The Journal of Molecular Diagnostics, Vol. 18, No. 5, September 2016

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the Journal of Molecular Diagnostics
jmd.amjpathol.org

Mutation Spectrum of the Survival of Motor Neuron 1 and Functional Analysis of Variants in Chinese Spinal Muscular Atrophy

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Accepted for publication May 10, 2016.

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Proximal spinal muscular atrophy (SMA) is a common fatal autosomal recessive disorder caused by deletion or mutation of the survival of motor neuron 1 (*SMN1*). Here, we studied SMA molecular pathology in 653 Chinese patients and found approximately 88.2% with homozygous *SMN1* exon 7 deletion and 6.3% with heterozygous exon 7 loss using multiplex ligation-dependent probe amplification. *SMN1* variants were detected in 34 patients with heterozygous *SMN1* loss by clone sequencing. In 27 of them, 15 variants were identified: five were unreported novel variants [c.-7_9del(p.0), p.Tyr109Cys, p.Ile249Tyrfs*16, p.Tyr272Trpfs*35, and c.835-5T>G], five were previously found only in Chinese patients (p.Ser8Lysfs*23, p.Gln14*, p.Val19Glyfs*21, p.Leu228*, and p.Tyr277Cys), and five were reported in other populations [p.Ala2Gly, p.Gln15*, p.Glu134Lys, p.Ser230Leu, and c.863G>T (r.835_*3del, p.Gly279Glufs*5)]. Variants p.Ser8Lysfs*23 and p.Leu228* were the most common in Chinese SMA. Five variants (p.Ser8Lysfs*23, p.Gln14*, p.Gln15*, p.Val19Glyfs*21, and p.Leu228*) resulted in premature stop codons, likely causing SMN1 mRNA nonsense-mediated decay. The novel variant c.-7_9del (p.0) caused deletion of the translation start codon (AUG), resulting in full-length SMN protein loss. The novel variant c.835-5T>G, located in a splice site, resulted in 90% exon 7 skipping. Our study could facilitate early diagnosis for SMA patients in mutation detection and revealed the specific mutation spectrum of *SMN1* in Chinese SMA and high genetic heterogeneity in subtle variants observed between patients from China and Caucasians. *(J Mol Diagn 2016, 18: 741—752; http://dx.doi.org/10.1016/j.jmoldx.2016.05.004)*

Proximal spinal muscular atrophy (SMA) is a common fatal autosomal recessive disorder with an incidence of 1 in approximately 6000 to 10,000 newborns,[1] and the carrier frequency in the Chinese population is approximately 1 in 42 live births.[2] It is characterized by α motor neuron degeneration in the anterior horn cells of the spinal cord, leading to progressive symmetrical limb- and trunk-muscle weakness and atrophy.[3] SMA is categorized into four clinical types (SMA I to IV) based on the age at onset and maximum attained motor functions.[4,5] Type I SMA (Werdnig-Hoffmann disease; MIM 253300, *http://www.ncbi.nlm.nih.gov/omim*, last accessed April 22, 2016) is the most severe form, with onset occurring before 6 months of age and mortality before age 2 years. Types II (MIM 253550) and III (Kugelberg-Welander disease; MIM 253400) are of intermediate and mild severity, with onset before or after 18 months, respectively, and differ in patient ability to stand and walk, whereas type IV onset occurs after age 30 years.

The survival of motor neuron 1 (*SMN1*), telomeric, located on 5q11.2 to 13.3, is identified as the SMA disease-determining gene. *SMN2* (centromeric) resembles *SMN1*,

Supported by the National Natural Science Foundation of China grants 81470056 (F.S.), 81100933 (Y.-j.Q.), 81500979 (Y.-y.C.), and 81501850 (J.-l.B.); the Beijing Municipal Natural Science Foundation grant 7112020 (F.S.); and the Organization Department of Beijing Talents Project grant 2014000021469G22B (Y.-y.C.).

Disclosures: None declared.

http://dx.doi.org/10.1016/j.jmoldx.2016.05.004

differing only in five nucleotides.[6] *SMN1* encodes the functional survival motor neuron (SMN) protein, whereas *SMN2* generates limited functional SMN protein owing to the c.840C>T substitution, which results in exclusion of exon 7 in 90% of SMN2 transcripts. *SMN2* copy number is negatively correlated with clinical phenotype.[7–10]

Most patients with SMA exhibit homozygous *SMN1* deletion or spontaneous *SMN1*-to-*SMN2* conversion. Some SMA cases are caused by compound variants, with one *SMN1* deletion and one variant allele. More than 70 *SMN1* variants have been identified to date.[11] Although variants are distributed along the entire *SMN1* coding sequence, most are located in exons 3 and 6 (47.5%). Since 2001, multiple variants associated with Chinese SMA have been successively identified.[12–16]

With increased discovery of novel variants, evaluation of the association between these variants and their pathogenesis for genetic disorders becomes necessary. As in SMA, several variants have been associated with SMN1 mRNA nonsense-mediated decay (NMD),[17] and those located in splicing sites or *cis*-elements might result in exon skipping.[18–20] Missense variants might also influence SMN protein interactions, thereby disrupting SMN-complex assembly.[21,22]

Here, our aim was to determine the spectrum of *SMN1* mutations in Chinese SMA and explore the functional influence of several *SMN1* variants. We analyzed *SMN1* and *SMN2* copy numbers in 653 patients with SMA and detected *SMN1* variants in patients carrying only a single *SMN1* allele. Our results indicated high genetic *SMN1* heterogeneity as well as differences between patients from China and Caucasians.

Materials and Methods

Patient Samples

Samples were collected from 653 unrelated patients with SMA from July 2003 to December 2014. All patients fulfilled the diagnostic criteria of the International SMA Consortium.[23] Of these, 95% were ethnically northern Han Chinese, and 298, 284, and 71 were diagnosed as type I, II, or III SMA, respectively. Total RNA was extracted from the peripheral blood of patients with *SMN1* subtle variants and from their parents. Informed consent was obtained from all participants, and this study was approved by the Ethics Committee of the Capital Institute of Pediatrics.

SMN Copy Number Analysis

Multiplex ligation-dependent probe amplification reactions were performed to detect *SMN1* and *SMN2* copy numbers using the SALSA MLPA Kit (P021-A2; MRC-Holland, Amsterdam, the Netherlands) as previously described.[14] Amplification products were analyzed on an ABI Prism 3730 automatic sequencing system (Applied Biosystems, Foster City, CA). For each sample, raw data were analyzed and compared with normal controls using GeneMarker software version 1.75 (SoftGenetics, State College, PA). Ratios <0.3, between 0.3 to 0.7, 0.7 to 1.3, or 1.3 to 1.6 indicated zero, one, two, or three gene copies, respectively. All samples were analyzed at least twice.

SMN1 Variant Analysis

RT-PCR amplicons were cloned and sequenced to analyze subtle variants. First-strand cDNA synthesis was performed using 0.5 μg total RNA, random primers, and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Specific PCR primers (SMN575[22] and 541C1120[6]) were used to amplify *SMN1* (exons 1 to 8) using LA Taq polymerase (TAKARA, Tokyo, Japan) and cDNA templates. Thermal cycling conditions were as follows: 5 minutes initial denaturation at 94°C; 30 cycles of 45 seconds at 94°C, 50 seconds at 60°C, and 60 seconds at 72°C; and 10 minutes final extension at 72°C. For the -7_9del variant, primers 5′-untranslated region (UTR)-F (Table 1) and 541C1120 were used. Amplicons were subcloned into the pGEM-T Easy Cloning vector (Promega, Madison, WI) according to the manufacturer's instructions. *SMN1* and *SMN2* subclones were differentiated using the restriction enzymes *Dra*I and *Dde*I.[24] *SMN1* (5 to 8) and *SMN2* (2 to 3) clones were sequenced for each patient. Variants were further confirmed by genomic sequencing of the mutant region and by PCR-based restriction enzyme analysis or allele-specific PCR of samples from core family members. Restriction endonuclease

Table 1 Oligonucleotide Sequences

Primer	Sequence (5′-3′)
5′-UTR-F	5′-GGGCGGCGGAAGTCGTCA-3′
SMN_mgb-F	5′-TGGTACATGAGTGGCTATCATACTG-3′
SMN_mgb-R	5′-GTGAGCACCTTCCTTCTTTTT-3′
SMN1 probe	5′-FAM-ATGGGTTTCAGAA-MGB-NFQ-3′
SMN2 probe	5′-FAM-ATGGGTTTTAGAA—MGB—NFQ-3′
hSMNE6F	5′-CGATCTCGAGATAATTCCCCCACCACCTC-3′
hSMNE8R	5′-GCTACCCGGGCACATACGCCTCACATACA-3′
835-5A-F	5′-TTCCTTTATTTTCCTAACAGGGTTTCAGACAA-3′
835-5A-R	5′-TAGGAAAATAAAGGAAGTTAAAAAAAATA-3′
835-5G-F	5′-TTCCTTTATTTTCCTGACAGGGTTTCAGACAA-3′
835-5G-R	5′-CAGGAAAATAAAGGAAGTTAAAAAAAATA-3′
835-5C-F	5′-TTCCTTTATTTTCCTCACAGGGTTTCAGACAA-3′
835-5C-R	5′-GAGGAAAATAAAGGAAGTTAAAAAAAATA-3′
835-1A-F	5′-TTCCTTTATTTTCCTTACAAGGTTTCAGACAAAT-3′
835-1A-R	5′-TTGTAAGGAAAATAAAGGAAGTTAAAAAAAATA-3′
-7_9del 16 F	5′-GAGCAGCGGCGGCAGTGGTG-3′
-7_9del 16 R	5′-CGCGGATAATCACTAGTGAATTCGC-3′
Y277C F	5′-TCATACTGGCTATTGTATGGGTTTCAGAC-3′
Y277C R	5′-CAATAGCCAGTATGATAGCCACTCATGTA-3′
pEasy-M2F	5′-TCTAGAGGATCGCCCTTCGA-3′
SMNE8R-FAM	5′-FAM-GTGGTGTCATTTAGTGCTGCT-3′

The underlined bases in primers indicate the mutant nucleotides for site-directed mutagenesis.

UTR, untranslated region.