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...
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, lymphoma, psoriasis vulgaris, developmental disorders, and inherited diseases. Ellard et al. 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. and Song et al. 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’-TGTTTGAAACAAATACTTT-3’; antisense: 5’-AAAAAGTCTGCTTGCTGCTGCT-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.](image-url)
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 (R²) 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
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
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*) for one case, 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
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...
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 \textit{SMN1} heterozygous deletion: Ser8Lysfs*23, Leu228*, Tyr276His, Ser143Phefs*5, and Pro218Hisfs*26. Those patients without \textit{SMN1} subtle variants were recommended to return to their clinician for reassessment.

Figure 6: Sanger DNA sequencing for screening \textit{SMN1} variation. (a) The ratio of \textit{SMN1}/\textit{SMN2} was 1.3. (b) The ratio of \textit{SMN1}/\textit{SMN2} was 1.2. For the first row sequencing chromatogram, the arrows indicate the differential site on exon 7 (C for \textit{SMN1} and T for \textit{SMN2}). The second row sequencing chromatogram shows \textit{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 \textit{SMN1}. \textit{SMN}: Survival motor neuron gene.
Among those five subtle SMN1 variants, Ser8Lysfs*23 and Leu228* were the two most common variants in Chinese SMA population. 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

![Figure 7: Diagnostic procedure for SMA based on Sanger DNA sequencing. SMA: Spinal muscular atrophy; SMN: Survival motor neuron gene.](image)

| 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)** |
| DNA extraction | 0.5 | 10 | RNA extraction | 1.0 | 50 | About 266.5 | 790 |
| MLPA combined with TA cloning | RA | 150 | RT-PCR reaction | 3.5 | 20 | | |
| MLPA reaction | About 20.0 | 170 | Cloning | 240.0 | 550 | | |
| Capillary electrophoresis | 1.5 | 10 | Sequencing | 244.5 | 620 | | |
| Total | About 22.0 | 170 | (others) | 5 × 6 | | 7.5 | 255 |
| DNA extraction | 0.5 | 10 | PCR reaction (exon 7) | 1.5 | 5 | | |
| Sanger-based method | | | Sequencing | 2.0 | 30 | | |
| PCR reaction (exon 7) | 1.5 | 5 | Sequencing | 2.0 | 30 | | |
| Total | 4.0 | 45 | Sequencing | 2.0 | 30 | | |
| 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.
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.Pro143Hisfs *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.