Original Article

Diagnosis of Spinal Muscular Atrophy: A Simple Method for Quantifying the Relative Amount of Survival Motor Neuron Gene 1/2 Using Sanger DNA Sequencing

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Abstract

Background: Spinal muscular atrophy (SMA) is caused by homozygous deletion or compound heterozygous mutation of survival motor neuron gene 1 (*SMN1*), which is the key to diagnose SMA. The study was to establish and evaluate a new diagnostic method for SMA.
Methods: A total of 1494 children suspected with SMA were enrolled in this study. Traditional strategy, including multiplexed ligation-dependent probe amplification (MLPA) and TA cloning, was used in 1364 suspected SMA children from 2003 to 2014, and the 130 suspected SMA children were tested by a new strategy from 2015 to 2016, who were also verified by MLPA combined with TA cloning. The *SMN1* and *SMN2* were simultaneously amplified by polymerase chain reaction using the same primers. Mutation Surveyor software was used to detect and quantify the *SMN1* variants by calculating allelic proportions in Sanger sequencing. Finally, turnaround time and cost of these two strategies were compared.
Results: Among 1364 suspected SMA children, 576 children had *SMN1* homozygous deletion and 27 children had *SMN1* compound heterozygous mutation. Among the 130 cases, 59 had *SMN1* homozygous deletion and 8 had heterozygous deletion: the *SMN1*-specific peak proportion on exon 7 was 34.6 ± 1.0% and 25.5 ± 0.5%, representing *SMN1:SMN2* to be 1:2 and 1:3, respectively. Moreover, five variations, including p.Ser8Lysfs *23 (in two cases), p.Leu228*, p.Pro218Hisfs *26, p.Ser143Phefs*5, and p.Tyr276His, were detected in 6/8 cases with heterozygous deletion, the mutant allele proportion was 31.9%, 23.9%, 37.6%, 32.8%, 24.5%, and 23.6%, which was similar to that of the *SMN1*-specific site on exon 7, suggesting that those subtle mutations were located in *SMN1*. All these results were consistent with MLPA and TA cloning. The turnaround times of two strategies were 7.5 h and 266.5 h, respectively. Cost of a new strategy was only 28.5% of the traditional strategy.
Conclusion: Sanger sequencing combined with Mutation Surveyor analysis has potential application in SMA diagnosis.

Key words: Mutation Surveyor Software; Quantitative Analysis; Sanger DNA Sequencing; Spinal Muscular Atrophy

Introduction

Spinal muscular atrophy (SMA), an autosomal recessive motor neuron disease, is the leading genetic cause of death in young children. The clinical features of SMA are progressive muscle weakness and atrophy, which result from degeneration of α motor neurons in the anterior horn of the spinal cord. Based on the age at onset and level of motor function of an individual, SMA in children is classified into types I, II, and III, reflecting decreasing severity of the disease. So far, SMA is incurable; however, several promising therapeutics are currently under early-phase clinical trials.[1]

The survival motor neuron gene 1 (*SMN1*) encodes the full-length, and functional SMN protein and mutations in this gene are associated with SMA. Approximately 95% of patients with SMA had homozygous deletion of *SMN1* and around 5% of patients carried compound heterozygous mutation with one allele deleted and a subtle variation in the other allele.[2] Molecular detection of *SMN1* variations is effective and specific for diagnosis of SMA; however, it is more challenging than other monogenic disorders

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because of the existence of the nearly identical *SMN2*, with only five nucleotides differentiating from *SMN1*. These two *SMN* genes are located at an inverted duplication region of chromosome 5q. Due to the intrinsic instability of this region, *SMN2* copy number is variable and is inversely correlated with the severity of SMA; however, only the genotype of *SMN1* is used for the clinical diagnosis of SMA.

In addition, Mutation Surveyor software (SoftGenetics LLC, PA, USA), a commercial program, can automatically complete sequence alignment and detect variants, including single-nucleotide variants, as well as insertions and deletions within Sanger sequencing traces. This software provides high accuracy and sensitivity in the analysis of DNA variants and has been validated for various genetic tests in clinical applications, such as tumors,[3] lymphoma,[4] psoriasis vulgaris,[5] developmental disorders,[6] and inherited diseases.[7] Ellard *et al*.[8] evaluated the sensitivity of Mutation Surveyor software by unidirectional sequence data obtained from patients referred for genetic testing to three clinical diagnostic laboratories in the UK. All 701 different heterozygous variations in 29 genes were detected by this software, suggesting the sensitivity of heterozygote detection more than 99.57% (with 95% confidence interval). In addition, 130 heterozygous insertions/deletions were identified, and none were missed.

Moreover, Mutation Surveyor software also has "Mutation Quantifier" function to quantify the wild type and variant alleles by calculating the peak proportions, which are suitable for variants associated with somatic mutations, heteroplasmy, and mosaicism. It provides two methods of quantifying peak proportions, including a simplified allele ratio based on peak relative fluorescence units (RFUs), and a standardized allele ratio for percentage drop of normal allele (SC drop) and percentage gain of mutant allele (SC gain). Dong *et al*.[9] and Song *et al*.[10] demonstrated that this software could successfully detect at least 10% of somatic mutations and more than 3% of heteroplasmy with mitochondrial DNA A11778G, based on detailed studies using different ratios of wild-type DNA and mutant DNA.

According to the features of multicopies and highly homologous of *SMN1* and *SMN2* genes, which are similar to heteroplasmy or mosaicism, this study aimed to establish and evaluate a simple method for the relative quantification of *SMN* genes, as well as simultaneous screening of *SMN1* subtle variations, based on Sanger DNA sequencing. Moreover, this study compared the performance of two SMA diagnostic strategies, multiplexed ligation-dependent probe amplification (MLPA) combined with TA cloning and this new method. Besides, this study attempted to report the successful development of a diagnostic procedure for SMA based on Sanger DNA sequencing.

Methods

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Ethics Committee of the Capital Institute of Pediatrics. Informed written consent was obtained from all patients' parents/guardians prior to their enrollment in this study.

Subjects

A total of 1494 unrelated suspected SMA children with lower-limb weakness and motor development retardation who underwent *SMN* screening at the Department of Medical Genetics, Capital Institute of Pediatrics (Beijing, China), were included in this study. Between 2003 and 2014, *SMN* genes of 1364 patients were genotyped using MLPA combined with TA cloning. From 2015 to 2016, 130 patients were screened by this method based on Sanger DNA sequencing, and then confirmed by MLPA combined with TA cloning. Peripheral blood samples were taken from all children as well.

Sample preparation and polymerase chain reaction

Genomic DNA was extracted using the proteinase K-phenol/chloroform method. *SMN1* and *SMN2* were simultaneously amplified by using the same pair of primers (sense: 5'-TGTCTTGTGAAACAAAATGCTT-3'; antisense: 5'-AAAAGTCTGCTGGTCTGCCTA-3'), which were located in sequences that were homologous between the two *SMN* genes. Polymerase chain reaction (PCR) was performed in a-50 μl volume containing 100 ng DNA, 10× PCR buffer, 0.2 mmol/L of each dNTP, primers (1.5 pmol/L each), and 2.5 units of Taq DNA polymerase (TransGen Biotech, Beijing, China). The reaction was carried out with an initial denaturation step for 3 min at 94°C, followed by 35 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 30 s, and then, accompanied with a final elongation step for 5 min at 72°C. Therefore, both *SMN1* and *SMN2* can be considered as an internal reference for each other in the PCR reaction. The amplicon covers four known differential sites in *SMN1* and *SMN2*, among which one is located in intron 6 and exon 7 and two in intron 7 [Figure 1].

Construction for survival motor neuron gene recombinant DNA plasmids

In our previous experience, we found the heights of base peaks at differential sites in *SMN* genes associated with a difference in relative amount between *SMN1* and *SMN2*. Therefore, we hypothesized that Sanger DNA sequencing might be used for relative quantitation of *SMN* genes to indicate *SMN1* heterozygous deletion. In order to evaluate

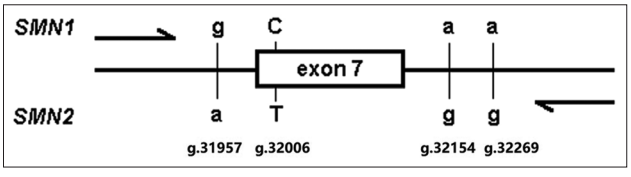

Figure 1: Schematic diagram showing the locations of PCR primers and the bases at four differential sites in *SMN1* and *SMN2*. The four bases above the gene are specific to *SMN1* and the four below are specific to *SMN2*. The arrows represent the sense and antisense primers used for the PCR reaction. *SMN*: Survival motor neuron gene; PCR: Polymerase chain reaction.

the accuracy of this method, we constructed *SMN1* and *SMN2* recombinant DNA plasmids and then mixed them at different molarities to represent various ratios of *SMN1*:*SMN2*. Amplified fragments of *SMN1* and *SMN2* were obtained using normal control DNA as described above. Additionally, we cloned them into the pEASY®-T1 cloning vector (TransGen Biotech, Beijing, China), according to the manufacturer's instructions. Cloned inserts were all verified by sequencing. Plasmid DNA was extracted using EasyPure® Plasmid MiniPrep kit (TransGen Biotech, Beijing, China) and quantified by Nanodrop 2000 (Thermo Fisher Scientific, Waltham MA, USA). According to the concentration and molecular weight of each plasmid, we calculated the molarity of the recombinant plasmids containing *SMN1* and *SMN2* fragments, respectively. Two *SMN* plasmids were mixed in molar ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 2:1, 2:3, and 3:2 to represent different ratios of *SMN1*:*SMN2*. Eventually, these mixed plasmids were used as the templates for PCR and subsequent Sanger DNA sequencing.

Relative quantification of survival motor neuron 1 genes by Sanger DNA sequencing and Mutation Surveyor software

The purified PCR products were analyzed by Sanger DNA sequencing using a DNA sequencer (ABI 3730XL DNA Analyzer; SeqGen Inc., CA, USA). There were four differential sites within the intron 6–intron 7 amplicon of the *SMN1* and *SMN2* genes [Figure 1]. The relative quantification of *SMN1* and *SMN2* was therefore undertaken by Mutation Surveyor software at the four differential sites by using standardized allele ratio method. Mutation Quantifier function quantified the *SMN1* allele that is decreased at a differential site in the sample and the *SMN2* allele that is gained at that site. The reference sequence is *SMN1* (NM_000344.3); *SMN2* and *SMN1* plasmids were considered as 0 and 100% allele ratio, respectively; the SC gain value represents the concentration of *SMN1*. In addition, MLPA was carried out using the SALSA MLPA probe mix P021 SMA (MRC-Holland, Amsterdam, the Netherlands), according to the manufacturer's instructions.

Screening survival motor neuron gene 1 subtle variations by Sanger DNA sequencing and Mutation Surveyor software

When a case was suspected of harboring a *SMN1* heterozygous deletion, screening for *SMN1* subtle variations was undertaken. After amplification of the entire *SMN1/SMN2* gene by PCR, as previously described,[11] all nine exons and flanking sequences were analyzed by Sanger DNA sequencing. Because we could not predict the *SMN1* subtle mutations, the simplified allele ratio method was used for quantitative analysis without need of the relative wild or variant plasmids. Mutation Surveyor software could automatically calculate the mutant peak percentages from RFU. Since the percentage is similar to that of differential site on exon 7, we could judge whether there was a point mutation in *SMN1*. Point mutations were then confirmed by TA cloning of the PCR products and sequencing.

Statistical analysis

The data were shown as mean ± standard deviation (SD). Pearson's correlation analysis was performed to assess the relationship between expected and observed ratios at four differential sites in *SMN* gene. Statistical analyses were performed by SPSS version 23.0 software (IBM, Armonk, NY, USA). A $P < 0.05$ was considered statistically significant.

Results

Characteristics of subjects

During July 2003 and December 2014, a total of 1364 patients (816 males and 548 females) underwent *SMN1* screening in our laboratory using MLPA combined with TA cloning. The median age was 16.5 months (ranging from 2 days to 16 years). Among those patients, *SMN1* homozygous deletion was found in 576 cases (42.2%), and *SMN1* heterozygous deletion was observed in 41 cases (3.0%) by MLPA analysis. Besides, 34 of 41 patients with *SMN1* heterozygous deletion consented to *SMN1* subtle variant analysis. Using TA cloning, 27 of 34 patients (79.4%) were found carrying *SMN1* subtle variants combined with *SMN1* heterozygous deletion. Therefore, according to SMA diagnostic evaluation recommended by Wang *et al*.,[12] the 5q SMA was confirmed in 603 cases (44.2%), *SMN*-related SMA was unconfirmed in 7 cases (0.5%), and the remaining 754 cases (55.3%) might be affected by other motor neuron disorders.

Establishment of a test for relative quantitation of survival motor neuron genes by Sanger DNA sequencing and Mutation Surveyor software

Accuracy evaluation

To evaluate the accuracy of the new method, we mixed *SMN1* and *SMN2* recombinant DNA plasmids at different molarities. As shown in Figure 2, the *SMN1*:*SMN2* ratio was accurately detected over a gene dosage range of 1:1–1:6, which was analyzed by Mutation Surveyor software. The correlation coefficient (R^2) between expected and observed ratios at four differential sites was 0.997, 0.999, 0.997, and 0.998, respectively [Figure 3]. Among the four differential sites in *SMN1/SMN2*, the C/T base peaks on exon 7 of the *SMN* genes showed the highest level of consistency with the *SMN1*:*SMN2* ratio not only in plasmid mixture, but also in blood DNA [Supplementary Figure 1].

Reliability assessment

From 1364 samples, 65 samples with known *SMN1* genotypes were used to assess the effectiveness of this method. All the samples were sequenced and quantified by Mutation Surveyor software in the following groups: (1) ten cases with *SMN1* homozygous deletion; (2) twenty cases with *SMN1* heterozygous deletion, in which ten cases with *SMN1/SMN2* = 1:2, the *SMN1*-specific peak proportion on exon 7 was expectedly 33.3% (1/3), and the proportion observed was 34.1 ± 1.5%; ten cases with

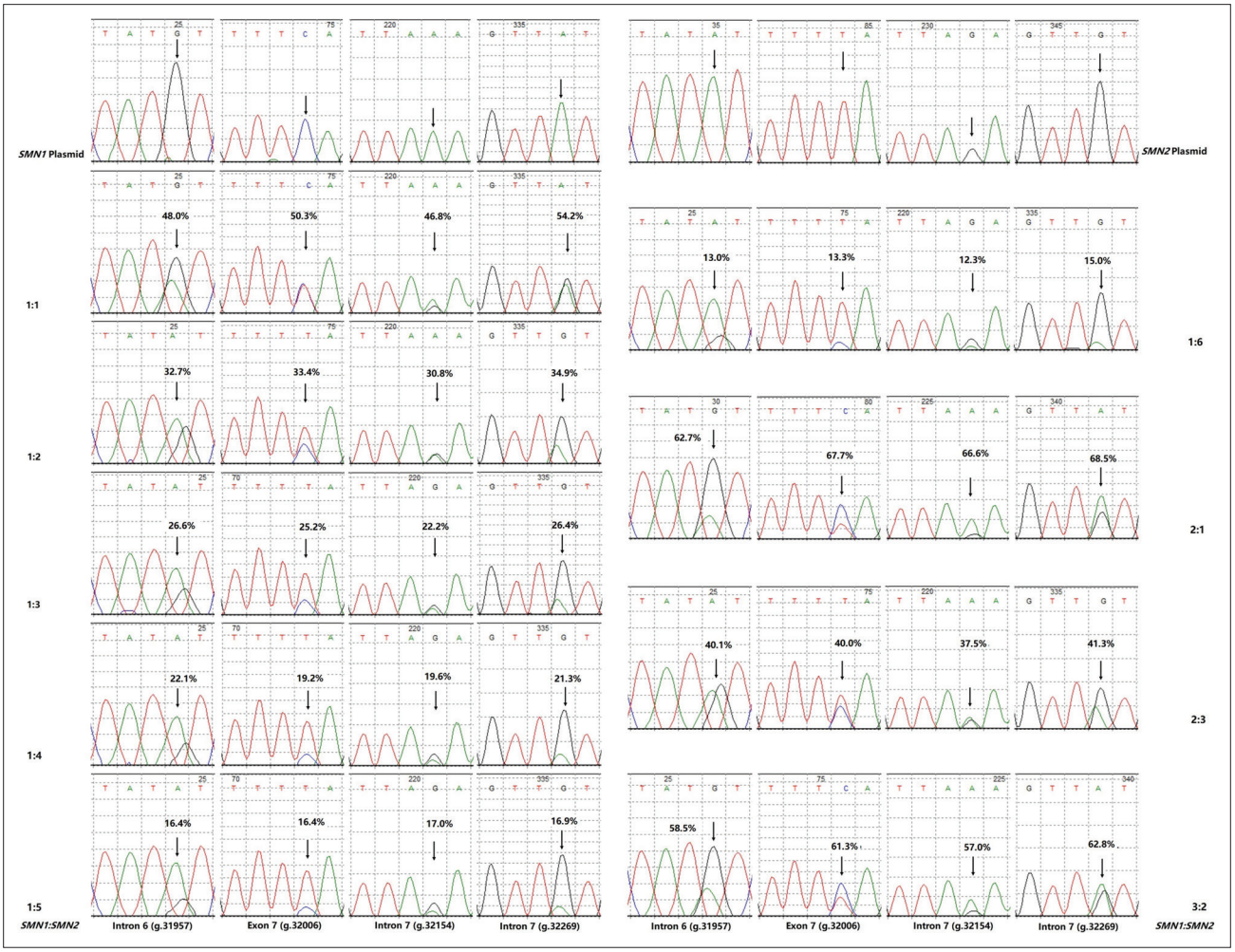

Figure 2: Sanger DNA sequencing for *SMN1* and *SMN2* mixed plasmids. The base at four differential sites (one each in intron 6 and exon 7 and two in intron 7) are indicated by arrows: G, C, A, and A for *SMN1*; A, T, G, and G for *SMN2*. The *SMN1* percentage was obtained by Mutation Surveyor software. The base height was calculated according to peak height of the same base upper and lower 8 bases by Mutation Surveyor software. *SMN*: Survival motor neuron gene.

SMN1/SMN2 = 1:3, the expected proportion was expectedly 25.0% (1/4), and the observed proportion was 25.8 ± 1.4%; (3) 15 cases with *SMN2* heterozygous deletion, in which ten cases with *SMN1/SMN2* = 2:1 (expected 66.7% vs. observed 66.6 ± 1.1%) and five cases with *SMN1/SMN2* = 3:1 (expected 75.0% vs. observed 75.2 ± 2.0%); and (4) twenty cases with *SMN* nondeletion, in which 15 cases with *SMN1/SMN2* = 2:2 (expected 50.0% vs. observed 50.3 ± 1.5%) and five cases with *SMN1/SMN2* = 2:3 (expected 40.0% vs. observed 40.8 ± 1.5%). These results were consistent with the results previously obtained by MLPA [Figure 4]. Subsequently, we screened the other exons for the four cases with *SMN1* heterozygous deletion by Sanger DNA sequencing. Mutation Surveyor software detected four pathogenic variations: c.40G>T (p.Glu14*), c.22dupA (p.Ser8Lysfs*23), c.683T>A (p.Leu228*), and c.56delT (p.Val19Glyfs*21). Moreover, the quantitative analysis showed the percentages of variant base peaks to be 31.7%, 24.6%, 24.1%, and 27.6%, suggesting the *SMN1:SMN2* ratios to be 1:3, 1:2, 1:2, and 1:2, respectively, which was matched with those of MLPA [Figure 5].

Validation of the test for relative quantitation of survival motor neuron genes by Sanger DNA sequencing and Mutation Surveyor software

To assess the validity of our approach, 130 suspected SMA patients were examined in our laboratory in parallel from January 2015 to December 2016 using Sanger DNA sequencing and MLPA combined with TA cloning. The Sanger DNA sequencing identified 59 (45.4%) cases with *SMN1* homozygous deletion, 8 (6.2%) with *SMN1* heterozygous deletion, and 63 (48.5%) with *SMN1* nondeletion. Additionally, MLPA also detected 59 cases with *SMN1* homozygous deletion (*SMN1*:*SMN2* = 0:2, 0:3, or 0:4), 8 with *SMN1* heterozygous deletion (*SMN1*:*SMN2* = 1:2, or 1:3), and 63 with *SMN1* nondeletion (*SMN1*:*SMN2* = 2:2, or 2:1). All the results were consistent with those of Sanger DNA sequencing.

In addition, among eight patients who were identified with *SMN1* heterozygous deletion by the Sanger DNA sequencing, the *SMN1* proportion in four cases was

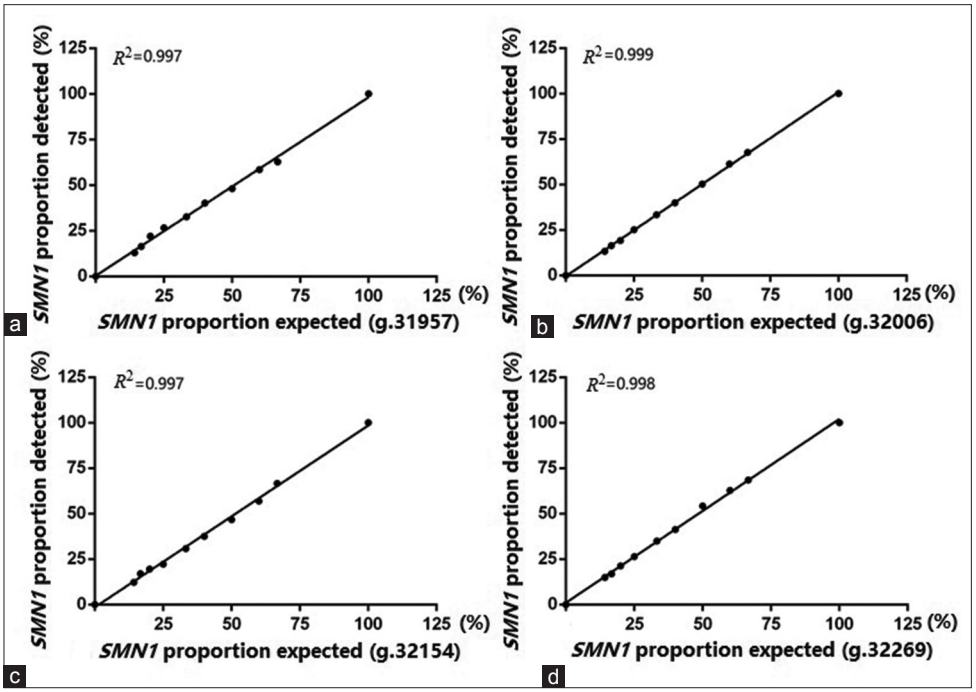

Figure 3: Correlation of expected and detected *SMN1* proportion by Mutation Surveyor software at four differential sites in 11 plasmid mixtures containing various known ratios of the *SMN1* and *SMN2*:*SMN2* plasmid, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 2:1, 2:3, 3:2, and *SMN1* plasmid. (a–d) Four differential sites between *SMN1* and *SMN2*. *SMN*: Survival motor neuron gene.

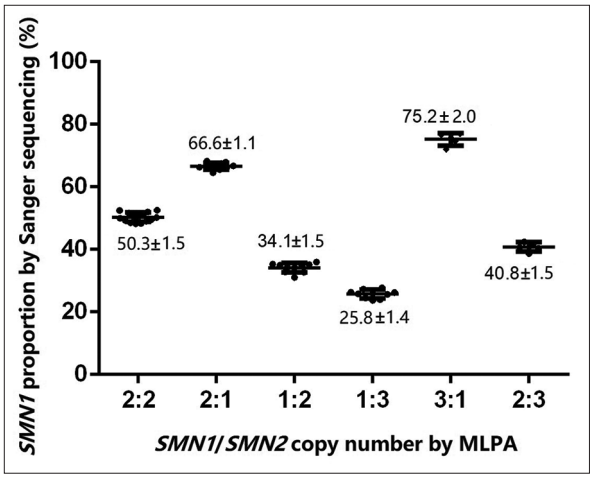

Figure 4: Comparison of *SMN1*/*SMN2* quantitation by MLPA and Sanger DNA sequencing in 65 samples with known *SMN1* genotypes. All data are presented as mean ± SD. *SMN*: Survival motor neuron gene; MLPA: Multiplexed ligation-dependent probe amplification; SD: Standard deviation.

34.6 ± 1.0% and 25.5 ± 0.5% in the rest four cases. MLPA showed a copy number ratio (*SMN1*:*SMN2*) of 1:2 in the former four cases and 1:3 in the later four cases. Moreover, six out of eight patients were found with five types of subtle variations in *SMN1*: c.22dupA (Ser8Lysfs*23) in two cases and c.683T>A (p.Leu228*), c.651_652dupAC (p.Pro218Hisfs*26), c.427dupT (p.Ser143Phefs*5), and c.826T>C (p.Tyr276His) each for one case. Mutation Surveyor software showed the mutant proportion to be 31.9%, 23.9%, 37.6%, 32.8%, 24.5%, and 23.6%, respectively. It was noteworthy that the proportion of variant base peak was similar to that of differential site on exon 7 [Figure 6 and Supplementary Figure 2]. These five *SMN1* variations were confirmed by TA cloning, and Tyr276His, Ser143Phefs*5, and Pro218Hisfs*26 were not previously reported.

Spinal muscular atrophy diagnostic procedure based on Sanger DNA sequencing

According to the data mentioned above, an appropriate diagnostic procedure was developed for SMA based on Sanger DNA sequencing [Figure 7]. In our opinion, Sanger DNA sequencing of the amplified *SMN* gene segment from intron 6 to intron 7 should be firstly undertaken for those suspected SMA patients based on clinical phenotype, biochemical tests, and electromyography. Based on the results of the Sanger DNA sequencing, especially for the differential site on exon 7, diagnosis was proceeded as follows: (1) if only bases specific to *SMN2* are detected at the four differential sites (T specific to *SMN2* on exon 7), meaning *SMN1* homozygous deletion, patients are diagnosed with 5q SMA. (2) When base peaks specific to *SMN2* are higher than those to *SMN1* (C specific to *SMN1* was lower than T specific to *SMN2* on exon 7), patients are considered with *SMN1* heterozygous deletion, and the *SMN1*:*SMN2* ratios can be calculated by Mutation Surveyor software. For those patients, all *SMN* coding exons and their flanking introns should be amplified and sequenced to screen the point mutations. If variant site is found and the variation peak is lower than that of wild peak, and the percentage of variant base peak is similar to that of differential site on exon 7, this case should be diagnosed

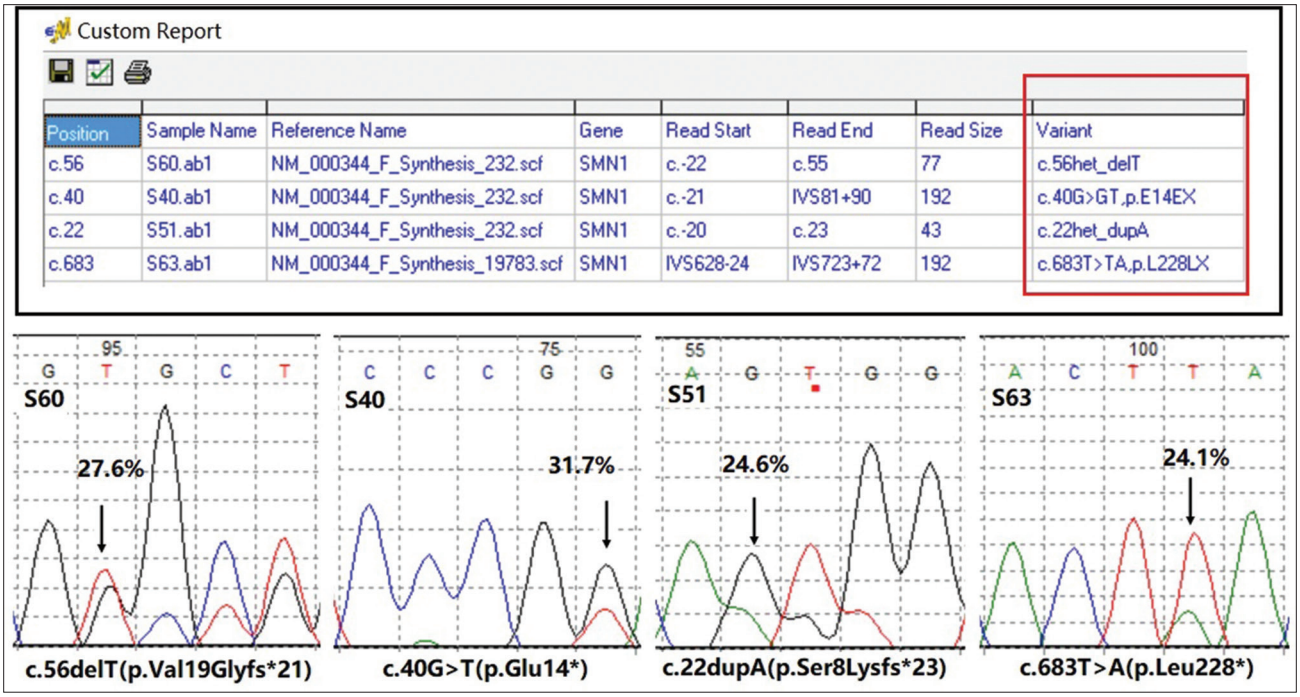

Figure 5: Mutation Surveyor software detects and quantifies the variations in Sanger DNA sequencing trace. The upper row is a custom report and variations detected were in a red frame. The arrows indicate the variations.

with 5q SMA; if there is no variation in screening, this case should be diagnosed with unconfirmed SMN-related SMA. (3) If the differential site peak specific to *SMN1* is almost equal or higher than that of *SMN2* (C equal to or higher than T on exon 7), this case should be considered as *SMN1* nondeletion, and patients should return to their clinician for reassessment of their disease symptoms and clinical follow-up.

Cost and time savings using Sanger DNA sequencing-based diagnostic strategy

The cost analysis suggested that the costs using traditional diagnosis strategy were RMB 170 Yuan and 620 Yuan per sample for *SMN1* deletion and *SMN1* subtle variation, respectively. While using Sanger sequencing-based strategy, the cost per sample was RMB 45 Yuan and 210 Yuan for *SMN1* deletion and variation, respectively [Table 1]. Moreover, the turnaround time of this strategy was significantly less than that of traditional method in total diagnostic procedure for SMA (about 7.5 h vs. about 266.5 h).

Discussion

Sanger sequencing has been considered as a gold standard for the identification of nucleotide sequence variation for performing qualitative analysis. Notably, by using Mutation Surveyor software, Sanger DNA sequencing was used for quantitative analysis, with sensitivity to the variant allele extending down to 5% of the primary peak. In this study, we established and assessed a Sanger DNA sequencing-based method for SMA diagnosis, not only for *SMN1* homozygous deletion, but also for compound heterozygous mutation. Analysis of the nearly 200 *SMN* fragments' sequences from 130 patients suspected to have SMA showed that 65 SMA patients got the molecular diagnosis, including 59 cases with *SMN1* homozygous deletions and 6 cases with *SMN1* compound heterozygous mutations. These results were consistent with those of MLPA and TA cloning; however, our method was remarkably faster and cheaper.

High homology between *SMN1* and *SMN2* made it possible to simultaneously amplify both *SMN* genes in one PCR reaction, and they served as an internal reference for each other to indicate the relative amount of the two genes. Therefore, according to the sequencing chromatogram combined with Mutation Surveyor software, we could easily deduce a change of *SMN* amount based on the heights of the base peaks at differential sites, especially in exon 7. Our plasmid mixtures' results showed high consistency between the measured results and the real sample compositions, indicating a very accurate analytical capability ($R^2 = 0.999$, on exon 7).

In addition to the relative quantization of *SMN* genes, our method can be used to screen intragenic subtle variants in those patients with *SMN1* heterozygous deletion. This is because in these cases, the *SMN1*-specific peak in exon 7 is lower than that of *SMN2* and the peak ratio is correlated with *SMN* copy number. This applies to any *SMN* mutated site; when the percentage of variant base peak is similar to that of differential site on exon 7, it implies that the mutation is in *SMN1* [Figure 6]. Moreover, Mutation Surveyor software can provide various descriptions according to nomenclature recommendations of the Human Genome Variation Society (http://www.hgvs.org). Meanwhile, this software also includes different databases, allowing users to

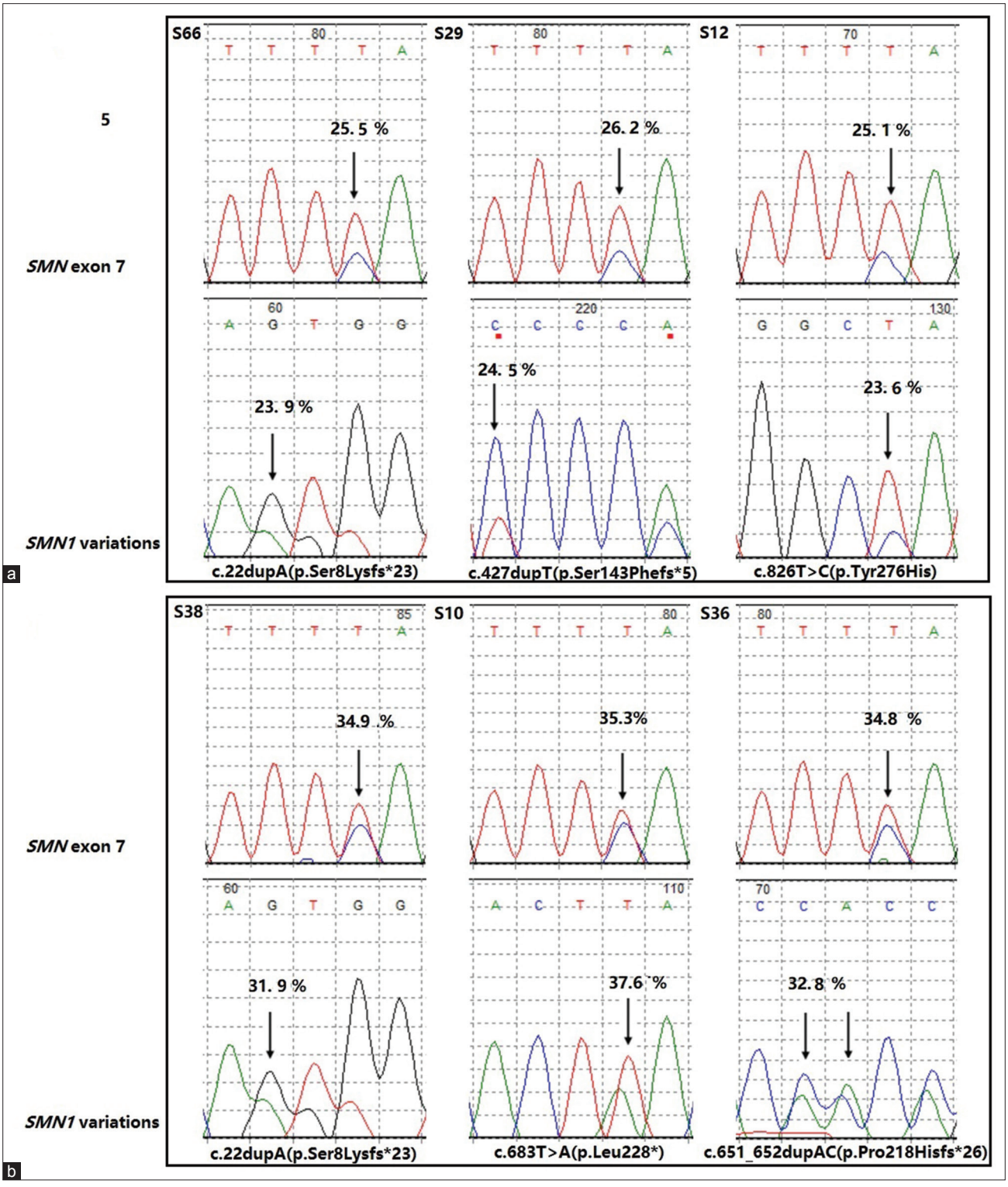

Figure 6: Sanger DNA sequencing for screening *SMN1* variation. (a) The ratio of *SMN1*/*SMN2* was 1:3. (b) The ratio of *SMN1*/*SMN2* was 1:2. For the first row sequencing chromatogram, the arrows indicate the differential site on exon 7 (C for *SMN1* and T for *SMN2*). The second row sequencing chromatogram shows *SMN1* variations: the variant base peak is under the wild-type sequence, and the percentage of variant peak is associated with that of differential site on exon 7, demonstrating that the mutation might be in *SMN1*. *SMN*: Survival motor neuron gene.

query mutation data from popular databases, such as Single Nucleotide Polymorphism Database and the Catalogue of Somatic Mutations in Cancer. All these automation features can effectively reduce the time required for conducting the analysis. Therefore, by using our diagnostic strategy, we identified five types of variants in eight SMA patients carrying *SMN1* heterozygous deletion: Ser8Lysfs*23, Leu228*, Tyr276His, Ser143Phefs*5, and Pro218Hisfs*26. Those patients without *SMN1* subtle variants were recommended to return to their clinician for reassessment.

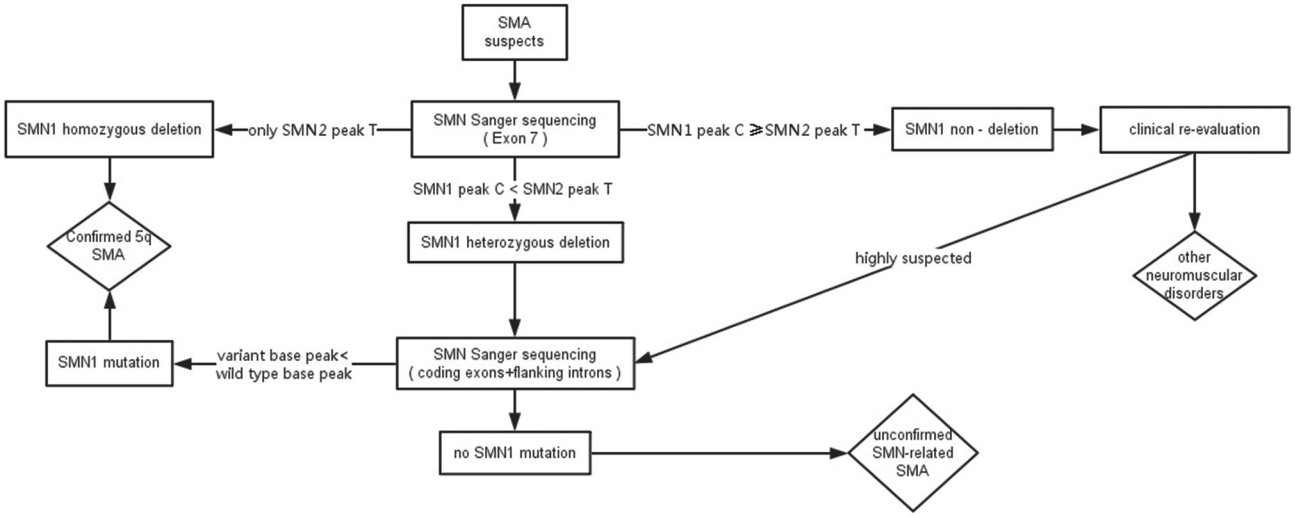

Figure 7: Diagnostic procedure for SMA based on Sanger DNA sequencing. SMA: Spinal muscular atrophy; *SMN*: Survival motor neuron gene.

Table 1: Comparison of cost and time between Sanger DNA sequencing and traditional strategy

Strategy	Detection of *SMN1* deletion			Screening of *SMN1* variations			Total	
	Items	Time (h)	Cost (RMB Yuan)	Items	Time (h)	Cost (RMB Yuan)	Time (h)	Cost (RMB Yuan)
MLPA combined with TA cloning	DNA extraction	0.5	10	RNA extraction	1.0	50	About 266.5	790
	MLPA reaction	About 20.0	150	RT-PCR reaction	3.5	20		
	Capillary electrophoresis	1.5	10	Cloning	240.0	550		
	Total	About 22.0	170	Sequencing	244.5	620		
Sanger-based method	DNA extraction	0.5	10	PCR reaction	1.5	5 × 6	7.5	255
	PCR reaction (exon 7)	1.5	5	(others)	2	30 × 6		
	Sequencing	2.0	30	Sequencing				
	Total	4.0	45		3.5	210		

SMN1: Survival motor neuron gene 1; MLPA: Multiplexed ligation-dependent probe amplification; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription-polymerase chain reaction.

Among those five subtle *SMN1* variants, Ser8Lysfs*23 and Leu228* were the two most common variants in Chinese SMA population.[13] Three novel variants, Tyr276His, Ser143Phefs*5, and Pro218Hisfs*26, were reported in this study for the first time. According to *the Standards and Guidelines for the Interpretation of Sequence Variants of American College of Medical Genetics and Genomics*,[14] we classified Tyr276His as pathogenic and weighted as moderate and Ser143Phefs*5 and Pro218Hisfs*26 were classified as pathogenic and weighted as very strong. For *SMN1* subtle mutation screening, our method is based on segmental PCR amplification; it is actually not strict in the quality and integrity of the DNA sample. However, if the PCR reaction is not effective, the variation on the primers should be considered. In summary, compared with long AS-PCR,[15] our method is easier to practice at the technical level.

Moreover, based on Sanger DNA sequencing, we also developed a simple diagnostic strategy for SMA [Figure 7]. For *SMN1* deletion, our strategy had shorter duration (4 h) and lower cost (45 Yuan), which were 1/5th and 1/4th of the traditional method, respectively. For screening *SMN1* variation, our strategy was significantly superior to the traditional one in terms of both turnaround time and cost.

However, limitation of the presented method should be considered. This was because the Sanger DNA sequencing used for analyzing the amount of *SMN* demonstrated the relative amount of *SMN1* and *SMN2* genes; when a case harbored *SMN1* and *SMN2* at equal amounts of gene, regardless of the copy number ratio of 1:1 or 2:2, Sanger DNA sequencing always showed equal peak heights for the two bases at the differential sites. Therefore, Sanger DNA sequencing is unable to detect the *SMN1* heterozygous deletion when the copy number ratio of *SMN1*:*SMN2* is equal to 1:1. However, in our study, none of this category was found in 130 SMA-suspected patients. Moreover, regarding patients with *SMN1* nondeletion using Sanger DNA sequencing, we recommended that those patients should return to their clinicians for re-evaluation. Subsequently, we would screen *SMN* variations for those highly suspected patients in order to avoid missed diagnosis.

In conclusion, this study suggested that Sanger DNA sequencing can be used in the relative quantification of copy number under certain conditions, not solely as the

gold standard for qualitative analysis of sequence variation. Based on Sanger DNA sequencing, we have developed a novel method for the relative quantification of *SMN* genes. It can be used not only to diagnose SMA associated with *SMN1* homozygous deletion, but also to screen *SMN1* subtle variations. We have also developed a diagnostic procedure based on this method, which has been validated as being appropriate for the diagnosis of SMA in Chinese patients and has potentially wider clinical application.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

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Conflicts of interest

There are no conflicts of interest.

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脊髓性肌萎缩诊断新方法：Sanger测序相对定量分析*SMN1/SMN2*基因

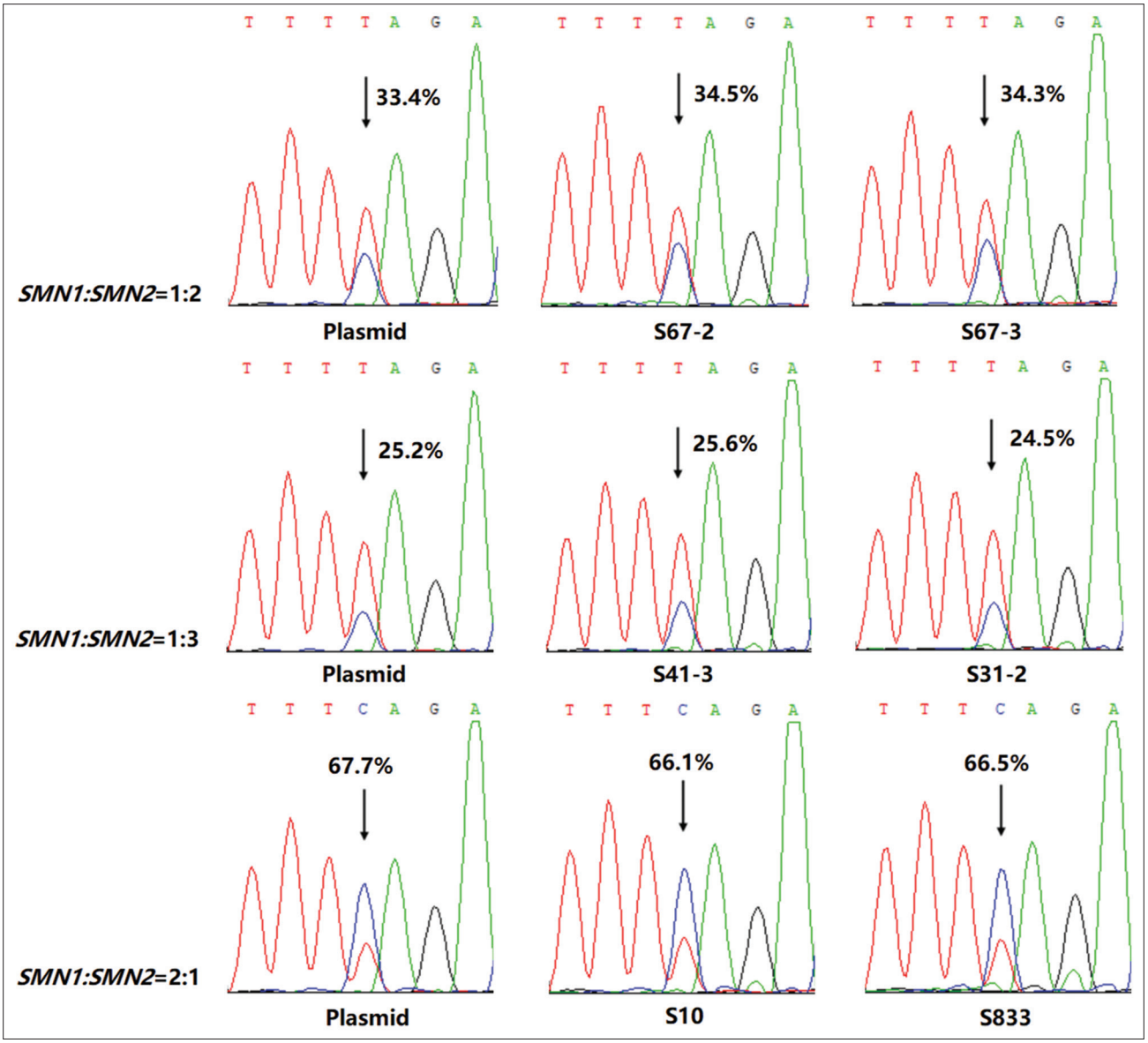
摘要

背景：脊髓性肌萎缩（SMA），可由运动存活基因1（*SMN1*）的纯合缺失或复合杂合突变导致。因此，*SMN1*基因检测是诊断SMA的关键。本研究旨在建立和评估一种新的适于缺失型和复合杂合突变型SMA的诊断方法。

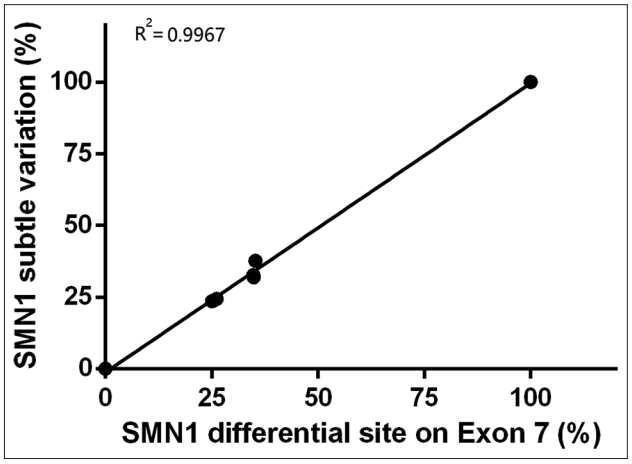
方法：本研究纳入2003年-2016年在首都儿科研究所遗传室进行基因检测的1494例SMA疑似患儿。1364例（2003年-2014年）疑似患儿应用传统诊断策略；130例（2015–2016年）采用新的诊断方法进行检测，并经MLPA和TA克隆测序验证：PCR同时扩增*SMN1*和*SMN2*基因外显子区域；Mutation Surveyor软件通过计算等位基因比例用于定量分析*SMN1*基因以及检测*SMN1*基因内变异。最终比较传统和新的诊断策略的检测周期和检测费用。

结果：1364例SMA疑似患儿中，576例为*SMN1*纯合缺失，27例为*SMN1*复合杂合突变。新的诊断策略显示130个SMA疑似患儿中，*SMN1*基因纯合缺失59例，杂合缺失8例：*SMN1*基因在外显子7上的特异碱基峰高比例在*SMN1:SMN2*为1:2的病例中为34.6%±1.0%，在*SMN1:SMN2*为1:3的病例中为25.5%±0.5%。此外，在8例杂合缺失的病例中检测到6例患儿携带5种微小变异，2例为p.Ser8Lysfs *23，p.Leu228*、p.Pro218Hisfs *26、p.Ser143Phefs*5和p.Tyr276His各1例，变异等位基因比例分别为31.9%、23.9%、37.6%、32.8%、24.5%和23.6%，且均与相应的外显子7上的*SMN1*基因特异碱基峰高比例一致，提示微小变异位于*SMN1*基因。上述结果均与MLPA和TA克隆测序结果一致。最后，新诊断策略和传统诊断策略的检测周期分别为7.5小时和266.5小时；新诊断策略的检测费用为传统的28.5%。

结论：Sanger测序结合Mutation Surveyor软件分析用于诊断SMA具有潜在应用性。



Supplementary Figure 1: Sanger DNA sequencing for *SMN* exon 7 both in plasmid and blood: The C/T base peaks on exon 7 of the *SMN* showed consistency with the *SMN1*:*SMN2* gene ratio not only in plasmid mixture, but also in blood DNA (the arrows indicate the differential site on exon 7: C for *SMN1* and T for *SMN2*; "Plasmid" represents recombinant plasmid and "Sx-x" demonstrates genomic DNA). The base height was calculated according to peak height of the same base upper and lower 8 bases by Mutation Surveyor software. *SMN*: Survival motor neuron gene.



Supplementary Figure 2: Correlation of percentage of *SMN1* subtle variation and *SMN1* differential site on exon 7. *SMN1*: Survival motor neuron gene 1.