific amplification was performed by real-time PCR using the LightCycler® 96 system (Roche Applied Science). 50ng of DNA (in 2 μl of TE buffer) was added to PCR mixture (total volume, 30 μl) containing 1× PCR buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, 1.0 U Fast Start Taq DNA polymerase, and 1.5 μl of 20× EvaGreen® Dye (Biotium, Hayward, CA, USA). The sequences of the primers are as follows: R111 (5'-AGA CTA TCA ACT TAA TTT CTG ATC A-3'), SMN1-COP (5'-TGT CTG AAA CC-3') and SMN2-COP (5'-TTG TCT AAA ACC-3'). 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 37°C for 1 min, and extension at 72°C for 1 min; and (3) melting analysis. Fluorescence signals were detected at the end of each extension procedure. Melting curve analysis was performed after PCR amplification, with 10 sec of denaturation at 95°C, 1 min of renaturation at 60°C, and then melting, which consisted of a continuous fluorescence reading from 65°C to 97°C at the rate of five data acquisitions per °C.

## RESULTS

We succeeded in gene-specific amplification of *SMN1* exon 7 and/or *SMN2* exon 7 in real-time PCR using fresh blood DNA, without the targeted pre-amplification step. We analyzed 12 samples that were assigned to each of the three genotype groups. Group 1 contained four samples from four control individuals with *SMN1* (+) and *SMN2* (+). Group 2 contained four samples from four control individuals with *SMN1* (+) and *SMN2* (-). Group 3 contained four samples from four patients with *SMN1* (-) and *SMN2* (-).

As shown in Figure 1, real-time PCR with SMN1-COP primer efficiently amplified *SMN1* exon 7 in the samples from control individuals of Groups 1 and 2, but not in the patients of Group 3 samples. On the other hand, real-time PCR with an SMN2-COP primer efficiently amplified *SMN2* exon 7 in the samples from control individuals of Group 1 and the samples from patients of Group 3, but not in the samples from control individuals of Group 2.

The amplification efficiency of *SMN1* or *SMN2* was assessed from the quantification cycle (Cq) values. In this study, a Cq value of less than 32 was judged to indicate the presence of *SMN1* or *SMN2*. At this Cq value,

positive and negative amplification can be clearly distinguished. The results based on the Cq values were completely matched to the results of PCR-RFLP.

In addition, the total procedures of extraction of DNA to detection of the presence or absence of *SMN1* took only 4 hours, while it took more than 12 hours in PCR-RFLP using fresh blood DNA.

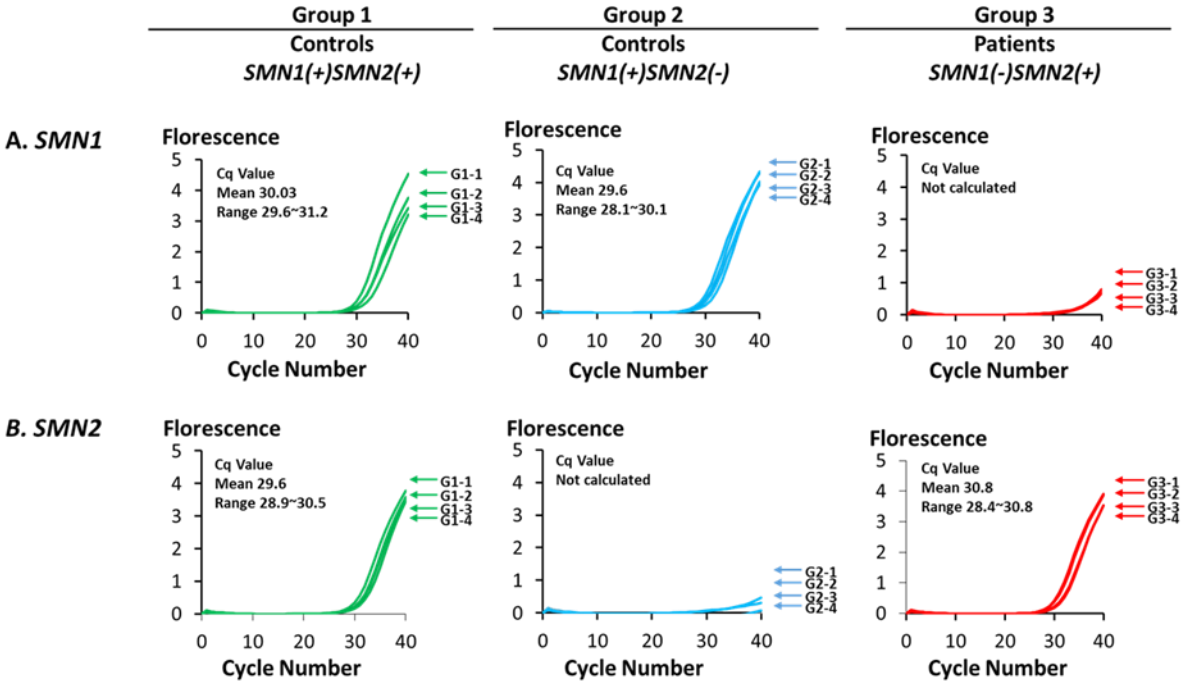


Figure 1. Selective amplification of *SMN1* and *SMN2* by real-time mCOP-PCR.
Gene-specific amplification of *SMN1*/ *SMN2* exon 7 by real-time mCOP-PCR. (A) Amplification curves of *SMN1* for the three different groups. The numbers shown next to each curve represents the sample number in each group. The samples with *SMN1*(+) showed significant amplification, while the samples with *SMN1*(-) showed no significant amplification. (B) Amplification curves of *SMN2* for the three different groups. The numbers shown next to each curve represents the sample number in each group. The samples with *SMN2*(+) showed significant amplification, while the samples with *SMN2*(-) showed no significant amplification.

## DISCUSSION

SMA has been considered an incurable disease. However, in 2016, clinical trial results of intrathecal administration of an antisense-oligo, nusinersen, demonstrated encouraging clinical efficacy of the drug [4]. We are now about to enter an era with the possibility that SMA can be treated and cured. Thus, diagnosis of SMA or detection of *SMN1*-deletion will become much more important in the near future. By foreseeing the future requirements, we have already engaged in the development of rapid and accurate *SMN1*-deletion detection system. In this study, we established a real-time mCOP-PCR system for detection of *SMN1* deletion using fresh blood DNA.

PCR-RFLP method, which was reported by van der Steege et al., 1995 has been widely used as the first-tier diagnostic method of SMA, but it is a time-consuming and effort-taking method, because it needs enzyme-digestion process and gel-electrophoresis. Compared with the conventional PCR-RFLP method, our new system is much more rapid: the genotyping results can be obtained in a short time.

It is difficult to discuss the superiority between the new real-time mCOP-PCR system in this study and the current form of mCOP-PCR system using DBS as a DNA source and gel-electrophoresis for the detection of amplified products [13]. The new mCOP-PCR system needs an expensive machine for real-time PCR, while the current form of mCOP-PCR system using DBS and gel-electrophoresis does not. However, the new system enables us to obtain the genotyping results in a shorter time. Replacement of DBS by fresh blood DNA can eliminate the pre-amplification step which is essential for preventing non-specific amplification. In addition, real-time PCR does not require gel-electrophoresis.

In conclusion, our real-time mCOP-PCR system using fresh blood DNA was rapid and accurate, suggesting it may be useful for the first-tier diagnostic method of SMA. To prove the practicability of our method, we are now planning to assay more than 100 samples including SMA patients.

## DECLARATION OF INTEREST

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

## ACKNOWLEDGMENTS

This research was supported in part by the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development, Grant No. 16ek0109086h0002 (title “Practical study for multicenter cooperative and investigator initiated clinical trial using valproic acid in childhood onset spinal muscular atrophy”).

## REFERENCES

1. **Brzustowicz, L.M., Lehner, T., Castilla, L.H., et al.** 1990. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. Nature **344**: 540-541.
2. **Burghes, A.H., and Beattie, C.E**. 2009. Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat Rev Neurosci. **10**: 597-609.
3. **Feldkötter, M., Schwarzer, V., Wirth, R., et al.**. 2002. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR; fast and highly reliable carrietest and prediction of spinal muscular atrophy. Am J Hum Genet **70**: 358-368.
4. **Finkel, R.S., Chiribaga, C.A., Vajsar, J., Day, J.W., Montes, J., De Vivo, D.C., et al.** 2016. Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. Lancet **388**:3017-26.
5. **Kato, N., Sa'Adah, N., Ar Rochmah, M., et al.** SMA screening system using dried blood spots on filter paper: application of COP-PCR to the SMN1 deletion test. 2014. Kobe J Med Sci. **60**: E78-85.
6. **Kolb, S.J., and Kissel, J.T.** 2011 Spinal muscular atrophy: a timely review. Arch Neurol **68**:979-84.
7. **Lefebvre, S., Bürglen, L., Reboullet, S., et al.** 1995. Identification and characterization of a Spinal Muscular Atrophy-determining gene. Cell **80**: 155-165.
8. **Melki, J., Abdelhak, S., Sheth, P., et al.** 1990. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. Nature **344**: 767-768.
9. **Nurputra, D.K., Lai, P.S., Harahap, N.I., et al**. 2013. Spinal Muscular Atrophy: From Gene Discovery to Clinical Trials. Ann Hum Genet. **77**: 435-463.
10. **Oskoui, M., Levy, G., Garland, C.J., et al.** 2007. The changing natural history of spinal muscular atrophy type 1. Neurology; **69**:1931-6.
11. **Prior, T.W.** 2011. Perspectives and diagnostic considerations in spinal muscular atrophy. Genet Med. **12**: 145-152.
12. **Schrank, B., Götz, R., Gunnersen, J.M., et al.** 1997. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci. **94**:9920-9925.
13. **Shinohara, M., Rochmah, A.M., Nakanishi, K., Harahap, N.I.F., Niba, E.T.E., et al.** 2017. New, Improved Version of the mCOP-PCR Screening System for Detection of Spinal Muscular Atrophy Gene (*SMN1*) Deletion. Kobe J Med Sci. **63**:E37-40
14. **Van der Steege, G., Grootscholten, P.M., Van der Vlies, P., et al.** 1995. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. The Lancet **345**: 985-986