INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive diseases causing infant mortality, with an incidence around 1 in 10,000 births. About 81%–95% of SMA patients have no detectable exon 7 of the SMN1 gene, which is located in a 1.5-Mb reverse-duplicated region containing multiple copies of homologous sequences. Survival motor neuron 2 (SMN2) gene is also located in the 5q13 region, the coding sequence of which differs from SMN1 only by the 6th nucleotide of exon 7, where a C-to-T transition leads to the alternatively spliced isoform translating the non-functional SMN△7 protein. Pre-natal diagnosis is an essential prevention for SMA. Conventional procedure involves invasive approaches for fetal genetic materials such as amniocentesis and chorionic villus sampling (CVS), which harbor risks for miscarriage and infection. Non-invasive prenatal diagnosis of SMA in earlier pregnancy would be timely and safer.

The discovery of cell-free DNA (cfDNA) in maternal plasma has enhanced the development of non-invasive prenatal testing (NIPT). Although NIPT for fetal aneuploidies has already been clinically applied, non-invasive prenatal diagnosis for many single-gene disorders remains on the developing stage. For NIPD of SMA, a technique by targeted sequencing of cfDNA in maternal plasma and relative haplotype dosage (RHDO) analysis has been
previously published. However, this haplotype-based strategy has several limitations. Firstly, there is a rigid demand for DNA of the probands and parents, as well as adequate informative genomic markers beside the SMN1 gene. This limitation restricts the scope of subjects applicable to this test. Secondly, a recombination event may result in incorrect fetal genotype classification if it occurs as a genomic location near the mutation. Thirdly, for de novo SMN1 mutations with a rate that is reported to be high, and for germline mosaicism, haplotyping would fail or come out with false-negative results.

Droplet digital PCR is a technology with high sensitivity, specificity, and accuracy to detect and analyze low-abundance nucleic acids. Its high resolution is guaranteed by millions of oil droplets generated per test. Utilizing digital PCR, the feasibility of non-invasive prenatal diagnosis (NIPD) for fetal monogenic disorders has been proved in several studies analyzing cfDNA. In particular, for maternally inherited single nucleotide mutations, the relative mutation dosage (RMD) analysis based on the sequential probability ratio test (SPRT) has enabled detection of a slight increase in the load of the mutant allele in the maternal plasma of heterozygous carriers. Digital PCR provides an ideal platform for the development of a haplotype-free test strategy for SMA-NIPD.

Unlike most other single-gene disorders, SMA harbors need and potential for a specific design of NIPD technique. The prominent hot spot mutation in the SMN1 gene, which is the loss of exon 7 copies but not point mutations, implies the utilization of single-base targeting strategy but disables the application of regular RMD algorithm. The pseudogene SMN2 that disturbs quantification of SMN1 proposes a major obstacle. In this article, we present a novel technique that directly analyses SMN1 gene dosage using droplet digital PCR, as well as the results of performance validation.

2 | MATERIALS AND METHODS

2.1 | Design of probes and primers

TaqMan MGB probes were designed at the 6th nucleotide in exon 7 of SMN1/SMN2 gene and intron 3 of the reference ALB gene and synthesized by Thermo Fisher Scientific. We designed the length of SMN1/SMN2 and ALB amplicons as short as 75 bp and 72 bp to reduce the bias caused by unbalanced PCR amplification in favor of fetal cfDNA, which is generally shorter than maternal cfDNA. Sequences of the probes and primers are listed in the Appendix S1. Quantitative PCR was conducted for samples with various SMN1/SMN2 copy numbers to validate the specificity of the probes.

2.2 | Droplet digital PCR

RainDrop droplet digital PCR should be performed following standard protocols, through processes including PCR mixture preparation, droplet sourcing, PCRs, and signal sensing. For each PCR, droplets with positive signal for SMN1/ALB should be counted using RainDrop Analyst II software.

2.3 | The digital relative SMN1 dosage method

Statistical analysis is essential for the determination of SMN1 copy number from digital PCR data. Based on the principle of Poisson distribution and hypothesis testing, we set up an algorithm called digital relative SMN1 dosage, as specified and illustrated in the Appendix S1. In short, a hypothesis that fetal SMN1 copy number equals 1 is established at first. Next, Pr(observed) value is generated for each test of one sample (one data set), which is determined solely by the number of reaction-positive droplets. Then, through comparing Pr(observed) to the upper and lower thresholds (derived from the number of reaction-positive droplets and FF) under a certain threshold likelihood ratio (a marker of statistical significance with a default value 2, a higher value represents higher reliability), the algorithm would return one of the three possible outcomes: accept the hypothesis (fetal SMN1 copy number = 1)/reject the hypothesis (fetal SMN1 copy number = 0 when n_{SMN1}/n_{ALB} < 0.5; fetal SMN1 copy number = 2 when n_{SMN1}/n_{ALB} > 0.5)/an unclassifiable result.

2.4 | Validation of the technique performance

2.4.1 | Participants and sample processing

For the validation, we recruited pregnant women seeking SMA prenatal diagnosis on 16 ~ 22 weeks of gestation for this study from the Hunan Jiahui Genetics Hospital and signed informed consent. All of the pregnancies had undergone non-invasive prenatal screening for fetal aneuploidies by next-generation sequencing (NGS). Approval was obtained from the Ethics Committee of The Center for Medical Genetics, School of Life Sciences, Central South University, Hunan, China. For each participant, 6 ~ 10 mL of peripheral blood was collected in BCT Cell-Free DNA Blood Collection Tube (Streck) and 10 mL of blood was collected into k3-EDTA acid tubes. Weeks of gestation when sampling blood are listed in Table 1. Plasma was separated after double centrifugation within 6 hours after collection, one at 1600 g for 10 minutes and the second at 16000 g for 10 minutes. We extracted cell-free DNA from maternal plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer’s instructions. The concentrations of cfDNA samples were tested on Qubit (Thermo Fisher Scientific). Amniotic fluid was obtained by amniocentesis, from which fetal genomic DNA was extracted by the phenol-chloroform method.

2.4.2 | Establishment of test sets

SMN1/SMN2 copy numbers of all participants and fetuses were quantified by the multiplex ligation-dependent probe amplification (MLPA) analysis using SALSA MLPA Kit (P060 MRC Holland) according to the manufacturer’s instructions. Fetal sex was determined by amplifying the SRY gene in amniocyte DNA.

We established test set A of cfDNA from 17 women with 1 copy of SMN1 (SMA carriers) and pregnant with male fetuses. A researcher
blind to fetal genotypes established test set B by randomly selecting 10 samples in the test set A. The other two researchers conducted digital PCR and data analysis for set A and set B independently and blinded to fetal genotypes. In addition, six genomic DNA samples with different SMN1/SMN2 copy numbers were also included in this study to validate probe specificity.

2.4.3 | Determination of fetal DNA fraction

We determined fetal DNA fraction (FF) of the samples based on the relative proportion of mapped chromosome Y (ChrY) sequencing reads, which is the golden standard method for FF determination. In brief, low-coverage (0.1×) whole-genome sequencing was performed for the cfDNA samples. FF in maternal plasma was calculated by comparing the sequence tag density of ChrY in maternal plasma with the sequence tag density of ChrY in male plasma.

3 | RESULTS

3.1 | Validation of probe specificity for SMN1 by quantitative real-time PCR

Results of TaqMan quantitative real-time PCR conducted on genomic DNA samples with various SMN1/SMN2 copy numbers were completely in accordance with the MLPA results. Samples with one or more SMN2 copies and no SMN1 copy did not produce fluorescence signal (FAM) of SMN1, which proved reliable specificity of the designed TaqMan MGB probes.

3.2 | Validation of performance

Fetal fraction determined by low-coverage (0.1×) whole-genome sequencing ranged from 6.58% to 16.21%, with an average of 11.27% (Table 1). For samples in test set A, 16 had a classifiable SMN1 copy number, while one sample had an unclassifiable result (Table 2). The concordance rate with the results of MLPA testing of amniocyte DNA in test set A was 94.12% (16/17). For samples in test set B, nine had a classifiable SMN1 copy number, while one sample had an unclassifiable result (Table 3). The concordance rate with the results of MLPA testing of amniocyte DNA in test set B was 90% (9/10). For all tests with a classifiable result, the percent of agreement with the results of MLPA testing of amniocyte DNA was up to 100% (25/25). The results showed considerable accuracy and precision of the technique to test fetal SMN1 copy number in cfDNA.

4 | DISCUSSION

A novel NIPD technique has been developed for SMA based on a distinct strategy, with probes and cfDNA-fit primers designed directly targeting the 6th base of exon 7 in the SMN1 gene of the fetus. It can detect the loss of SMN1 exon 7 copy caused by either deletion of DNA fragment containing SMN1 exon 7 or SMN1-to-SMN2 gene conversion. It could address the problems encountered by the haplotype-based methods. In other words, this technique would be applicable to SMA families without available patient samples or in the conditions that de novo mutations/
| Sample number | Number of droplets produced | Number of droplets positive for SMN1 ($n_{SMN1}$) | Number of droplets positive for ALB ($n_{ALB}$) | $n_{SMN1}/n_{ALB}$† | Pr(observed)‡ | Upper threshold | Lower threshold | Fetal SMN1 copy number by cell-free DNA | Fetal SMN1 copy number by amnio-cyte DNA |
|---------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|------------------------|----------------|----------------|----------------|--------------------------------------|-------------------------------------|
| G3313         | 7 509 922                   | 558                                           | 1172                                          | 0.501706485            | 0.65909091     | 0.660974755     | 0.642899527 | 1                          | 1                                  |
| G3507         | 7 589 437                   | 641                                           | 1287                                          | 0.498057498            | 0.667531120     | 0.679454682     | 0.668897741 | 1                          | 1                                  |
| G3515         | 7 476 559                   | 919                                           | 1840                                          | 0.499456522            | 0.666908300     | 0.684909300     | 0.667741972 | 1                          | 1                                  |
| G3562         | 7 214 377                   | 508                                           | 976                                           | 0.520491803            | 0.657681941     | 0.65580450      | 0.649001031 | 2                          | 2                                  |
| G3567         | 6 510 824                   | 1219                                          | 2452                                          | 0.497145188            | 0.669378922     | 0.688729633     | 0.682159827 | 1                          | 1                                  |
| G3612         | 7 093 361                   | 431                                           | 1024                                          | 0.42098438             | 0.703780069     | 0.693910900     | 0.656060557 | 0                          | 0                                  |
| G3673         | 7 068 369                   | 900                                           | 2107                                          | 0.427147603            | 0.700698370     | 0.684916625     | 0.672097403 | 0                          | 0                                  |
| G3731         | 7 406 514                   | 1339                                          | 2905                                          | 0.460929432            | 0.684495759     | 0.682411499     | 0.672372092 | 0                          | 0                                  |
| G3736         | 7 903 682                   | 545                                           | 1328                                          | 0.410391566            | 0.709022958     | 0.689770750     | 0.673605329 | 0                          | 0                                  |
| G3780         | 4 182 515                   | 526                                           | 897                                           | 0.586399108            | 0.630358398     | 0.669983619     | 0.640830268 | 2                          | 2                                  |
| G3846         | 7 288 518                   | 305                                           | 613                                           | 0.497553018            | 0.66755991      | 0.698248252     | 0.669674650 | 1                          | 1                                  |
| G3854         | 6 577 602                   | 480                                           | 926                                           | 0.518358531            | 0.658605974     | 0.658261929     | 0.649566182 | 1                          | 1                                  |
| G3978         | 7 811 029                   | 266                                           | 528                                           | 0.503787879            | 0.664987406     | 0.661969747     | 0.646302039 | 1                          | 1                                  |
| G4007         | 7 611 593                   | 1124                                          | 2052                                          | 0.547758285            | 0.646095718     | 0.667492232     | 0.649494269 | 2                          | 2                                  |
| G4032         | 4 607 289                   | 189                                           | 457                                           | 0.41356674             | 0.707430341     | 0.704116199     | 0.667720307 | 0                          | 0                                  |
| G4185         | 7 294 012                   | 1141                                          | 2314                                          | 0.493085566            | 0.66975398      | 0.689684443     | 0.675104247 | 1                          | 1                                  |
| G4223         | 7 198 643                   | 706                                           | 1345                                          | 0.524907063            | 0.655777669     | NA              | NA             | unclassifiable                      | 2                                  |

Note: †$n_{SMN1}/n_{ALB}$: It is the only index determining hypothesis testing H1. H1: fetal SMN1 copy number = 0 (in cases that $n_{SMN1}/n_{ALB}$ < 0.5) or fetal SMN1 copy number = 2 (in cases that $n_{SMN1}/n_{ALB}$ > 0.5). ‡Pr(observed): $Pr_{\text{observed}} = n_{ALB}/(n_{ALB} + n_{SMN1})$. It is a value entirely depending on the data of one single test on one sample. Fetal SMN1 copy number is determined by comparing the value of $Pr_{\text{observed}}$ with the upper/lower thresholds. Find details in the Appendix S1.
TABLE 3  Results of cfDNA samples in test set B

| Sample number | Number of droplets produced | Number of droplets positive for SMN1 (n_{SMN1}) | Number of droplets positive for ALB (n_{ALB}) | Pr(\text{observed}) \textsuperscript{3} | Upper threshold | Lower threshold | Fetal SMN1 copy number by cell-free DNA | Fetal SMN1 copy number by amniocyte DNA |
|---------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------|----------------|----------------|--------------------------------------|-------------------------------------|
| G3507         | 6 288 548                   | 440                                           | 882                                           | 0.498866123                         | 0.667170953  | NA             | NA                                  | unclassifiable                      | 1                                 |
| G3515         | 7 220 904                   | 745                                           | 1501                                          | 0.496335776                         | 0.668291999 | 0.67983664 | 0.672809351 | 1                                  |
| G3562         | 5 588 717                   | 556                                           | 941                                           | 0.590860786                         | 0.628590514 | 0.66406216 | 0.641520503 | 2                                  |
| G3612         | 7 479 914                   | 385                                           | 908                                           | 0.424008811                         | 0.702242846 | 0.696283100 | 0.653687012 | 0                                  |
| G3673         | 6 958 213                   | 657                                           | 1562                                          | 0.420614597                         | 0.703920685 | 0.687192209 | 0.669817422 | 0                                  |
| G3731         | 6 295 878                   | 1100                                          | 2352                                          | 0.434439179                         | 0.697136564 | 0.683257462 | 0.671526720 | 0                                  |
| G3846         | 7 289 053                   | 279                                           | 551                                           | 0.506332087                         | 0.663855422 | 0.645140279 | 0.657350939 | 1                                  |
| G3854         | 6 813 333                   | 321                                           | 636                                           | 0.504716981                         | 0.664576803 | 0.66030065 | 0.647525672 | 1                                  |
| G4007         | 6 527 871                   | 936                                           | 1772                                          | 0.528216704                         | 0.654357459 | 0.662329211 | 0.655114379 | 2                                  |
| G4223         | 5 705 388                   | 670                                           | 1155                                          | 0.58008658                          | 0.632876712 | 0.669258109 | 0.643905623 | 2                                  |

Note: \(n_{SMN1}/n_{ALB}\): It is the only index determining hypothesis testing H1. H1: fetal SMN1 copy number = 0 (in cases that \(n_{SMN1}/n_{ALB} < 0.5\)) or fetal SMN1 copy number = 2 (in cases that \(n_{SMN1}/n_{ALB} > 0.5\)).

\(Pr(\text{observed}) = n_{ALB}/(n_{ALB} + n_{SMN1})\). It is a value entirely depending on the data of one single test on one sample. Fetal SMN1 copy number is determined by comparing the value of \(Pr(\text{observed})\) with the upper/lower thresholds. Find details in the Appendix S1.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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