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ORIGINAL RESEARCH ARTICLE

A rapid molecular diagnostic method for spinal muscular atrophy

Kai-Chen Wang[a,b], Chiao-Yuan Fang[c], Chi-Chang Chang[d], Chien-Kuan Chiang[d] and Yi-Wen Chen[c]

[a]Cheng-Hsin General Hospital, Taipei, Taiwan; [b]School of Medicine, National Yang Ming University, Taipei, Taiwan; [c]Cold Spring Biotech Corp, New Taipei City, Taiwan; [d]YuJian Biotech, Taipei, Taiwan

ABSTRACT

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder which has been considered as the second common cause of infant death, with an estimated prevalence of 1 in 10,000 live births. The disorder is caused by survival motor neuron 1 gene (*SMN1*) deficiency leading to limb weakness, difficult swallowing and abnormal breathing. Here, a fast and accurate method for SMA detection has been developed. Genomic DNA sample collected from whole blood, amniotic fluid, or dried blood spots can be analysed by using the Clarity™ Digital PCR (dPCR) System for determining the copy numbers of *SMN1* and *SMN2* genes. Two hundred and fourteen clinical samples determined by qPCR-based method were enrolled and used to establish the cut-off ranges for unaffected individual, SMA carrier and SMA patient categories. After setting the cut-off range for each group, 12 samples were analyzed by both dPCR-based method and MLPA (multiplex ligation-dependent probe amplification), the current testing golden standard for SMA, and 100% concordant results between the two testing methods were performed. CSB SMA Detection Kit combined with dPCR platform provides a robust and precise approach to distinguish unaffected individuals, SMA carrier and SMA patients. This rapid molecular diagnostic method can be adapted to pre-pregnancy eugenics inspection, prenatal testing as well as newborns screening and help physicians or genetic counselors to improve population SMA incidence.

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KEYWORDS
Digital PCR; SMA; SMN1; SMN2

Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disease which is considered as the second common cause of infant death, with an estimated prevalence of 1 in 10,000 live births (Pearn, 1978; Su *et al.*, 2011). This disorder is caused by deficiency in survival motor neuron 1 (SMN1) gene, leading to limb weakness, difficult swallowing and abnormal breathing. Two nearly identical genes SMN1 and SMN2, located on 5q13, differ by only five nucleotides. With a single nucleotide polymorphism (c.840C > T) in exon 7 of the SMN2 gene, SMN1 gene can encode functional SMN proteins, while SMN2 gene produces most of degraded proteins (Lefebvre *et al.*, 1995). Approximately 95% of SMA patients arise from homozygous absence of SMN1 gene, while 5% from *de novo* deletions or mutations. Absence of SMN1 gene leads to SMA, but an increase of SMN2 copy numbers can compensate SMN1 function partially (Ogino & Wilson, 2004). Therefore, though determining SMN1 gene copy number itself is crucial to classify patients as SMA affected, SMA carrier and normal group which lays the foundation for SMA genetic testing. On the hand, determining SMN2 copy number also plays an important role for SMA typing and therapeutic approaches.

Recently, carrier screening for SMA has been highly recommended by obstetrician in basic prenatal inspection because of the high carrier frequency (1/40–1/60) as well as the genetic risk of this disease. Generally, an unaffected individual has two or more copies of SMN1, SMA carrier possesses one copy which is symptom-free, and SMA patient lacks SMN1 gene. Mendel's law states that a 25% incidence rate would be expected to inherit SMA, if both parents were SMA carriers (Figure 1). Up to date, molecular diagnosis of SMA is performed using real-time PCR, denaturing high performance liquid chromatography (DHPLC), or multiplex ligation-dependent probe amplification (MLPA) mostly. But an important limitation of the current established assays is the need for parallel-run standard curve to align with the value of unknown sample so as to obtain exact copy numbers of SMN1 and SMN2 genes.

A relatively new technology called Digital PCR (dPCR)-based method offers a precise approach for quantitative target molecule without the need for standard curve. Our dPCR system which is called tube-in-tube JN Clarity dPCR system (Low, Chan, Soo, Ling, & Tan, 2017), enables the PCR reaction mixture to be evenly distributed into 10,000 or more partitions first and followed by PCR amplification. Sample partitioning can achieve 0 or 1 target nucleotide in each partition. By counting the number of positive partitions and negative partitions, the absolute DNA copies of the target molecule will be determined. In this study, we demonstrate and establish a novel SMA detection kit based on dPCR technology. The accuracy and feasibility of this assay is evaluated through comparison the quantitative results to

CONTACT Yi-Wen Chen ellen.chen@csbiotech.com.tw

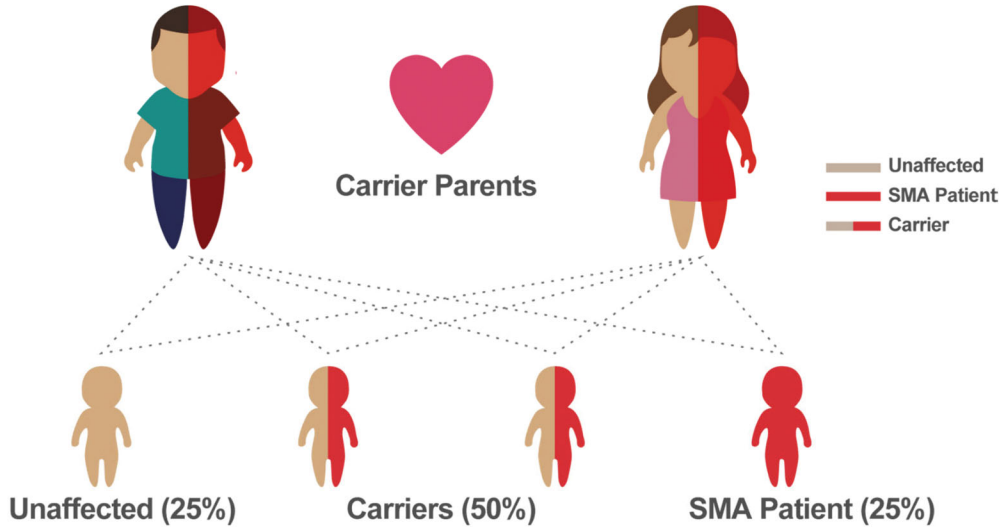

Figure 1. SMA inheritance by Mendel's laws. According to Mendel's law, a 25% chance that a child is a SMA patient is expected and 50% a SMA carrier given that both parents are SMA carrier.

another PCR-based High Resolution Melting (HRM) assay and MLPA assay.

Materials and methods

Sample collection and preparation

214 samples were collected from Dr. Wang. Genomic DNA (gDNA) was isolated from whole blood using TANBead® Blood DNA Auto Kit and Smart LabAssist-32 (Taiwan Advanced Nanotech Inc., Taiwan) following the manufacturer's instructions. For subsequent usage, gDNA concentration should be greater than 15 ng/µl. To cut long fragments of gDNA into pieces, gDNA was first digested into 5 ng/µl using EcoRI enzyme at 37 °C for 20 min and 80 °C for 5 min before PCR. This study was approved by the Institutional Research Board and carried out according to the Helsinki Declaration Principles. Written informed consent was collected from all participating subjects.

Detection of SMN1 and SMN2 genes

Both SMN1 and SMN2 genes were detected using a home-brew kit, CSB SMA Detection Kit (Cold Spring Biotech., Taiwan). Primers and hydrolysis probes (Integrated Device Technology, Inc.) were targeted at SMN1, SMN2 and ribonuclease RNA component H1 (RPPH1) as the reference gene. The two Locked Nucleic Acid (LNA) probes were designed to specifically recognize a single polymorphism (c.840C > T) at exon 7 of SMN1 and SMN2 genes. With two separate FAM-labelled SMN1 and SMN2 probes, reference gene RPPH1 labelled with HEX fluorescence was accompanied in each reaction. Each sample requires two reactions for detection of both SMN1 and SMN2 genes, respectively. PCR reaction mix per each was then prepared with Clarity™ Digital PCR Mastermix (JN Medsys, Singapore), 20X JN solution (JN Medsys, Singapore), 400 nM primers, 150 nM probes, and 15 ng digested-gDNA in a final volume of 15 µL.

Digital PCR analysis

CSB SMA Detection Kit was used with Clarity™ Digital PCR system (JN Medsys, Singapore). The prepared PCR reaction mix was first partitioned into Clarity™ Chip-in-a-Tube, which contains approximately 10,000 partitions per chip using Clarity™ Auto Loader. By this step, 0 or 1 targeted nucleotide was delivered into each partition. The partitions were than sealed by Clarity™ Sealing Enhancer, and Sealing fluid was added into tube. The Chip-in-a-Tube was then placed into PeqSTAR 2X Gradient Thermocycler (peqLAB, Germany) for PCR amplification. PCR was performed with initial denaturation at 95 °C for 5 min, followed by 40 cycles in two steps at 95 °C for 50 s and 63 °C for 90 s, and final extension at 70 °C for 5 min. After amplification, the fluorescent signals were detected using Clarity™ Reader. The exact numbers of DNA copies were analyzed by Clarity 2.1 software. As a result, the copy numbers of SMN1 and SMN2 genes were determined by normalization to the intra-reaction copies of reference gene (Low *et al.*, 2017).

High resolution melting (HRM) analysis

The flanking region of exon 7 in both SMN1 and SMN2 genes were amplified by PCR. The 20 µL PCR reaction mixture contained 5 µL gDNA and 15 µL RI-SMN Master Mix (MBGEN Biosciences, Taiwan) including 10 µM SMNQ primer and 10 µM internal control KIRT1 primer. The whole PCR condition and the melting analysis were described before (Wang *et al.*, 2015).

Multiplex ligation-dependent probe amplification (MLPA) assay

The MLPA assay was performed using SALSA MLPA P021 KIT (MRC-Holland, The Netherlands) following the manufacturer's instructions. The commercial kit contains 32 probes with 4 probes specific for sequences in exon 7 or 8 of either SMN1 or SMN2, 17 probes detecting sequences

Table 1. Cohen's kappa coefficient (κ) between HRM method and dPCR.

		Rater 1 (HRM)		
		SMA carrier	Unaffected	Row Marginals
Rater 2 (dPCR)	SMA carrier	23	0	23
	Unaffected	0	191	191
Column Marginals		23	191	214

presented in every exon of both SMN1 and SMN2 genes, and other reference probes (Huang *et al.*, 2007).

Results

Identical results between HRM method and dPCR

Two hundred and fourteen blood samples analyzed by high resolution melt (HRM) method were used for a parallel comparison with dPCR. The concordance between these two methods was presented by Cohen's kappa coefficient (κ). The result indicated that 23 SMA carriers diagnosed by HRM method were considered as SMA carriers by dPCR (Table 1). Further, 191 unaffected individuals were also determined as unaffected by dPCR. These analyses result in a κ value of 1.00, i.e. a perfect agreement between HRM method and dPCR for determining SMA (Table 1).

Establishment of a clear separation between unaffected individuals and SMA carriers

Two hundred and fourteen blood samples analyzed by CSB SMA Detection Kit were used to establish the cut-off ranges for unaffected individual and SMA carrier. Copy numbers of SMN1 and SMN2 genes from 214 samples were applied to distribution analysis and a clear distinction, so called a distinguishing window (Figure 2, gray zone), was exhibited between unaffected individuals and SMA carriers. This distinguishing window showed that unaffected individuals and SMA carriers can be separated clearly by using CSB SMA Detection Kit.

A parallel comparison of MLPA and dPCR

Quantitative PCR-based method may cause controversial results in unaffected individuals and SMA carriers due to the similar SMN1/SMN2 ratio. Digital PCR methodology provides absolute quantification for copy number variation and rare mutation detection. Therefore, we selected 12 blood samples that have been analyzed by CSB SMA Detection Kit for multiplex ligation-dependent probe amplification (MLPA) analysis, the standard testing method for SMA diagnosis clinically now. As indicated in Table 2, 12 blood samples showed the identical SMN1:SMN2 ratio in both testing methods (1:1, 1:2 or 1:3 in SMA carriers and 2:3 in unaffected individuals).

Discussion

Digital PCR is an absolute quantitative method that detects DNA copy number without need to use extra standard like

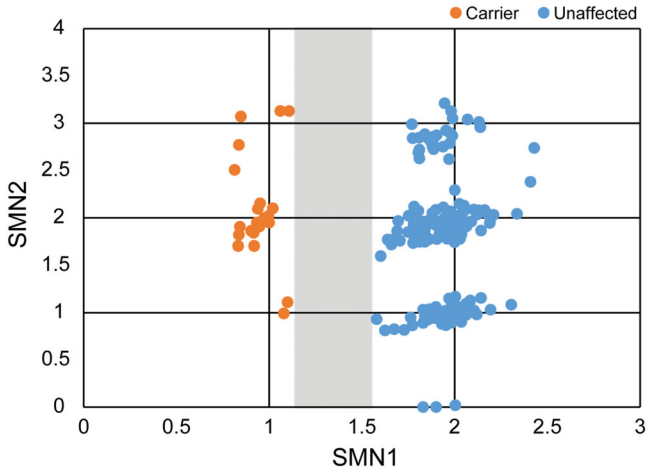

Figure 2. Distribution analysis on SMN1 and SMN2 genes copy number in unaffected individuals and SMA carriers. SMN1 and SMN2 genes copy numbers in unaffected individuals and SMA carriers were plotted and a distinguishing window was rendered in gray.

Table 2. A parallel comparison between MLPA and dPCR for SMA detection.

Sample	SMN1:SMN2		
	MLPA	dPCR	Report
A	1:1	1:1	Carrier
B	1:1	1:1	Carrier
C	1:1	1:1	Carrier
D	1:2	1:2	Carrier
E	1:2	1:2	Carrier
F	1:2	1:2	Carrier
G	1:3	1:3	Carrier
H	1:3	1:3	Carrier
I	1:3	1:3	Carrier
J	2:3	2:3	Unaffected
K	2:3	2:3	Unaffected
L	2:3	2:3	Unaffected

qPCR. In dPCR, the sample mixture is partitioned into a large number of small-volume PCR sub-reactions which contains very few target nucleic acid in each partitions. After PCR amplification, the positive partitions are quantified by Poisson's statistics. The sub-reactions format concentration effect not only resulting in detecting rare mutation easily in wild type nucleic acid by reducing target nucleic acid competition, but also increasing the tolerance of inhibitor in a sample. Due to these advantages, dPCR has been applied for monitoring rare mutation gene, copy number variation and low abundant pathogen. In the market, there are two main digital PCR platforms, droplet-(BioRad and RainDance)(Mazaika & Homsy, 2014) and chip-base (QuantStudio) systems (Laig, Fekete, & Majumdar, 2020) which means that the sample mixture is partitioned into oil droplets or distributed in micro-well on the chip individually. Although droplet dPCR system can generate a lot partitions of a sample that allows a wide dynamic range of detection, it has the disadvantage of droplet merge or break when the sample is transferred or during PCR amplification, which may result in significant sample loss when the nucleic acid level is low .

On the other hand, the chip-based system is quite stable and a quick data acquiring platform. Furthermore, compared the droplet system with the chip-based system, the uncertainty of droplet volume and partition number affects the

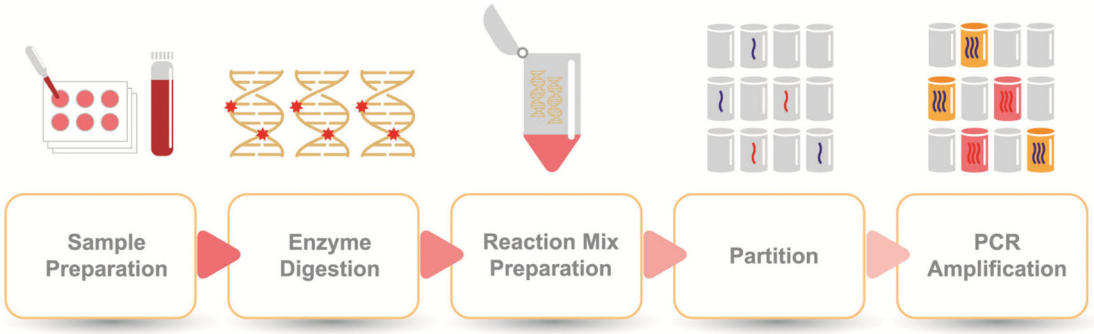

Figure 3. Workflow of CSB SMA Detection Kit.

accuracy of dPCR measurement in the single copy or rare mutation (Dong *et al.*, 2015). But the shape of the chips used in the QuantStudio chip-based platform is square that the PCR amplification can only be performed on specific type of thermos-cycler which limits the throughput of the platform.

Here, we use the chip-in-tube partition technology from JN Clarity dPCR that combined with the advantages of droplet and chip-based dPCR. The sample can be partitioned on a high density chip with 10,000 micro-wells. Due to its chip-in-tube, the PCR amplification is performed in conventional thermos-cycler and therefore it allows higher throughput in a single experiment. The accuracy and precision of gene copy number in JN Clarity dPCR system was also confirmed in parallel comparison with the current method using QX100 ™ droplet dPCR system (Low *et al.*, 2017).

Currently, the two main methods adapted in SMA screening are the multiplex ligation-dependent probe amplification (MLPA) and the high-resolution melting PCR (HRM-PCR). MLPA is a gold standard for SMA detection which is accurate, specific and a multiplex method, but it is time-consume (24 h) and the cost is relatively high.(Zhang *et al.*, 2018). On the other hand, the HRM-PCR method developed by Wang *et al.* (2015) had developed a fast (2 h) and cheap, but the disadvantage of HRM is that the melting curve is interpreted manually. Therefore, we developed a new methodology using JN Clarity dPCR system as a quick (only 3 h required), low–cost and auto-interpretation platform for accurate SMA detection.

Following the workflow of CSB SMA Detection Kit (Figure 3), genomic DNA was first extracted and digested. The prepared PCR reaction mix was partitioned, amplified and analyzed. After normalization to intra-reaction reference gene, the copy numbers of SMN1 and SMN2 genes were determined.

The CSB SMA Detection Kit is developed to determine SMN1 and SMN2 genes copy number from blood specimens, amniotic fluid and dry blood spots using JN Clarity™ Digital PCR (dPCR) System. The CSB SMA Detection Kit is as capable as HRM method and MLPA and produces a clear distinction between unaffected individuals and SMA carriers. Owing to a high carrier frequency and incidence rate of SMA, the CSB SMA Detection Kit is a rapid and precise molecular testing for SMA which can be adapted to pre-pregnancy eugenics inspection, prenatal testing as well as newborns screening to help physicians or genetic counsellors for diagnosis and therapeutics approaches.

Disclosure statement

The authors report no declarations of interest.

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