Subtle mutations in the SMN1 gene in Chinese patients with SMA: p.Arg288Met mutation causing SMN1 transcript exclusion of exon7

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Abstract

Background: Proximal spinal muscular atrophy (SMA) is a common neuromuscular disorder resulting in death during childhood. Around 81 ~ 95% of SMA cases are a result of homozygous deletions of survival motor neuron gene 1 (SMN1) gene or gene conversions from SMN1 to SMN2. Less than 5% of cases showed rare subtle mutations in SMN1. Our aim was to identify subtle mutations in Chinese SMA patients carrying a single SMN1 copy.

Methods: We examined 14 patients from 13 unrelated families. Multiplex ligation-dependent probe amplification analysis was carried out to determine the copy numbers of SMN1 and SMN2. Reverse transcription polymerase chain reaction (RT-PCR) and clone sequencing were used to detect subtle mutations in SMN1. SMN transcript levels were determined using quantitative RT-PCR.

Results: Six subtle mutations (p.Ser8LysfsX23, p.Glu134Lys, p.Leu228X, p.Ser230Leu, p.Tyr277Cys, and p.Arg288Met) were identified in 12 patients. The p.Tyr277Cys mutation has not been reported previously. The p.Ser8LysfsX23, p.Leu228X, and p.Tyr277Cys mutations have only been reported in Chinese SMA patients and the first two mutations seem to be the common ones. Levels of full length SMN1 (fl-SMN1) transcripts were very low in patients carrying p.Ser8LysfsX23, p.Leu228X or p.Arg288Met compared with healthy carriers. In patients carrying p.Glu134Lys or p.Ser230Leu, levels of fl-SMN1 transcripts were reduced but not significant. The SMN1 transcript almost skipped exon 7 entirely in patients with the p.Arg288Met mutation.

Conclusions: Our study reveals a distinct spectrum of subtle mutations in SMN1 of Chinese SMA patients from that of other ethnicities. The p.Arg288Met missense mutation possibly influences the correct splicing of exon 7 in SMN1. Mutation analysis of the SMN1 gene in Chinese patients may contribute to the identification of potential ethnic differences and enrich the SMN1 subtle mutation database.

Keywords: Spinal muscular atrophy, Survival motor neuron gene-1, Subtle mutation, Transcript
7 owing to a conversion (C→T) at position 840 in exon 7 [5]. Mutations in SMN1 result in the SMA phenotype, whereas SMN2 copy numbers determine the severity of SMA.

The majority of SMA patients have been found to have a homozygous deletion in exon 7 of SMN1. Some SMA cases are caused by compound mutations, with a SMN1 deletion on one allele and a subtle mutation on the other. Since the first several mutations were identified at 1995 by Lefebvre et al. [4] and Bussaglia et al. [6], More than 60 subtle mutations of SMN1 gene have been continuously identified worldwide [7-24]. Although mutations are distributed along the entire coding sequence of SMN1, the majority are located in exons 3 and 6 (47.5%). Tsai et al. reported the first subtle mutation in SMN1 of Chinese SMA patients in 2001 [18]. To date, eight subtle mutations have been successfully identified in Chinese SMA patients [18-23]. In this article, our aim was to identify subtle mutations in SMN1 of Chinese SMA patients and detect the SMN1 transcript levels of these patients based on the quantitative RT-PCR.

Methods

Patients and materials

Fourteen SMA patients and their parents, from 13 unrelated families, were enrolled in this study. All patients met the diagnostic criteria of proximal SMA, with their clinical data provided in Table 1. Of these cases, three were diagnosed as type I SMA, nine were type II SMA, and two were type III SMA. Case 9 was the younger sister of case 8. Genomic DNA and total RNA from these individuals were isolated from peripheral venous blood using phenol-chloroform extraction and an RNeasy Kit (Qiagen, Germany), respectively. Samples of the parents of cases 1 and 7 were not available. This study was approved by the Ethics Committee of the Capital Institute of Pediatrics, and informed consent was obtained from all subjects.

Analysis of subtle mutations in SMN1

Cloning and sequencing of reverse transcription polymerase chain reaction (RT-PCR) amplicons was carried out to analyze subtle mutations. First-strand cDNA synthesis was performed with 0.5 μg of total RNA, random primers, and M-MLV Reverse Transcriptase (Invitrogen, USA) in accordance with the manufacturer's instructions. Specific PCR primers (SMN575 [13] and 541C1120 [4]), were used to amplify the SMN gene (exons 1–8) using LA Taq polymerase (TAKARA, Japan) and cDNA template. Thermal cycling conditions involved an initial denaturation step for 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 50 s at 60°C, and 60 s at 72°C, with a final extension step at 72°C for 10 min. Amplicons were subcloned into the pGEM-T Easy cloning vector (Promega, USA) according to the supplier's protocol. SMN1 and SMN2 subclones were differentiated using restriction enzymes (DraI and DdeI) [25]. Around 5 ~ 8 SMN1 and 2 ~ 3 SMN2 clones for each case were sequenced. Mutations were further confirmed by direct sequencing of the amplified products from SMN genomic DNA samples.

Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA analysis was performed to detect copy numbers of SMN1 and SMN2 in all cases using a SALSA MLPA kit (P021-A1; MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's recommendations. This SALSA kit contained 16 probes specific for the SMA critical region (5q12.2-q13.3). Among these, two specific probes for the C→T transition in exon 7 (C for SMN1 and T for SMN2) and two specific probes for the G→A transition in exon 8 (G for SMN1 and A for SMN2) were included. In addition, the SALSA kit contained 21 control probes mapping to other autosomes. After MLPA treatment, products were run on the ABI 3730 automatic sequencing system (Applied Bio-Systems, USA). Four healthy individuals carrying two SMN1 copies were the normal controls, with eight carriers (parents of the patients with a homozygous SMN1 deletion) as the single copy SMN1 reference. For each sample, raw data (relative peak area (RPA)) were analyzed and compared with normal controls using Gene marker version 1.75 software. This software is able to calculate the RPA for each probe and to compare RPA with those derived from normal controls. All samples were analyzed at least twice. A ratio with normal controls in the range 0.7–1.3 indicated a normal copy number (two copies), a ratio less than 0.7 indicated one copy, a ratio between 1.3–1.6 was indicative of three copies, and a ratio equal to 0 indicated zero copy.

Analysis of the novel p.Tyr277Cys mutation

The allele-specific primer, Y277CR (5′-CTG AGT GAT TAC TTA CCA TAC-3′), was designed to identify only the mutant allelic sequence, and was coupled with the upstream primer SMNE6F [12]. This was applied in an allele-specific PCR (AS-PCR) to screen for the p.Tyr277Cys mutation in 150 control individuals. Simultaneously, alignment analysis of SMN proteins from six different species was performed using Clustal X version 1.8 to analyze levels of conservation for tyrosine 277.

Restriction endonuclease digestion of SMN transcripts

Total RNA (0.5 μg) was isolated from the peripheral blood of patients and controls, and used to synthesize first-strand cDNA as described earlier. The cDNAs were amplified using primers SMN575 [13] and 541C1120 [4]
## Table 1  Genotype and phenotype in patients with a subtle mutation of SMN1 gene

| Family No. | Case No. | PhenoType | Gender | Age at last examination | Age of onset | Attained motor function | SMN1 genotype | Point Mutation location | SMN2 copies | FI-SMN2 transcript | FI-SMN transcript | Parental origin |
|------------|----------|-----------|--------|-------------------------|-------------|-------------------------|----------------|-------------------------|--------------|-------------------|------------------|-----------------|
| 1          | 1        | I         | M      | 3y6m                    | 4 m         | +                       | Deletion       | p.Ser8LysfsX23          | Exon 1       | 2                 | -                | ND              |
| 2          | 2        | II        | M      | 1y7m                    | 11 m        | +                       | Deletion       | p.Ser8LysfsX23          | Exon 1       | 2                 | 6.33 ± 1.72 6.76 ± 1.94 | Paternal        |
| 3          | 3        | II        | M      | 6y8m                    | 1y2m        | +                       | Conversion     | p.Ser8LysfsX23          | Exon 1       | 3                 | 20.36 ± 11.09 21.01 ± 10.4 | Paternal        |
| 4          | 4        | II        | M      | 5y                      | 11 m        | +                       | Deletion       | p.Glu134Lys             | Exon 3       | 2                 | 7.73 ± 5.44 12.46 ± 5.47 | Paternal        |
| 5          | 5        | II        | F      | 2y6m                    | 1y1m        | +                       | Deletion       | p.Glu134Lys             | Exon 3       | 2                 | 6.41 ± 5.78 11.21 ± 3.45 | Paternal        |
| 6          | 6        | I         | F      | 2y3m                    | 4 m         | +                       | Deletion       | p.Leu228X               | Exon 5       | 2                 | 9.24 ± 6.6 10.277 ± 6.8 | Maternal        |
| 7          | 7        | II        | M      | 4y1m                    | 8 m         | +                       | Conversion     | p.Leu228X               | Exon 5       | 3                 | -                | ND              |
| 8          | 8        | II        | M      | 14y                     | 10 m        | +                       | Deletion       | p.Ser230Leu             | Exon 5       | 2                 | 8.04 ± 7.27 12.22 ± 7.07 | Paternal        |
| 9          | 9        | III       | F      | 9y8m                    | 2y          | +                       | Deletion       | p.Ser230Leu             | Exon 5       | 2                 | 9.35 ± 5.74 14.83 ± 4.45 | Paternal        |
| 10         | 10       | II        | M      | 6y4m                    | 1y          | +                       | Deletion       | p.Tyr277Cys9            | Exon 6       | 2                 | 4.53 ± 3.47 6.54 ± 4.33 | Maternal        |
| 11         | 11#      | I         | F      | 2y10m                   | 5 m         | -                       | Conversion     | p.Arg288Met             | Exon 7       | 3                 | 13.1 ± 10.4 13.71 ± 10.6 | Paternal        |
| 12         | 12#      | II        | F      | 4y4m                    | 1y6m        | +                       | Conversion     | p.Arg288Met             | Exon 7       | 3                 | 9.46 ± 7.84 12.46 ± 8.01 | Paternal        |
| 13         | 13       | II        | M      | 5y                      | 10 m        | +                       | Deletion       | -                       | -            | 2                 | 8.02 ± 1.97 9.86 ± 2.01 | ND              |
| 14         | 14       | III       | M      | 15y                     | 1y5m        | +                       | Conversion     | -                       | 3            | 21.67 ± 11.0 25.17 ± 11.8 | ND              |

Conversion means deletion of SMN1 owing to conversion of SMN1 sequences to SMN2 with the copy number of SMN2 increasing. ND, not detected. §Novel mutation; #, these patients had been reported [22]; †, Walk in waddling gait; case 8 and case 9 are siblings.
in a 50-μl reaction with a 60°C annealing temperature for 30 cycles as the section of "Analysis of subtle mutations in SMN1" described. In general, SMN transcripts yielded three products, full-length SMN1 (fl-SMN1, 1259 bp), full-length SMN2 (fl-SMN2, 1259 bp) and SMN2 isoform lacking exon 7 (Δ7-SMN2, 1205 bp). The SMN1 transcripts could be distinguished from SMN2 transcripts by digestion with the restriction enzyme DdeI. Following digestion, there was a 1259-bp fragment corresponding to fl-SMN1, a 1136-bp fragment corresponding to fl-SMN2, a 1082-bp fragment indicative of Δ7-SMN2 and a 123-bp fragment from SMN2. The transcripts and their products after DdeI digestion were separated on a 6% polyacrylamide gel at 500 volts for 2 h. Gels were stained with silver stain.

Quantitative RT-PCR (qRT-PCR)
Three plasmids (fl-SMN1, fl-SMN2 and GAPDH) were constructed as external standards. Amplified SMN1 and SMN2 were obtained using normal control cDNA and primers SMN-F (5'-GCT GAT GCT TTG GGA AGT ATG TTA-3') and SMN-R (5'-TCA ACT GCC TCA CCG TGC TGGCAGG-3'), specific for exons 6 and 8, respectively. The primer pair for amplification of GAPDH was described by Tiziano FD et al. [26].

Table 2 Information of Primers and Probes Used for qRT-PCR

| Fragment | Primers | Probe | Amplify length |
|----------|---------|-------|----------------|
| SMN1     | SMN_mgb-F: 5'TGTTACATGAGTGGCTATCACTG3' | 5'FAM-ATGGGTTTCAGAA-MGB-NFQ | 75 bp |
|          | SMN_mgb-R: 5'GTGACCCCTTCTTCTTTT3' |       |
| SMN2     | SMN_mgb-F: 5'TGTTACATGAGTGGCTATCACTG3' | 5'FAM-ATGGGTTTCAGAA-MGB-NFQ | 75 bp |
|          | SMN_mgb-R: 5'GTGACCCCTTCTTCTTTT3' |       |
| GAPDH    | GAPDH_abs-F: 5'GCTGGTGATACGATCGAGATGA-3' | 5'FAM-CAAGATCATCAGCAATGC-NFQ | 73 bp |
|          | GAPDH_abs-R: 5'CTAAGCGATTGTGTCAGCGG-3' |       |

The bases C and T in bold are specific for SMN1 and SMN2, respectively. The primers and probe sequences of GAPDH are described by Tiziano FD et al. [26].

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Figure 1 Analysis of the novel p.Tyr277Cys mutation. (A) Sequencing map of the p.Tyr277Cys (TAT > TGT) mutation. Underlined bases indicate codons affected by subtle mutations. Black arrows indicate the converted base peak. (B) AS-PCR screening results for the p.Tyr277Cys mutation in normal controls. M, DNA marker; P, case 10; Fa and Ma are the father and mother of case 10, respectively; N1–6 are normal controls. Case 10 and his mother carrying the p.Tyr277Cys mutation showed two products, a 251-bp fragment corresponding to the internal control and a 182-bp fragment corresponding to fl-SMN2. (C) Alignment of the SMN protein. * indicates highly conserved amino acids. The colon (:) is an indicator of conserved amino acids. The period (.) indicates amino acids that are not conserved. P.Glu134 and p.Tyr277 located in the Tudor domain and Y/G box, respectively, are highly conserved.
was GAPDH_ext-F and GAPDH_ext-R, as previously described [26]. Amplicons for SMN1/SMN2 (395 bp) and GAPDH (133 bp) were cloned into the pGEM-T Easy cloning vector (Promega, USA). Cloned inserts were all verified by sequencing. Plasmid DNA was extracted and quantified by absorbance using a NanoDrop 2000 (Thermo, USA). Based on plasmid length and concentration, the copy number of each plasmid can be calculated. These plasmids were serially diluted across a range (10³–10⁸ copies) and used as external standards to construct the standard curve.

A qPCR assay to quantify SMN transcripts was conducted as described by Tiziano et al. [26]. The primers and MGB-probes were designed using Primer Express v1.5 software (Applied Biosystems, USA), with sequences provided in Table 2. The fl-SMN1 and fl-SMN2 transcripts were amplified using the same primer pair (SMN_mgb-F and SMN_mgb-R). Full-length transcripts of the two genes were distinguished by two different Taqman MGB probes on the basis of the C→T transition located in exon 7. For GAPDH, the primers (GAPDH_abs-F and GAPDH_abs-R) and MGB probe sequence were the same as those described in Tiziano et al. [26] described. All reactions (20 μl) were carried out using a 7500 Real-Time PCR System (Applied Biosystems, USA) and contained 2× GoldStar TaqMan Mixture (KANGWEI, China), 20 ng of cDNA, 0.4 μl of each primer (10 pmol/μl), and 4 pmol of the SMN1, SMN2 or GAPDH probe. The thermal cycling conditions involved 2 min at 50°C then 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was assayed in duplicate and repeated at least twice. Evaluation of data was performed using 7500 Software SDS version 1.4.

Statistical analysis
Transcript levels for fl-SMN1, fl-SMN2 and GAPDH were expressed as copies per nanogram of total RNA. Expression levels of fl-SMN1, fl-SMN2, and fl-SMN were normalized to GAPDH. Statistical analysis was carried out using SPSS 19.0. A parametric test (t-test) was used to compare the transcript levels between normal controls and carriers, as well as between carriers and patients. Correlation between SMN2 gene copy numbers and fl-SMN2 transcript levels were analyzed by a general linear model(one-way ANOVA). A p-value less than 0.05 was regarded as significant.

Results
SMN1 and SMN2 copy numbers
The numbers of SMN1 and SMN2 copies in the 14 patients analyzed by MLPA are presented in Table 1. All patients carried only one copy of SMN1. Patients 3, 7, 11, 12, and 14 carried three copies of SMN2. The remaining patients had two copies of SMN2.

SMN1 subtype mutation
Six subtle mutations were identified in the present study (Table 1). Five mutations (p.Ser8LysfsX23, p.Leu228X, p.Ser230Leu, p.Glu134Lys, and p.Arg288Met) were detected in more than one patient. A novel mutation (p.Tyr277Cys) located in exon 6 of SMN1 was identified for the first time (Figure 1A). The p.Arg288Met mutation has been previously described [22]. No subtle mutations were detected in two of the patients carrying one copy of SMN1.

Screening for the p.Tyr277Cys mutation
AS-PCR was performed to screen for the novel p.Tyr277Cys mutation in control individuals (Figure 1B). This mutation was not observed in the 150 control individuals. Sequence alignment of six different species showed that the Tyr277 residue was highly conserved (Figure 1C).

SMN transcripts analysis of patients with subtle mutations
A Ddel digest of SMN transcripts assay was carried out to qualitative analysis the SMN transcript in all the patients with subtle mutations. Following Ddel digestion, three obvious products (fl-SMN1, fl-SMN2 and Δ7-SMN2) were detected, without obviously truncated
or prolonged transcripts in most patients (Figure 2). While in patients carrying the p.Arg288Met mutation, the fl-SMN1 transcript (1259 bp) was rare, but the undigested Δ7-SMN1 fragment (1205 bp) was more prominent (Figure 2). An undigested Δ7-SMN1 fragment was also observed in their father with the p.Arg288Met mutation.

Clone sequencing of the patients with p.Arg288Met mutation
The SMN cDNA (from exon 1 to exon 8) of patients with p.Arg288Met were amplified and subclone to the pGEM-T vector. After screening the SMN1 clones and sequencing, we found that all 5 subclones of SMN1 from these patients had lost the entire sequence of exon 7 (Figure 3), while three full-length SMN clones belong to the sequences of SMN2. This result was consistent to the result of Ddel digest of SMN transcripts assay. The p.Arg288Met mutation produces a transcript of Δ7-SMN1 other than the transcript of fl-SMN1, it implies that it might cause the skip of exon 7 of SMN1 gene.

Comparison of fl-SMN1 transcripts
To evaluate the effect of subtle mutations on fl-SMN1 levels, we compared fl-SMN1 levels in patients with that of normal controls, and with healthy carriers (Table 3 and Figure 4). The fl-SMN1 levels in normal controls and healthy carriers were 23.77 ± 7.74 and 9.47 ± 5.39, respectively. The difference between these two groups was statistically significant (t = 5.296, P = 0.000). The fl-SMN1 transcript levels in all patients were significantly decreased compared with normal controls (t = 7.839, P = 0.000). Compared with healthy carriers carrying one copy of SMN1, patients with the p.Glu134Lys or p.Ser230Leu mutation showed no significant difference (t = 1.769, P = 0.094 and t = 1.660, P = 0.115, respectively), patients with the novel p.Tyr277Cys mutation presented a slight decrease in fl-SMN1 transcript levels (t = 2.337, P = 0.032), while the patients with the p.Ser8-LysfsX23, p.Leu228X, and p.Arg288Met mutations were significantly reduced (t test, P = 0.000). Especially for the patients carrying p.Arg288Met, fl-SMN1 transcript levels were almost undetectable (0.017 ± 0.15).
### Table 3 fl-SMN1 transcript levels of controls, carrier, and patients with SMN1 subtle mutations

| Type     | Control | Carrier | p.Ser8LysfsX23 | p.Glu134Lys | p.Leu228X | p.Ser230Leu | p.Tyr277Cys | p.Arg288Met |
|----------|---------|---------|----------------|-------------|-----------|-------------|-------------|-------------|
| fl-SMN1  | Mean ± SD | 26.49 ± 13.88 | 185.71 ± 148.00 | 0.61 ± 0.30 | 6.30 ± 2.02 | 1.57 ± 0.38 | 3.54 ± 0.95 | 7.19 ± 2.72 | 0.05 ± 0.10 |
| Min-Max  | 11.16-50.8 | 29-470 | 0.22-0.95 | 3.58-8.26 | 1.24-1.98 | 2.94-4.64 | 4.90-10.20 | 0-0.19 |
| GAPDH    | Mean ± SD | 2322.2 ± 1174.9 | 4857.3 ± 2452.5 | 3720 ± 1969 | 2742 ± 8.26 | 1695 ± 456 | 8173 ± 2.56 | 3002 ± 1801 |
| Min-Max  | 1232-4700 | 1976-8660 | 2000-6500 | 1788-3680 | 2040-4120 | 1388-2220 | 5340-11240 | 1315-5300 |
| corrected fl-SMN1 | Mean ± SD | 23.77 ± 7.74 | 9.47 ± 5.39 | 0.52 ± 0.46 | 4.59 ± 0.68 | 1.04 ± 0.39 | 4.18 ± 0.20 | 2.01 ± 0.89 | 0.07 ± 0.15 |
| Min-Max  | 15.92-34.74 | 1.05-18.73 | 0.15-1.15 | 3.98-5.29 | 0.69-1.46 | 3.98-4.38 | 1.23-2.98 | 0-0.29 |
| P value (t-test) | 0.000 a | 0.000 b | 0.094 b | 0.000 b | 0.011 b | 0.032 b | 0.000 b |

fl-SMN1, GAPDH, and corrected fl-SMN1 indicate transcript levels, measured as no. of molecules per nanogram of total RNA. Superscripted a means that the t-test was carried out between controls and carriers; Superscripted b means the t-test was performed between carrier and the patients with subtle mutations.
Figure 4 Levels of fl-SMN1 transcripts in controls, carriers and patients with subtle mutations. The dark horizontal lines indicate the 95% confidence interval. The empty circle indicate the median. Fl-SMN1 transcript levels were significantly different between normal controls and healthy carriers. The levels of transcripts in patients were significantly reduced compared with those in controls. Fl-SMN1 transcript levels in patients with the p.Arg288Met mutation were almost undetectable. In patients with p.Ser8LysfsX23 or p.Leu228X mutations, transcript levels were severely reduced. In the patient carrying the novel p.Tyr277Cys mutation, transcript levels were decreased, while patients with missense mutations (p.Ser230Leu and p.Glu134Lys) showed no significantly decrease compared with carriers.

Figure 5 fl-SMN2 transcript levels among the individuals with different SMN2 copy numbers. Mean fl-SMN2 transcript levels in healthy individuals (normal controls and healthy carriers) with a single copy of SMN2 were 6.3895 ± 1.56, 11.48 ± 8.01 for those with two copies of SMN2, and 15.61 ± 11.39 for patients with three SMN2 copies. No correlation was observed between fl-SMN2 transcript level and SMN2 copy numbers (F = 0.391, P = 0.679).
Transcript levels of fl-SMN in different clinical cases

Although there was no correlation between fl-SMN2 transcript level and SMN2 copy numbers (one-way ANOVA, F = 0.391, P = 0.679) in normal controls and healthy carriers, the fl-SMN2 transcript level was increased along with SMN2 copy numbers (Figure 5). The fl-SMN2 transcript levels in patients are presented in Table 1, with no significant differences (F = 1.029, P = 0.430). Total fl-SMN transcript levels (fl-SMN1 + fl-SMN2) were used to assess the correlation between the fl-SMN levels and the clinical severity. The fl-SMN transcript levels in normal controls and healthy carriers were 34.4 ± 7.1 and 21.7 ± 12.7, respectively, with a significant difference (t = 2.596, P = 0.017). The fl-SMN transcript levels in all patients were significantly decreased compared with normal controls (t = 7.060, P = 0.000). Mean fl-SMN transcript levels in type I (n = 2) and type II (n = 8) were 11.27 ± 6.8 and 11.67 ± 6.34, respectively. For type III (n = 2) patients, a slight increase in levels could be observed (20.00 ± 9.43). Because the number of type I and III patients were low, statistical analysis of fl-SMN transcript levels was only carried out between type II patients and healthy carriers, with a significant difference observed (t = 3.046, P = 0.009).

Discussion

Including the results from this study, 61 SMN1 subtle mutations have been detected among diverse populations worldwide. Nine subtle mutations have been identified in Chinese SMA patients [18-23] (Table 4). Among these, the c.-39A > G, p.Ser8LysfsX23, p.Tyr277Cys, and c.835-1 G > A mutations were only found in Chinese SMA patients, p.Arg288Met mutation had been reported in another East Asian country-Korea [17], only the two mutation (p.Glu134Lys [27], and p.Ser230Leu [28]) were found in Caucasian population. In East Asian country, there was still another mutation p.Trp92Ser only reported in Japanese SMA patient [15]. These reports showed the kinds of SMN1subtle mutation in the Chinese even East Asian population are distinct from those in Caucasian populations. In addition, the type of common mutations differed between Chinese and Caucasian populations. In Chinese SMA patients, p.Ser8LysfsX23 and p.Leu228X seem to be the common mutations, which were detected in seven families (33.3%, 7/21) and four families (19.0%, 4/21), respectively. While in Caucasian populations, Mutations p.Arg133fsX15, p.Gly261LeufsX8, p.Tyr272Cys and p.Thr274Ile were commonly detected [4,6-8,10,11,16,25,29-31]. Our research implies that the SMN1 subtle mutations show high heterogeneity in various populations.

Our results show that the level of fl-SMN1 transcripts in normal controls was significantly higher than in healthy carriers, which corresponded to the different number of SMN1 copies carried in these individuals. Although the patients with subtle mutation carried only one SMN1 copy, the effect of these mutations on the fl-SMN1 transcript levels were different. Based on the qPCR analysis, fl-SMN1 transcript levels in patients with p.Ser8LysfsX23 and p.Leu228X mutations were much lower than in healthy carriers. We presumed the reason for this might be that these two premature termination mutations initiate nonsense-mediated mRNA decay.

Table 4 Subtle mutations of SMN1 gene identified in Chinese SMA patients

| Exon/ intron | cDNA mutation | Protein prediction | Mutation type | References | Number of families | Phenotype |
|--------------|---------------|--------------------|---------------|------------|-------------------|-----------|
| 5UTR         | c.-39A > G    | -                  | -             | Wang et al. [21] | 1 | NA         |
| Exon 1       | c.22dupA      | p.Ser8LysfsX23     | Frameshift    | Tsai et al. [18] (first report) | 1 | I         |
|              |               |                    |               | Zeng J et al. [23] | 1 | I         |
|              |               |                    |               | Wang et al. [21] | 2* | NA        |
|              |               |                    |               | This work | 3 | I, II     |
| Exon 2       | c.84 C > T    | p.Ser28Ser         | Silence       | Wang et al. [21] | 1 | NA        |
| Exon 3       | c.400 G > A   | p.Glu34Lys         | Missense      | This work | 2 | II        |
| Exon 5       | c.683 T > A   | p.Leu228X          | Nonsense      | Tsai et al. [18] (first report) | 1 | I         |
|              |               |                    |               | Zeng J et al. [23] | 1 | I         |
|              |               |                    |               | This work | 2 | I, II     |
| Exon 5       | c.689C > T    | p.Ser230Leu        | Missense      | Zeng J et al. [19] (first report) | 1 | I         |
|              |               |                    |               | This work | 1* | II, III |
| Exon 6       | c.830A > G    | p.Tyr277Cys        | Missense      | This work (first report) | 1 | II        |
| Intron 6     | c.835-1 G > A | -                  | Splice site   | Zhu et al. [20] | 1 | I         |
| Exon 7       | c.863 G > T   | p.Arg288Met        | Missense      | Qu et al. [22] | 2 | I, II     |

*, indicate these families have two patients who were siblings. NA, not available; mutations in bold are common mutations in Chinese SMA patients.
(NMD). NMD procedure can result in rapid degradation of SMN1 mRNA [32]. A lack of any significant difference between carriers and patients with misense mutations (p.Glu134Lys and p.Ser230Leu) implied that these mis-sense mutations do not affect transcription of SMN1, or degradation of SMN1 mRNA. However, all the patients with subtle mutations significantly reduced fl-SMN transcript levels compared with normal controls. These results were similar to those seen in patients with a homoygous deletion of SMN1 [26]. We have attempted to assess the correlation between the fl-SMN levels and the clinical severity, while the difference of fl-SMN transcript levels in these patients were not significant. A direct relationship could not be observed between fl-SMN transcript levels and phenotypic severity in this study.

The p.Arg288Met mutation was firstly reported in 2009 [17], and was also found in two unrelated Chinese patients in a previous study [23]. Kang et al. predicted that this mutation was likely to be deleterious to protein structure and function [17]. In our study, an interesting finding was that the SMN1 transcript carrying the p.Arg288Met mutation skipped exon 7 entirely. According to sequence analysis and restriction digestion assays, these two patients with p.Arg288Met mutation produce a transcript corresponding toΔ7-SMN1 other than a transcript of fl-SMN1. Subsequent qPCR analysis verified that fl-SMN1 transcripts were almost undetectable (0.017 ± 0.15). The effect of this mutation was similar to that seen with the C to T conversion in SMN2 exon 7 producing Δ7-SMN mRNA. This transcript encodes a truncated SMN protein that fails to undergo self-oligomerization of SMN, is unstable, and degrades easily. The pathogenic effect in a patient with one copy of SMN1 with the p.Arg288Met mutation is the same as for the homozygous deletion of SMN1. The phenotypes in patients carrying the p.Arg288Met mutation were type I SMA in the two patients and type II SMA in another patient. These results were consistent with a phenotype in SMA patients that have a homozygous deletion of SMN1.

Rare missense mutations were reported to affect the splicing of SMN1. There were two variations that occurred in exon 7 of SMN2 that were described to affect the splicing of SMN2. The C→T conversion at position six of SMN2 exon 7 was found to cause the entire exon to be skipped [33,34]. The missense mutation p.Gly287Arg (c.859 G > C) in SMN2, recently described as a positive disease modifier, could improve SMN2 exon 7 inclusion [35]. Many studies have revealed that multiple cis-elements and splicing factors participate in the regulation of SMN exon 7 splicing. Singh et al. [36] showed that the Conserved tract is a positive element located in the middle of exon 7 at positions 16–44. Hofmann et al. [37] revealed binding of exonic splicing enhancers (ESEs) with Htra2-b1 in the middle of exon 7 at positions 19–27. In this study, our results show that the p.Arg288Met mutation may influence splicing of exon 7, causing the entire exon to be skipped. Serine/arginine-rich protein-binding ESE elements were not observed in wild-type or mutant sequences at c.863 using ESE finder 2.0 (http://rulai.cshl.edu/tools/ESE2). However, the p.Arg288Met mutation at position 29 of exon 7 was located within the Conserved tract element, and near where the ESE binder bind Htra2-b1. We speculate that an unascertained ESE, regulating SMN exon 7 splicing, might exist in the Conserved tract element and that this variant of G>T in c.863 may influence the ESE site or produce a new exonic splicing silencer element (ESS).

The tyrosine/glycine-rich sequence (Y/G box), containing highly conserved residues Tyr268 and Gly279 (YXXGXXGYXXG) in the C-terminus of SMN, is an essential self-oligomerization domain of SMN. It plays role in assembling of the SMN complex and participating in pre-mRNA splicing [38,39]. The aromatic amino acids Tyr268, Tyr272, and Tyr276, each at three-base intervals, seem to be more important than other amino acids in the Y/G box because two SMN monomers might form a stable dimer through aromatic stacking of these tyrosines [16]. When these three tyrosines mutate, SMN self-oligomerization could be severely disturbed. To date, several missense mutations in the Y/G box, including p.Tyr272Cys, p.His273Arg, p.Thr274Ile, p.Gly275Ser, p.Gly279Cys and p.Gly279Val, have been reported [4,7,10-14,16,40-44]. Among these, p.Tyr272Cys and p.Gly279Val were reported to be usually associated with the more severe type I form of SMA [4,11-13,44]. The p.Thr274Ile, p.Gly275Ser and p.Gly279Cys mutations were generally associated with the milder, type II and III phenotypes [7,10-13,16,41-43].

In this paper, the p.Tyr277Cys mutation within the Y/G box is reported for the first time. Although Tyr277 is an aromatic amino acid, it is near the critical Tyr276 residue. Both Tyr276 residues from two SMN monomers require adequate space to form aromatic stacking. We speculate that Tyr277 replaced by a Cys residue might disturb the space structure of aromatic stacking, thereby affecting the stability of the SMN dimer. The patient carrying the p.Tyr277Cys mutation also had two copies of SMN2. The mean fl-SMN transcript level in this patient was 6.54 ± 4.33, which was much lower than that in normal controls (34.4 ± 7.1) and healthy carriers (21.7 ± 12.7). The phenotype of this patient in our study was indicative of type II SMA. Although Tyr277 is a highly conserved residue, we predicted that the p.Tyr277Cys mutation might be a milder mutation affecting the structure of the Y/G box.

It was worth to note that two siblings (cases 8 and 9) with the same p.Ser230Leu mutation displayed different
This preliminary study revealed that p.Ser8LysfsX23 and p.Leu228X mutations seem to be the field in 12 patients, including the novel p.Tyr277Cys. We hypothesized that there might be other modifying factors that affect phenotypes, such as Plastin 3, which has been reported as a sex-specific protective modifier of SMA [45-47].

Conclusion
In conclusion, six SMN1 subtle mutations were identified in 12 patients, including the novel p.Tyr277Cys mutation. Based on our research and other studies, p.Ser8LysfsX23 and p.Leu228X mutations seem to be the common SMN1 subtle mutations in Chinese patients. This preliminary study revealed that SMN1 subtle mutations in Chinese SMA were different to those observed in Caucasian populations. A qRT-PCR assay implied that p.Ser8LysfsX23, p.Leu228X, and p.Arg288Met mutations affected fl-SMN1 transcript levels. Besides, we discovered that the p.Arg288Met mutation disturbed the splicing of exon7 SMN1 pre-mRNA, resulting in SMN1 transcripts skipping exon 7.

Competing interests
The authors declare no potential competing interests with respect to the authorship and/or publication of this article.

Authors’ contributions
SF conceived of, designed and organized the study, contributed to obtaining the funding, carried out the experiments, analyzed data, and wrote the initial draft of the manuscript. DJ helped to carry out the experiments, analyzed data, and wrote the initial draft of the manuscript. LJ helped to carry out the experiments, analyzed data, and wrote the initial draft of the manuscript. LE was responsible for diagnosis and management of patients; BJ contributed to the writing of the manuscript; JY and WH contributed to the sequencing experiments. All authors approved the final version of the manuscript submitted for publication.

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