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Research article

Variants located in intron 6 of *SMN1* lead to misdiagnosis in genetic detection and screening for SMA

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ABSTRACT

Accurate genetic diagnosis is necessary for guiding the treatment of spinal muscular atrophy (SMA). An updated consensus for the diagnosis and management of SMA was published in 2018. However, clinicians should remain alert to some pitfalls of genetic testing that can occur when following a routine diagnosis. In this study, we report the diagnosis of three unrelated individuals who were initially misdiagnosed as carrying a homozygous deletion of *SMN1* exon 7. MLPA (P060 and P021) and qPCR were used to detect the copy number of *SMN*. *SMN1* variants were identified by *SMN1* clone and next-generation sequencing (NGS). Transcription of *SMN1* variants was detected using qRT-PCR and ex vivo splicing analysis. Among the three individuals, one was identified as a patient with SMA carrying a heterozygous deletion and a pathogenic variant (c.835-17_835-14delCTTT) of *SMN1*, one was a healthy carrier only carrying a heterozygous deletion of *SMN1* exon 7, and the third was a patient with nemaline myopathy 2 carrying a heterozygous deletion of *SMN1* exon 7. The misdiagnosis of these individuals was attributed to the presence of the c.835-17_835-14delCTTT or c.835-17C > G variants in *SMN1* intron 6, which affect the amplification of *SMN1* exon 7 during MLPA-P060 and qPCR testing. However, MLPA-P021 and NGS analyses were unaffected by these variants. These results support that additional detection methods should be employed in cases where the *SMN1* copy number is ambiguous to minimize the misdiagnosis of SMA.

1. Introduction

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive genetic diseases of infancy and early childhood, with a high incidence of 1 in 10 000 live births and a worldwide carrier frequency of 1/51 [1,2]. SMA is characterized by muscle weakness, hypotonia, and ultimately muscle atrophy. Based on the age of onset and achieved motor milestones, SMA is classified into five groups from heavy to light, or type 0 through 4 [3,4].

SMA results from a biallelic deletion or occurrence of pathogenic variants in the survival motor neuron 1 gene (*SMN1*), which lies on chromosome 5q13 [5]. Approximately 95% of patients with SMA carry either a homozygous deletion of *SMN1* or *SMN1* to *SMN2*

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conversion. However, a heterozygous deletion alongside an intragenic pathogenic variant within the coding region or splicing sites is responsible for the disease in the remaining 3–5% of patients [6,7]. *SMN2*, a nearly identical paralog of *SMN1*, is currently the most important modifier of disease phenotype [8,9]. Multiplex ligation-dependent probe amplification (MLPA) and quantitative real-time PCR (qPCR) are most commonly recommended as methods for detecting the copy numbers of *SMN1* and *SMN2* [10]. In this study, we report the results of the genetic testing of three unrelated individuals who received false-positive MLPA and qPCR test results due to the presence of two variants in the 3′ end of intron 6 in *SMN1*. These cases did subsequently receive an accurate diagnosis using comprehensive detection technology, including MLPA P021 and P060 kits, real-time PCR, RT-PCR binding clone-sequencing, in vitro splicing experiments, and next-generation sequencing (NGS).

2. Materials and methods

2.1. Study participants

Three individuals with an ambiguous diagnosis of SMA were referred to our center for further genetic testing (Table 1).

Case 1 was a 4-year and 10-month-old female. She presented with symptom onset of severe hypotonia at 4 months, followed by progressive muscular weakness, delayed motor development, progressive decline in pulmonary function, and scoliosis. She was first diagnosed with SMA with homozygous deletion of *SMN1* exon 7*,* but heterozygous deletion of exon 8 after screening with the MLPA P060 kit in another lab at 10 months. Following she entered into our SMA register system, her molecular genetic analysis was

Table 1
Clinical manifestation and genetic testing results of the three cases.

	Case 1	Case 2	Case 3
Age	4y10 m	30y	6y9m
Sex	Female	Female	Female
Onset age	4 m	No manifestation	15d
Age developed head control	3 m	3 m	12 m
Age could sit independently	un-sitting	5 m	18 m
Age could walk independently	un-walking	14 m	4y
Age developed respiratory insufficiency	1y10 m	Normal	2 m
Age developed dysphagia	Normal	Normal	1 m +
Orthopedic diagnoses	Scoliosis，18 m	Normal	Kyphosis，1y
Myopathic face	No	No	Yes
Muscle tone	Hypotonia	Normal	Hypotonia
Motor powers	Grade 3 of upper limbs and grade 2 of lower limbs, Proximal > distal and lower > upper	Normal	Grade 4 of upper and lower limbs, Proximal > distal and lower > upper
Other manifestation	Areflexia	Normal	Facial muscle weakness, small jaw, high-arched palate, short tongue, funnel chest, joint contractures, Areflexia, Gowers sign
EMG	Neurogenic damage	NA	Myopathic changes early in disease seen on EMG
Muscle biopsy	NA	NA	Nemaline bodies (rods) seen on Gomori trichrome staining
CK levels	Slightly elevated	NA	Normal
MLPA-P060	0 copies of *SMN1* exon 7; 1 copy of *SMN1* exon 8	Ambiguous results: FR of *SMN1* exon 7 < 0.3; 1 copy of *SMN1* exon 8	Ambiguous results: FR of *SMN1* exon 7 < 0.3, 1 copy of *SMN1* exon 8
qPCR	0 copies of *SMN1* exon 7	0 copies of *SMN1* exon 7	0 copies of *SMN1* exon 7
MLPA-P021	1 copy of *SMN1* exon 7 and exon 8	1 copy of *SMN1* exon 7 and exon 8	1 copy of *SMN1* exon 7 and exon 8
SMN1 Clone sequencing (NM_000344.4)	c.835-17_c.835-14delCTTT	c.835-17C > G	c.835-17C > G
WES	*SMN1:* c.835-17_c.835-14delCTTT	*SMN1*: c.835-17C > G	*SMN1*: c.835-17C > G *NEB* gene: c.25177_25189del (p. Arg8393Valfs*20); c.24604G > T (p. Glu8202*); c.16817A > G (p. Tyr5606Cys)
Initial diagnosis, age	SMA with homozygous deletion of *SMN1* exon7,10 m	SMA with homozygous deletion of *SMN1* exon7, 30y	SMA with homozygous deletion of *SMN1* exon7, 21 m
Final diagnosis, age	SMA (compound heterozygous variants), 22 m	SMA carriers, 30y	Nemaline myopathy 2, 6y9m
Prognosis	Alive	Healthy, alive	alive

y, year; m, month; NA, not available; FR, final ratio.

crosschecked as regular.

Case 2 was a healthy 30-year-old pregnant female who was initially screened for carrier detection of SMA. Genetic screening in another prenatal center using the MLPA P060 kit identified a homozygous deletion of *SMN1* exon 7 and only one copy of *SMN2*. The pregnant female presented with no SMA phenotype and was therefore referred to our center for her *SMN1* status reconfirmation.

Case 3 was a 6-year and 9-month-old female. She presented with limb hypotonia, weak crying, and severe dysphagia since birth and was generally hypotonic with poor head control. She was diagnosed with SMA at 21 months of age based on the detection of a homozygous deletion of *SMN1* exon 7 using the MLPA P060 kit in another hospital. She was referred to our hospital for therapeutic consultation and was in the SMA follow-up cohort at 3 years of age. Regular follow-up revealed that her clinical presentation gradually deviated from the disease characteristics of SMA instead of showing typical characteristics of congenital myopathy. However, electromyography (EMG) identified the presence of myopathic changes. Muscle biopsy revealed nemaline bodies, intranuclear rods, and type I fiber predominance (Supplementary Fig. 1). Subsequently, testing with two MLPA kits (P060 and P021) and whole-exome sequencing (WES) were performed to confirm her diagnosis.

In total, 3 mL of peripheral blood in EDTA-anticoagulant tubes was collected from each case and their parents. Genomic DNA was extracted from all blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) and stored at −20 °C for subsequent use. All participants or their legal guardians provided written informed consent. This study was approved by the ethics committee of the Capital Institute of Pediatrics (approval no. SHERLL2017007).

2.2. *SMN1* and *SMN2* copy number analysis using MLPA

We performed routine MLPA assays for all three cases using a SALSA MLPA P021–B1 kit. The copy numbers of *SMN1* and *SMN2* were analyzed using the Coffalyser. Net™ software (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. Additionally, the SALSA MLPA P060–B2 kit (MRC-Holland) was used to verify the test results of other hospitals and compare results with those obtained using the SALSA MLPA P021–B1 kit. Genomic DNA (100 ng) from each patient was used in all experiments and capillary electrophoresis was performed using an ABI 3730 automatic sequencing system (Applied Biosystems, USA). All samples were analyzed at least twice. Healthy individuals with a normal copy number (two copies) were defined as having a final ratio (FR) between 0.80 and 1.20 for all probes included in the Probemix. A FR value between 0 and 0.1 indicated a homozygous deletion (zero copy), between 0.4 and 0.65 indicated a heterozygous deletion (one copy), between 1.3 and 1.65 indicated a heterozygous duplication (three copies), and between 1.75 and 2.15 indicated a heterozygous triplication or homozygous duplication (four copies). All other FR values were considered ambiguous results for copy number.

2.3. *SMN1* exons 7 and 8 copy number analysis using qPCR

In instances where the copy number of *SMN1* exon 7 differed between the results of the SALSA MLPA P021–B1 and P060–B2 kits, we performed qPCR assays (Chromysky Medical Research, Shanghai, China) to verify the copy number. This kit contains specific probes for exons 7 and 8 of *SMN1*. *RPP40*, the reference gene, was amplified at the same time as *SMN1*. Each PCR reaction (total volume of 20 μL) was performed alongside five controls (no-DNA control, homozygous deletion of *SMN1* control, and three gradients (1:2:4) of normal control with two copies of *SMN1*) using an ABI StepOne plus real-time PCR system (Thermo Fisher, USA). The copy number of *SMN1* exon 7 or exon 8 was evaluated using the 2-ΔΔCt method. For exon 7, $\Delta\Delta Ct > 0.8$ indicated a homozygous deletion, $0.45 \geq \Delta\Delta Ct > -0.45$ indicated a heterozygous deletion, while $\Delta\Delta Ct \leq -0.55$ indicated no deletion. For exon 8, $\Delta\Delta Ct > 1.5$ indicated a homozygous deletion, $0.45 \geq \Delta\Delta Ct > -0.45$ indicated a heterozygous deletion, while $\Delta\Delta Ct \leq -0.55$ indicated no deletion. If the ΔΔCt of exon 7 ranged from −0.55 to −0.45 or from 0.45 to 0.8, and the ΔΔCt of exon 8 ranged from −0.55 to −0.45 or from 0.45 to 1.5, the results were regarded as ambiguous, and the experiments were repeated.

2.4. Identification of *SMN1* variants by genomic DNA PCR plus clone sequencing

The genomic region spanning intron 6 to exon 8 of *SMN1* and *SMN2* was amplified from genomic DNA using the R111 and 541C1120 primers to yield a PCR product of approximately 1 kb [5,7]. The PCR products were then purified by 1% agarose gel electrophoresis and subcloned into the pEasy-T1 cloning Vector System (TRANSGEN, China) according to the manufacturer's instructions. Between 5 and 10 subclones of *SMN1* and *SMN2* were sequenced. Variants were detected by Sanger sequencing on the ABI 3730 XL DNA Sequencer (Applied Biosystems, USA). Sequencing results were analyzed using the Chromas Lite v2.01 software (Technelysium, Australia).

2.5. Next-generation sequencing (NGS) analysis

Whole-exome sequencing was performed to identify the disease-associated variants in the three cases (Fujungenetics Technologies Co., Ltd, Beijing, China). Genomic DNA was extracted from each patient, and exome sequence capture was performed using the IDT xGen Exome Research Panel v1.0 (Integrated DNA Technologies, USA). DNA libraries were prepared and sequenced using paired-end 300 bp reads on an Illumina Novaseq 6000 platform (Illumina, USA) following the standard manual. The raw data were subjected to quality control and aligned to the human genome hg19 (GRCh37) reference sequence using the FLIMS software. The identified variants were interrogated with several databases, including the dbSNP, gnomAD, 1000 genomes project, Human Gene Mutation Database (HGMD), Aggregation Consortium (ExAC), and ClinVar databases. Any variants detected in the parents of the participants were

identified using Sanger sequencing. The pathogenicity of variants was predicted using the AGVGD, FATHMM, FATHMMMKL, LRT, MAPP, METALR, METASVM, MUTATIONASSESSOR, SIFT-Alamut/PROVEAN, SIFT, Polyphen-2, and mutation taster programs. *SMN* gene copy numbers were estimated based on misalignment, as described by Lee et al. [11].

2.6. Transcription of SMN1

Total RNA was isolated from peripheral blood samples from the three cases. First-strand cDNA synthesis was performed using 0.5 μg total RNA, random primers, and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Specific PCR primers (SMNE6F and 541C1120) were used to amplify exons 6–8 (for both *SMN1* and *SMN2*) with the LA Taq polymerase (TAKARA, Kyoto, Japan). These *SMN* transcripts were subjected to restriction digestion (DdeI) and sequenced. Real-time PCR was used to quantify the *SMN* transcripts as previously described [7,12].

2.7. Plasmid construction, transfection, and ex vivo splicing analysis of two variants in SMN1 intron 6

Two *SMN1* mutant plasmids (*SMN1*-c.835-17C > G and *SMN1*-c.835-17_c.835-14del CTTT) were constructed using PCR-based site-directed mutagenesis with fly-pfu DNA polymerase (TRANSGEN, China) with minigene templates (pEasy-M2-*SMN1*) as previously described [7]. In total, 2 μg of each minigene plasmid was mixed with 10 μL TransLipid Transfection Reagent (TRANSGEN, China) and transfected into HEK293 cells. After 24 h, the transfected cells were harvested, and the total RNA was extracted. Reverse transcription was performed using 5 μg of total RNA. To calculate the splicing efficiency of these wild-type and mutant-type plasmids, the plasmid-derived transcripts were specifically amplified using a plasmid-specific forward primer (pEasy-M2F) and a FAM-labeled SMN-specific reverse primer SMNE8R. The PCR products were then run on the ABI 3730 automatic sequencing system (Applied Biosystems, USA), and the raw data were analyzed using the Gene marker version 1.75. Splicing efficiency was calculated as the full-length transcript read count divided by the sum of the full-length and truncated transcript read counts. These assays were

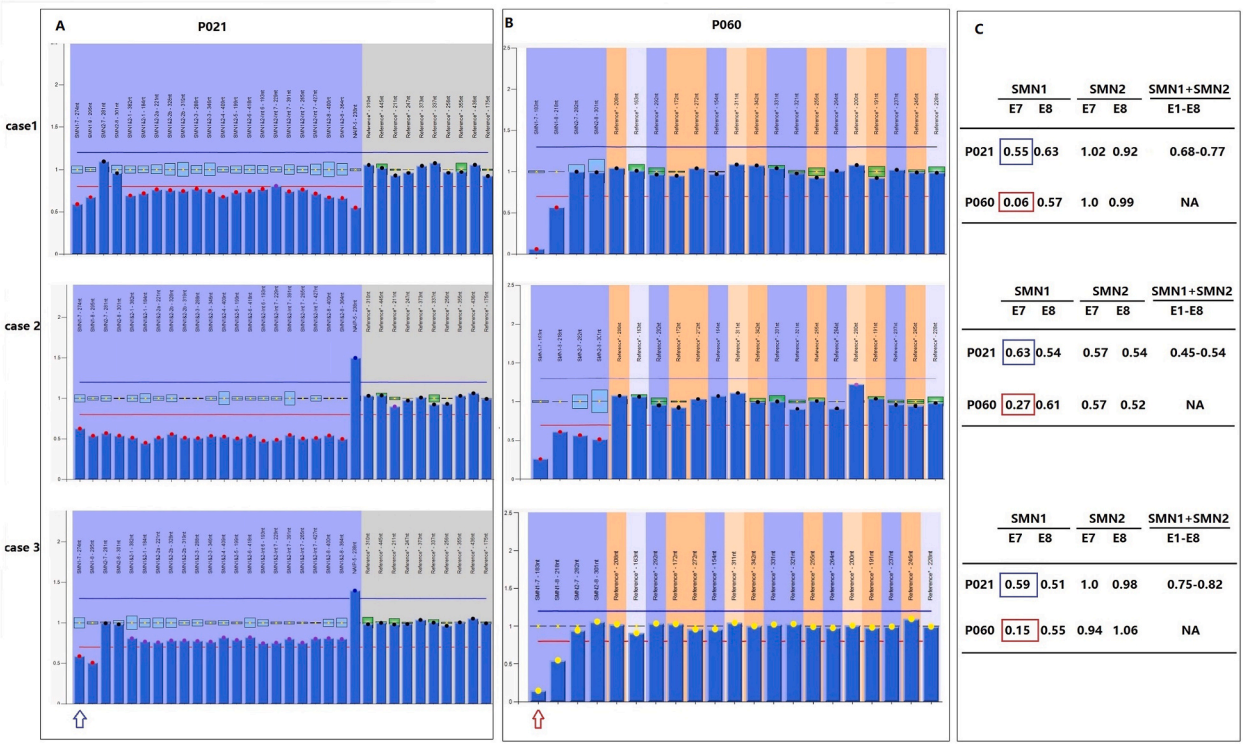


Fig. 1. Copy number analysis of *SMN* in three cases using the MLPA P021 and MLPA P060 kits. A, Copy number analysis using the MLPA P021 assay. The blue arrow indicates the *SMN1* exon 7. B, Copy number analysis using the MLPA P060 assay. The red arrow indicates *SMN1* exon 7. C, The final ratio (FR) results of three cases using the MLPA P021 assay and MLPA P060 assay. The results from the MLPA P021 assay indicated that the copy number of *SMN1* exon 7 in the three cases was one because the final ratio (FR) values of *SMN1* exon 7 all fell within the range of 0.4–0.65 (blue box). Conversely, the results from the MLPA P060 assay showed that the copy number of *SMN1* exon 7 in case 1 was zero, indicating a homozygous deletion, as the FR of *SMN1* exon 7 was below 0.1. The results for cases 2 and 3 were considered ambiguous as the FR values (red box) were 0.27 and 0.15, respectively. The FR value of *SMN1* exon 8 was between 0.4 and 0.65 for all cases when using either of the MLPA kits, indicating a heterozygous deletion (one copy). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

performed in triplicate.

3. Results

3.1. Clinical information of the three cases

Clinical information, including the age of onset, clinical characteristics, motor milestones, results of the auxiliary examination, complications, prognosis, and results of the genetic analysis were obtained for all three cases. This information is presented in Table 1.

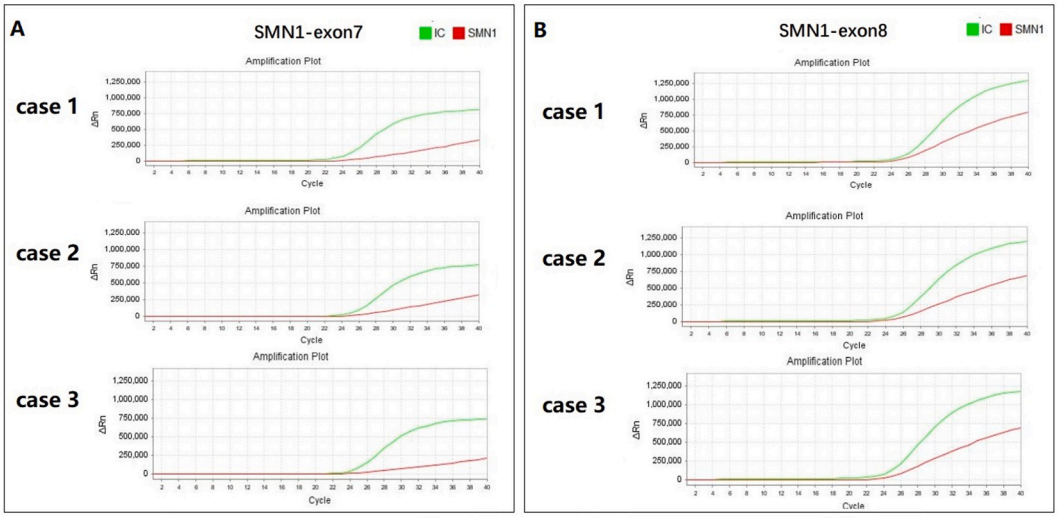
3.2. Inconsistent copy number of SMN1 exon 7 using MLPA-P021 and MLPA-P060

We observed that in all three cases, the MLPA P021 assay gave a FR range of 0.55–0.63 and 0.51–0.63 for *SMN1* exon 7 and exon 8, respectively (Fig. 1). These values indicated that all individuals carried one copy of *SMN1* exons 7 and 8. Conversely, the MLPA P060 analysis for case 1 revealed a FR value of 0.06 for exon 7, indicating a homozygous deletion of *SMN1* exon 7 (Fig. 1), as reported previously by other laboratories. Regarding cases 2 and 3, we found that the FR values of *SMN1* exon 7 were 0.15 and 0.27, respectively. Therefore, we categorized them as ambiguous results. Notably, both MLPA kits resulted in consistent copy number values of *SMN1* exon 8 and *SMN2* exons 7 and 8 in all three cases (Fig. 1).

To investigate the effect of PCR amplification in the MLPA-P060 assay of the variants located on or near the binding site of the PCR probe for exon 7, we reviewed rare variants reported near the intron 6-exon 7 junction in patients from our center. We identified five variants (c.835-5G > C, c.835-1G > A, c.835G > C, c.863G > T, and c.869C > T) located within this region and in the *SMN1* exon 7 probe sequence in six patients from our center, with two of them carrying the c.863G > T variant. Accordingly, we determined that the copy numbers of *SMN1* exon 7 and exon 8 were consistent when using these two MLPA kits (P060 and P021, Supplementary Fig. 2).

3.3. SMN1 exon 7 and exon 8 copy numbers detected using qPCR

We also used qPCR as a second method to determine the copy numbers of *SMN1* exon 7 and exon 8. In all three cases, our qPCR analysis indicated a homozygous deletion of *SMN1* exon 7, as the ΔΔCt values were in the range of 1.39–3.05. Regarding *SMN1* exon 8, our analysis indicated one copy number in case 1 (ΔΔCt = 0.07), whereas ambiguous results were observed for cases 2 and 3 (ΔΔCt values in the range 0.59–0.91) (Fig. 2). Therefore, we concluded that our qPCR results for *SMN1* exon 7 were in agreement with those



C

SMN1-exon7			SMN1-exon8		
sample	ΔΔCt	Result	sample	ΔΔCt	Result
case 1	2.49	Homozygous deletion	case 1	0.07	Heterozygous deletion
case 2	1.39	Homozygous deletion	case 2	0.59	Ambiguous
case 3	3.05	Homozygous deletion	case 3	0.91	Ambiguous

Fig. 2. Results of copy number of *SMN1* by using qPCR in the three cases. A, The amplification plot of *SMN1* exon 7. No amplification was detected in the amplification plot of *SMN1* exon 7 in all cases. B, The amplification plot of *SMN1* exon 8 in each of the three cases. C, The detecting results of *SMN1* exon 7 and exon 8 based on the ΔΔCt value. The ΔΔCt value of *SMN1* exon 7 in three cases all exceed 0.8 (cut-off value), indicating a homozygous deletion of *SMN1* exon 7. For *SMN1* exon 8, the results of the amplification plot of *SMN1* exon 8 indicate a heterozygous deletion of *SMN1* exon 8 (ΔΔCt = 0.07) in case 1, whereas the amplification results for cases 2 and 3 were ambiguous.

of MLPA-P060 but not MLPA-P021.

3.4. *SMN1* variant detection using clone sequencing

We performed Sanger sequencing and identified a small deletion variant (c.835-17_c.835-14del CTTT) in *SMN1/SMN2* intron 6 in case 1 and her father, and another variant (c.835-17C > G) in *SMN1/SMN2* intron 6 in cases 2 and 3, which was maternally inherited in both cases. We then performed clone sequencing to validate the origin of these two variants from the *SMN1* gene (Fig. 3).

3.5. *Whole exome sequencing (WES): SMN variant detection and estimation of SMN copy number*

To assist our diagnosis, we performed WES to screen for variants in *SMN* and other candidate genes in the three cases (Fig. 4). Our analysis identified 18 reads containing cytosine at c.840, which were therefore assigned to *SMN1* that carried the c.835-17_c.835-14del CTTT variant in case 1. Conversely, we noticed that the reads from *SMN2* did not carry this variant. This deletion variant was classified as a variant of uncertain significance according to the guidelines of the American College of Medical Genetics (ACMG). In addition, we found that 23 reads from case 2 and 20 reads from case 3 carried the c.835-17C > G variant and encompassed the paralogous sequence variant at c.840, which is indicative of the *SMN1* gene. Based on information from the WES database of local non-SMA individuals (Fujungenetics Technologies Co., Ltd, Beijing, China), the c.835-17C > G variant of *SMN1* was characterized as a single nucleotide polymorphism (SNP, rs 1749768663) with low frequency (0.00048679). According to the ACMG guidelines, the c.835-17C > G variant was classified as benign (PM2_supporting + BS3 + BP4 + BP5). Following WES, we further estimated the copy numbers of exon 7 in *SMN1* and *SMN2* using misalignment. The results from this analysis were in close agreement with the MLPA P021 results.

Furthermore, WES analysis revealed that case 3 carried three heterozygous variants in the nebulin (***NEB***) gene: c.24604G > T (p. Glu8202*), c.25177_25189del (p.Arg8393Valfs*20), and c.16817A > G (p.Tyr5606Cys). *NEB* has been reported to be responsible for nemaline myopathy type 2 (NEM2). We validated this finding by conducting Sanger sequencing across all members of the family for case 3 (Fig. 5). We determined that the c.24604G > T (p. Glu8202*) variant was paternally inherited, whereas the other two variants c.25177_25189del (p. Arg8393Valfs*20) and c.16817A > G (p. Tyr5606Cys) were in-cis and maternally inherited. According to the ACMG guidelines, the nonsense variant c.24604G > T (p. Glu8202*) and deletion variant c.25177_25189del (p. Arg8393Valfs*20) were classified as pathogenic (PVS1 + PM2_Supporting + PM3), whereas the missense variant c.16817A > G (p.Tyr5606Cys) was classified as a variant of uncertain significance (PM2_Supporting + PP3).

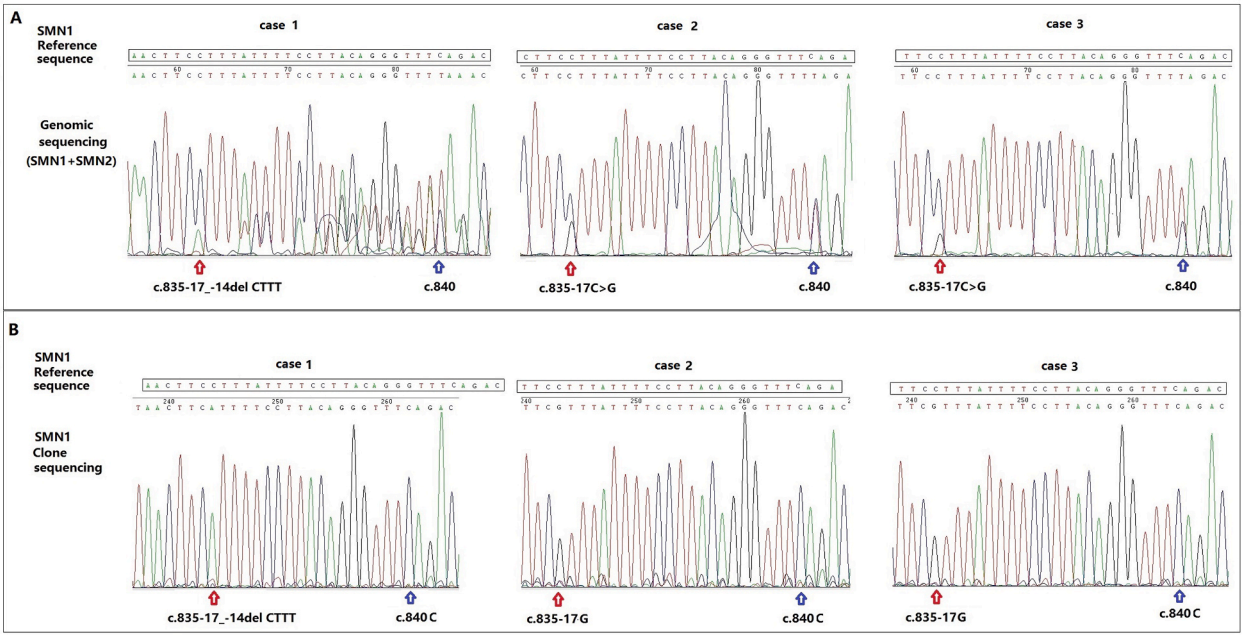


Fig. 3. Sanger sequencing of *SMN1* for each case. A, The Sanger sequencing diagram of genomic sequence for the region spanning intron 6 to exon 7 in *SMN1* and *SMN2*. Analysis revealed the presence of a deletion variant (c.835-17_c.835-14delCTTT) in intron 6 in case 1 and the c.835-17C > G variant in intron 6 in cases 2 and 3. The red arrow indicates the variant sites and the blue arrow indicates the c.840 site. B, The sequencing diagram of the *SMN1* clones. The red arrow indicates the variant sites and the blue arrow indicates the c.840 site. The results confirmed that the identified variants (c.835-17_c.835-14delCTTT and c.835-17C > G) were present in the *SMN1* and not in the *SMN2* gene in these three cases, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

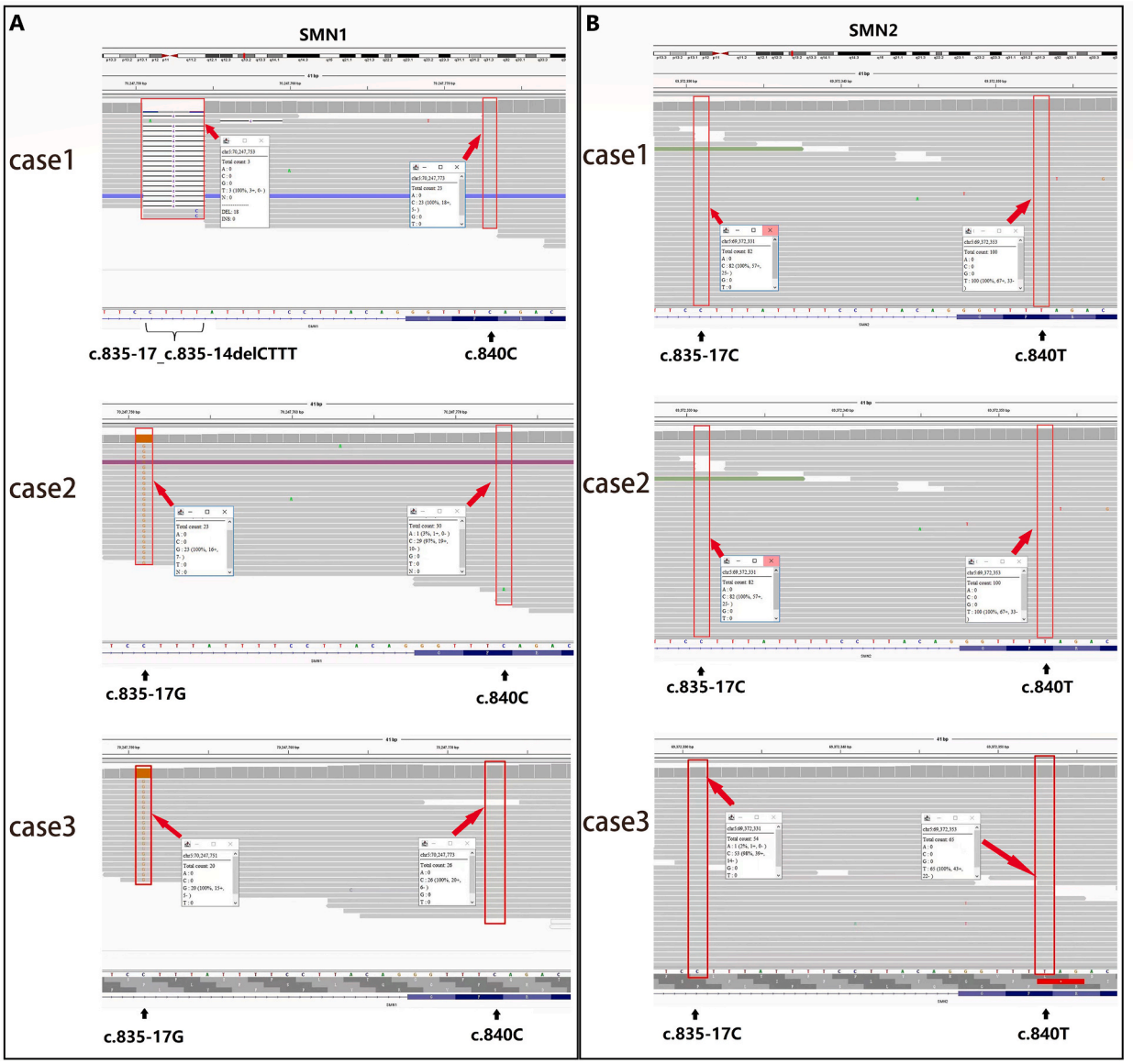


Fig. 4. *SMN1* genetic variants in each case detected using WES. A, The *SMN1* sequences in the region surrounding the c.840 paralogous sequence variant as visualized in the Integrative Genomics Viewer. The deletion variant (c.835-17_c.835-14delCTTT) was detected in case 1, whereas the c.835-17C > G variant was detected in cases 2 and 3, confirming the Sanger sequencing results. The sequence at paralogous sequence variant confirmed that these variants were all in the *SMN1*. B, The *SMN2* sequences in the region surrounding the c.840 paralogous sequence variant as visualized in the Integrative Genomics Viewer. These two variants were not detected in the *SMN2* gene.

3.6. c.835-17_835-14del CTTT variant causes SMN1 exon 7 skipping in contrast to c.835-17C > G

To qualitatively analyze the SMN transcripts in peripheral blood samples from the three cases, the transcripts (from exon 6 to exon 8) were digested using the restriction enzyme DdeI, and the fragments were separated using gel electrophoresis. We observed that an undigested Δ7-SMN fragment was the most abundant fragment in case 1 that carried the c.835-17_835-14delCTTT variant. Sanger sequencing of this fragment confirmed that the undigested segment resulted from *SMN1* due to exon 7 skipping.

We found that the levels of *fl-SMN1* transcripts in normal controls and healthy carriers were 921 ± 101.7 and 428.9 ± 77.2, respectively. However, the level of *fl-SMN1* transcripts in case 1 was significantly lower than that in normal controls and healthy carriers, at 113.06 ± 1.9, whereas the levels of *fl-SMN1* transcripts in cases 2 and 3 were similar to those of healthy carriers, at 402.1 ± 27.6 and 436.7 ± 11.4, respectively.

We further conducted ex vivo splicing analysis to detect whether these two variants, c.835-17C > G and c.835-17_835-14delCTTT, perturb the splicing of *SMN1* exon 7. Our analysis showed that the c.835-17C > G variant did not increase the incidence of exon 7

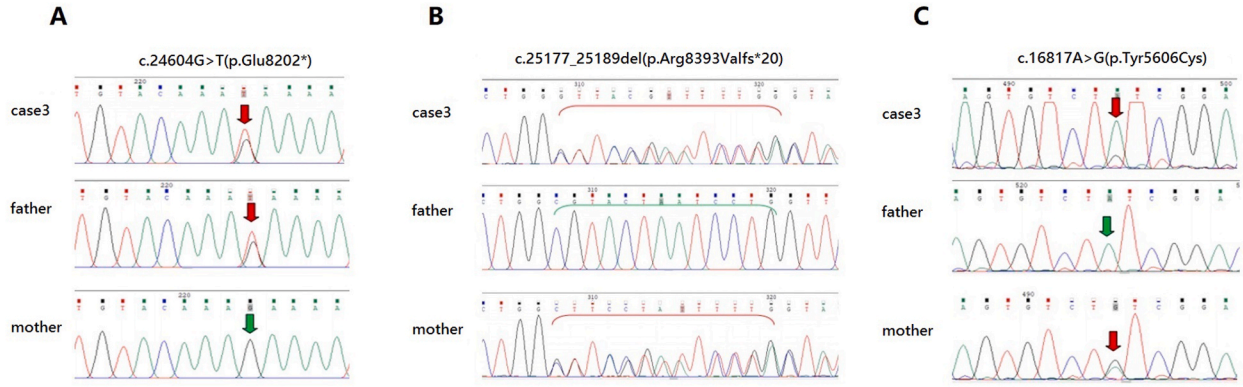


Fig. 5. *NEB* Sanger sequencing results in the family of case 3. A, Pathogenic variant of c.24604G > T (p. Glu8202*). B, Pathogenic variant of c.25177_25189del (p. Arg8393Valfs*20). C, Variant of uncertain significance of c.16817A > G (p.Tyr5606Cys). Three variants were detected within *NEB* in case 3. The red arrow or red line indicates the mutant sites and the green arrow or green line indicates the wild sites. One variant, c.24604G > T (p. Glu8202*), was paternally inherited, whereas the other two variants, c.25177_25189del (p. Arg8393Valfs*20) and c.16817A > G (p. Tyr5606Cys), were both in cis and maternally inherited. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

skipping, exhibiting a skipping rate comparable to that of wild-type *SMN1* plasmids at 7.7%. However, the c.835-17_835-14delCTTT variant caused substantial exon 7 skipping, with a skipping rate over 95% (Fig. 6, Supplementary Fig. 3), similar to that of the wild-type *SMN2* plasmid (90.3%). Therefore, due to its ability to disturb the normal splicing of exon 7 of *SMN1,* the c.835-17_835-14delCTTT variant was classified as likely pathogenic (PS3 + PM2_Supporting + PM3 +PP4).

3.7. Precision diagnosis of the three cases

Based on the molecular genetic analysis and ex vivo splicing analysis, we updated the clinical and genetic diagnoses of the three cases. Case 1, who presented with typical SMA characteristics, was accurately diagnosed as SMA type 1c with one *SMN1* allele deletion and another *SMN1* allele carrying the likely pathogenic variant c.835-17_c.835-14delCTTT. Case 2 carried only one *SMN1* gene with a benign variant c.835-17C > G on it and was therefore diagnosed as a SMA carrier. Case 3 was diagnosed as a nemaline myopathy 2 patient and also a SMA carrier having only one *SMN1* copy hiding a benign variant c.835-17C > G.

4. Discussion

SMA is a neuromuscular disease caused by biallelic mutations in *SMN1*. Therefore, accurate genetic diagnosis can provide the foundation for the implementation of the appropriate clinical therapy. With the approval of various drugs, such as Nusinersen [13–16],

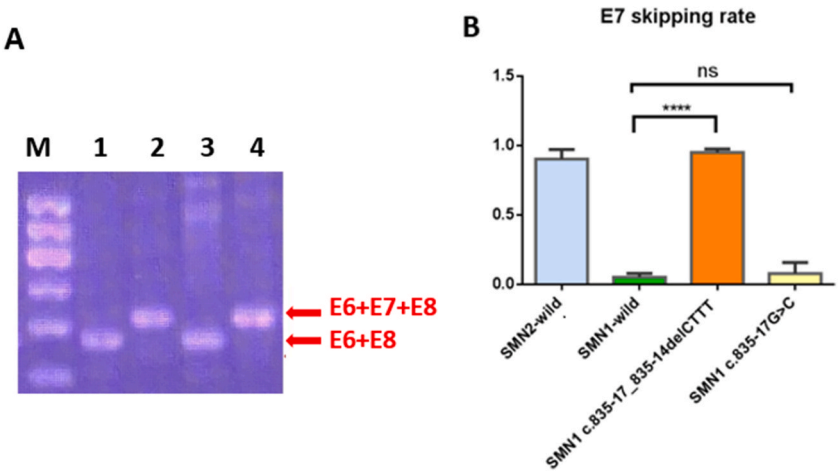


Fig. 6. Splicing analysis of the two variants in vitro. A, The figure shows the products of RT-PCR on ethidium bromide-stained agarose gel. M, marker; lane 1, *SMN2*-wild plasmid; lane 2, *SMN1*-wild plasmid; lane 3, *SMN1* c.835-17_c.835-14del CTTT plasmid; and lane 4, *SMN1* c.835-17C > G plasmid. B, The quantitative analysis results of RT-PCR products. The error bars indicate the SD; P-values were calculated by Student's t-test (****P < 0.0001).

as well as newborn screening (NBS) in the general population [17,18], the management of SMA has entered an era characterized by early diagnosis and pre-symptomatic treatment [19,20]. Accordingly, developments in therapeutic approaches will help to improve the survival time, quality of life, and motor function of patients with SMA [13–16,21–23]. Furthermore, universal SMA carrier screening may act as a primary preventative measure, allowing carrier couples to perform informed reproductive choices, thus eventually reducing the burden of the disease [23,24]. Therefore, obtaining an accurate genetic diagnosis of SMA probands will provide the foundation for the early treatment and multidisciplinary management of SMA. Accurate genetic testing of carriers will improve the outcomes of genetic counseling, and allow for prenatal diagnosis and preimplantation genetic testing, benefiting the families [25–28]. In our study, case 1 was newly diagnosed as having SMA, characterized by a heterozygous deletion of *SMN1* and the presence of a *SMN1* pathogenic variant. This improved diagnosis can thus guide better treatment and multidisciplinary management, improving survival time and the quality of life of patients. Moreover, the patient's family can obtain appropriate genetic counseling and recurrence risk assessment. In case 2, the patient was newly diagnosed as a SMA carrier. Her husband was also screened and characterized as a normal individual with two copies of *SMN1* and no pathogenic variants in this gene. Her fetus was deemed low-risk for SMA. Case 3 was newly diagnosed as having NEM2, not SMA, and therefore avoided inappropriate disease-modifying treatment. Importantly, this genetic information can be used for future prenatal diagnosis within the third family.

The gene responsible for SMA is located in the 5q13 locus, a highly complex and inverted duplication region [5,29,30]. *SMN1* and *SMN2* are identical except for 16 paralogous sequence variants located in intron 6, exon 7, intron 7, and exon 8 [31]. This high degree of homology may lead to difficulties in accurately detecting the copy number of *SMN1* and *SMN2* separately, especially in cases where the genes carry single base variants. To date, two paralogous sequence variants, c.840C > T of exon 7 and c.*239G > A of exon 8, have been used to distinguish *SMN1* and *SMN2* in detection methods. Traditionally, MLPA was considered the gold standard technique for detecting the number of *SMN* copies for SMA diagnosis, SMA carrier screening, and NBS. In addition, qPCR and droplet digital PCR (ddPCR) are also commonly used. MLPA and qPCR cannot easily differentiate between copy numbers greater than three [32,33]. However, ddPCR can accurately measure a large range of SMN copy numbers (0–6 copies). NGS approaches have recently been found to be useful for SMA carrier detection [34–36]. While they can detect intragenic variants, they cannot distinguish *SMN1* from *SMN2* except in the regions surrounding the paralogous sequence variants. In our study, the copy number of *SMN1* and *SMN2* were accurately measured with the MLPA P021 assay but not with MLPA P060 and qPCR. WES (NGS) identified two variants (c.835-17_c.835-14delCTTT and c.835-17C > G) near the c.840 site in intron 6 of *SMN1*.

Errors in genetic diagnosis may hinder the acquisition of accurate and early molecular diagnosis. Although MLPA, qPCR, and ddPCR have been widely used to quantitatively analyze the copies of *SMN1* and *SMN2*, many factors can affect the determination of the *SMN* gene dosage, such as DNA quality, the presence of variants in the target sequence of binding of primers or probes, and the choice of control samples or references, causing inaccurate estimation of copy number. Among these, genetic testing errors due to base variations at the primer or probe binding positions are not uncommon when using established PCR-based qualitative and quantitative technologies [25–27,37–39]. False-positive results have also been reported in the diagnosis of other diseases, such as DMD and hereditary breast cancer, for which the MLPA assay was used [27,37,38]. Regarding SMA, a patient carrying the c.863G > T variant and a heterozygous deletion of *SMN1* was previously diagnosed as carrying a homozygous deletion of *SMN1* based on PCR-restriction fragment length polymorphism (RFLP) testing. As the c.863G > T variant is located in the target sequence of the reverse primer of PCR-RFLP, it inhibits the amplification of *SMN1* exon 7 [39,40]. In our study, qPCR and MLPA P060 kit analyses in the three cases, all of which carried an intron 6 variant (c.835-17_c.835-14delCTTT or c.835-17C > G), showed contradictory and ambiguous results due to weak or no amplification, marking a diagnostic pitfall. The c.835-17 site is located in the middle of the P060 upstream probe binding region (from c.835-30 to c.840, a total of 35 nt) in *SMN1* exon 7 (https://www.mrcholland.com). The c.835-17C > G variant may therefore obstruct the binding efficiency of the upstream probe to *SMN1* exon 7, whereas the c.835-17_c.835-14delCTTT variant, a four-base deletion, may completely disrupt probe binding (resulting in a FR closer to zero). However, we found that several rare variants near the intron 6–exon 7 junction (c.835-5G > C, c.835-1G > A, c.835G > C, c.863G > T, and c.869C > T), which lie off the center of the P060 *SMN1* exon 7 probe binding site, may not influence probe binding. The discrepancies observed between different methods in our study strongly suggest that ambiguous results for copy number detected with the MLPA P060 SMA Carrier Kit should be confirmed using a different technique, such as MLPA P021, NGS, or Sanger sequencing.

The SALSA MLPA Probemix P060–B2 SMA Carrier and SALSA MLPA Probemix P021–B1 SMA kits are both semiquantitative assays used for the detection of deletions or duplications of the *SMN* genes in genomic DNA. Compared with P060–B2, P021–B1 contains 16 additional probes, which span the genomic region from exon 1 to exon 8, for detecting the combined copy numbers of *SMN1* and *SMN2*. Therefore, this more comprehensive kit can detect the deletion or repetition of one or several exons of *SMN* genes. Notably, in our study, MLPA P060 analysis in the three cases showed that the FR of *SMN1* exon 7 was abnormally low, especially falling below 0.1 in case 1, indicating homozygous deletion in all cases, thus resulting in misdiagnosis. As this pattern was not observed with the MLPA P021 kit assays, it appears that the two variants (c.835-17C > G and c.835-17_c.835-14delCTTT) are more problematic for determination of the copy number with the SALSA kit (P060–B2). Therefore, the SMA P021 kit is better suited for the diagnosis of SMA compared with the MLPA P060 SMA Carrier Kit.

Previous studies have shown that more than fifty *cis*-elements are clustered between intron 6 and exon 8, and trans-acting factors are involved in the regulation of *SMN* exon 7 splicing [41,42]. For example, a critical C-to-T mutation at the sixth position (C6U substitution in RNA) of exon 7 can trigger *SMN2* exon 7 skipping [10], whereas a G-to-A substitution at the −44th position (G-44A) of intron 6 [43] and an A-to-G substitution at the 100th position (A100G) of intron 7 was also associated with *SMN2* exon 7 skipping [44]. In addition, a G-to-C substitution at the 25th position of *SMN2* exon 7 (c.859G > C) was reported as a positive disease phenotype modifier that can improve the inclusion of exon 7 [45]. Any variant located in or near these cis-elements should be considered as having the potential to influence the splicing of exon 7 [41,42]. Previous studies have found that the poly-U tract (UUUU,

tetranucleotide) at position −9 to −12 in intron 6 is essential for the binding of hnRNP C1/C2 [46], which modulates the splicing of *SMN* exon 7. In this study we identified two variants, c.835-17C > G and c.835-17_835-14delCTTT, located within intron 6 near this hnRNP C1/C2 binding poly-U tract. Ex vivo splicing assays showed that the c.835-17_835-14delCTTT variant caused exon 7 skipping, whereas the 835-17C > G variant did not. This implied that the c.835-17_835-14delCTTT variant lies closer to the poly-U tract than the 835-17C > G variant as it may interfere with the binding of the splice suppressor hnRNP C1/C2. Further studies on splicing and the influence of variants at positions from −14 to −17 are needed to explore the differences between these two variants. The c.835-17_835-14delCTTT variant was classified as likely pathogenic (PS3 + PM2_Supporting + PM3 +PP4) according to the ACMG guidelines.

Delayed diagnosis is a common problem relating to SMA and other rare diseases. Cao et al. [47] reported that the diagnostic window for SMA types 1–3 with *SMN1* homozygous deletion was 3.38, 4.08, and 11.37 months, respectively, in China, and found that the main factors influencing the time of diagnosis were the SMA type and clinical diagnosis at the first visit. In our study, case 1 presented with disease onset at 4 months old and was finally diagnosed as type 1c SMA at 22 months old, while case 3 presented with possible SMA manifestation at birth but was subsequently diagnosed with NEM2 at the age of 6 years and 9 months. These patients, therefore, experienced delayed diagnosis beyond the typical times for SMA, possibly due to the presence of compound heterozygous *SMN* variants and other myasthenia-related diseases. After case 3 first entered our center and was enrolled in the SMA follow-up cohort, the clinical manifestations of congenital myopathy gradually became clearer. Subsequently, muscle biopsy identified Nemaline myopathy, and WES analysis resulted in a diagnosis of NEM2 characterized by *NEB* mutations. This case highlighted the importance of regular follow-up and lifelong management in patients with SMA and SMA-like disorders. Furthermore, WES proved an extremely valuable tool for differential and accurate diagnosis [48,49]. The pathogenicity of variants can be assessed based on the ACMG guidelines and the FAVOR can provide a functional annotations for non-coding variants across the human genome [50].

Our study had certain limitations. One limitation is that it did not address the population frequency of the c. 835-17C > G variant. We obtained the frequency (0.00048679) of variant c. 835-17C > G based on information from the WES database of local non-SMA patients (Fujungenetics Technologies Co., Ltd, Beijing, China). Understanding the frequency of the c.835-17C > G variant of specific ethnic groups is significant for carrier and neonatal screening by using PCR-based detection techniques. In addition, future improvements in detection methods, such as MLPA-P060 and qPCR, have the potential to reduce the effect of the c.835-17C > G variant during testing, thus minimizing the ambiguous results.

In summary, two intron 6 variants of *SMN1* lead to confusion in SMA diagnosis and screening when using MLPA P060 and qPCR assays but not when using MLPA-P021 or NGS analyses. The c.835-17_ 835-14del CTTT variant was identified as pathogenic in contrast to the c.835-17C > G variant. This study demonstrated the need for awareness of possible molecular misdiagnosis when variants lie within the primer or probe target sequences and recommends that additional detecting methods are needed in cases to minimize misdiagnosis in clinical practice.

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Data availability

The data generated in the current study are available from the corresponding author on request.

CRediT authorship contribution statement

Yujin Qu: Funding acquisition, Investigation, Validation, Writing – original draft. **Jinli Bai:** Methodology, Validation. **Hui Jiao:** Investigation, Resources. **Wenchen Huang:** Methodology, Validation. **Shijia OuYang:** Methodology, Validation. **Hong Qi:** Resources. **Xiaoyin Peng:** Resources. **Yuwei Jin:** Methodology, Validation. **Hong Wang:** Methodology, Validation. **Fang Song:** Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28015.

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