High-throughput screening reveals novel mutations in spinal muscular atrophy patients

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Research article

Keywords: spinal muscular atrophy (SMA), high-throughput sequencing, SMN1, onset

Posted Date: August 15th, 2019

DOI: https://doi.org/10.21203/rs.2.12903/v1

Abstract

Background Some spinal muscular atrophy (SMA) cases are caused by either compound heterozygosity with a point mutation in one allele and a deletion in the other or compound heterozygous point mutations in SMN1 or other genes. Methods To explore more genes and mutations in the onset of SMA, 83 whole blood samples were collected from 28 core families of clinically suspected SMA, and multiplex ligation probe amplification (MLPA) was firstly performed with a SALSA MLPA Kit P021 for preliminary diagnosis. Afterwards, the complete gene sequence of SMN1 gene was detected with the high-throughput sequencing platform of Illumina HiSeq-2500 to find more mutations in the 28 core families. Furthermore, 20 SMA patients were selected from the 28 prodands, and 5 non SMA children as controls. The Life Technologies SOLiD™ technology with mate-pair chemistry was utilized to conduct the whole exome high-throughput sequencing. Results MLPA results showed that 22 probands were SMA patients, 3 probands carriers, and 3 probands normal individuals. Moreover, 2 parents from 2 SMA families were with 3 SMN1 exon7 copies. 6 SMN1 single nucleotide variants (SNVs) were identified in the 83 samples, and c.[84C>T], c.[271C>T], c.[-39A>G] and g.[70240639G>C] were novel. Compared with control group, 9102 mutation were selected out in SMA patients. SPTA1 mutation c.[-41_-40insCTCT], FUT5 SNV c.[1001A>G], and MCCC2 SNV c.[-117A>G] were the 3 most frequent mutations in SMA group (95%, 85% and 75%, respectively). Conclusions We identified some mutations in both SMN1 and other genes, and c.[271C>T], c.[-41_-40insCTCT], c.[1001A>G] and c.[-117A>G] might be associated with the onset of SMA.

Background

Spinal muscular atrophy (SMA) is an autosomal recessive hereditary disease characterized by degeneration of spinal cord motor neurons, atrophy of skeletal muscles, and generalized weakness (1). It affects 1 in 10 000 live births, and often leads to early death (2). SMA manifests over a wide range of severity, affecting infants through adults. According to the onset time and severity of the disease, SMA is divided into 4 types (SMA1, SMA2, SMA3 and SMA4), and SMA 1, with onset before age six months; SMA 2, with onset between age 6 and 18 months; SMA 3, with onset in childhood after age 12 months; and SMA 4, with adult onset (3). Nusinersen (trade name: Spinraza) is the only approved drug to treat spinal muscular atrophy, which is administered directly to the central nervous system using an intrathecal injection (4). SMA is caused by homozygous disruption of the survival motor neuron 1 (*SMN1)* gene by deletion, conversion, or mutation (1). Since SMA is one of the most common lethal genetic disorders, with a carrier frequency of 1 in 40 to 1 in 60, direct carrier dosage testing has been beneficial to many families (5). About 96% SMA are caused by a homozygous deletion of *SMN1* exon7, and the remaining 4% of cases are caused either by compound heterozygosity with a point mutation in one allele and a deletion in the other or by compound heterozygous point mutations in *SMN1(*6). *SMN2* is a homologous gene of *SMN1* and functions as a SMA modifier. In general, the copy number of *SMN2* is substantial variation in SMA patients, and a high *SMN2* copy number tends to a milder type (7). Furthermore, more and more new genes or novel mutations have been reported to be related to the morbidity, severity, treatment and prognosis of SMA with the development of gene sequencing technology. A study revealed seven different mutations of *SMN1,* and among them c.824G>C, and c.825–2A>T were described for the first time (8). Another study found the *NAIP* copy number was inversely correlated with the clinical severity of SMA (9). *GTF2H2* and *H4F5* have been proved to be associated with the onset and type of SMA (10). In this study, multiplex ligation probe amplification (MLPA) was firstly used for preliminary diagnosis in 28 core families of suspected SMA patients, and then the complete gene sequence of *SMN1* gene was detected by high-throughput sequencing to find more mutations in the 28 core families. Afterwards, 20 children diagnosed with SMA and 5 children diagnosed with non SMA were enrolled, and the whole exome screening of other related genes was performed to explore more genes and mutations involved in the onset of SMA.

Methods

Patients and samples

From December 2013 to May 2017, 28 probands of clinically suspected SMA, 15 males and 13 females, were accepted by our hospital (Tianjin Children Hospital, China, Tianjin) because of unstable walking, and their age ranging from 1 month to 12 years. The phenotypes of their parents were normal. 3–5 ml peripheral blood samples from the 83 enrolled cases (probands and their parents) were collected. All the subjects signed the informed consent forms for genetic testing routinely, and all procedures were in accordance with the ethical standards of the institutional and/or national research committee.

MLPA

was Extracted Genomic DNA from 1–2 ml peripheral blood samples with the salting out method. The nucleic acid quantitative instrument NADO DROP 2000 (Thermo Fisher Scientific Inc., Waltham, USA) was utilized to determine the quality and quantity of the extracted DNA. MLPA was performed using a SALSA MLPA Kit P021 (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's protocol. MLPA products were run on an ABI PRISM 3130 genetic analyzer (Appliced Biosystems International Inc., California, USA). and analyzed using Gene Mapper version 3.5 software (Thermo Fisher Scientific Inc., Waltham, USA). For each sample relative peak heights were calculated and compared with 4 normal controls using the Coffalyser version 9 software (Coffalyser MLPA, Amsterdam, Netherlands). The evaluation criteria were based on the kit instructions: in normal individuals, SMN1 exon7 is 2 copies; the *SMN1* gene of patients with homozygous deletion and carriers with heterozygous deletion is 0 copies and 1copy, respectively(11,12).

SMN1 gene screening by high-throughput sequencing

The complete genome sequence of *SMN1* gene was detected from above 83 enrolled cases via high-throughput sequencing. Firstly, high-molecular-weight genomic DNAs were extracted from 2–3 ml blood samples with the DNeasy Blood and Tissue kit (QIAGEN, Dusseldorf, Germany) and 10 μg genomic DNA was used for library generation according to the manufacturer's recommendations. Secondly,. Briefly, the genomic DNA was mechanically sheared to an average fragment size of 1.5 kb. These size-selected fragments were then end repaired and added a special joint sequence at both ends. The probes (Roche, Basel, Switzerland) then were used to capture genomic regions including *SMN* gene. Secondly, the Illumina HiSeq–2500 platform (Illumina, California, USA) was used to conduct the sequencing reaction based on the manufacturer's protocol. The average sequencing depth was 100×, and more than 96% regions was up to 20×. Thirdly, the raw data were compared with human genome (NCBI build 36, hg18), and marked repeated reads and filtered out low-quality data. Fourthly, the base quality of reads was calibrated again by the genome analysis toolkit (GATK) algorithm, and ultimately all the a single base of DNA (point mutation) or a loss of base pairs (deletion) were screened out with the GATK software V3.0 (Eli and Edythe L. Broad Institute, Massachusetts, USA)(13). The QC_{30} of the raw data was more than 85% in all samples, and the allele frequency of all mutations was more than 20%.

The whole exome screening by high-throughput sequencing

Based on previous results, 20 SMA patients with homozygous deletion of *SMN1* exon7 were selected from the 28 prodands, and 5 non SMA children were as controls (2 children carried with heterozygous deletion of *SMN1* exon7 and 3 children with 2 *SMN1* exon7copies). was utilized to perform whole exome high-throughput sequencing was performed by The Life Technologies SOLiD™ (version 3) technology with mate-pair chemistry. according to the manufacturer's recommendations (Carlsbad, CA, USA). 20 μg high-molecular-weight genomic DNAs was used for library generation. Briefly, DNA was broken into 1.5kb fragments, repaired the ends and circularized around a long mate-pair adaptor by nicked ligation. SOLiD™ sequencing-specific sequencing adaptors were ligated to the ends of these fragments. Following PCR amplification, these mate-pair libraries were then used as templates in emulsion PCR reactions using SOLiD™ proprietary sequencing beads to generate clonal single molecule templated beads. The raw data were analyzed with GATK software. The average sequencing

depth was 100×, and more than 96% regions was up to 20×. The QC_{30} of the raw data was more than 85% in all samples, and the allele frequency of all mutations was more than 40%.

Results

MLPA

MLPA results showed that 22 probands were with homozygous deletion of *SMN1* exon7 (SMA patients), 3 probands carried with heterozygous deletion of *SMN1* exon7 (carriers), and 3 probands with 2 *SMN1* copies (normal individuals). However, the mother of a SMA patient had 3 *SMN1* exon7 copies and the father was carrier, and the father of another SMA patient had 3 *SMN1* exon7 copies and the mother was carrier. Here, we recorded the above 2 SMA patients as Proband–1 and Proband–2, respectively. Furthermore, 1 carrier, a 2-year old girl, had some clinical features that correspond to SMA, such as atrophy of skeletal muscles, generalized weakness and extensive neurogenic injury by electromyography. Here, the carrier was named as Proband–3. Besides, the father of Proband–3 was without heterozygous deletion of *SMN1* exon7 and exon8, and the mother was a carrier.

Novel mutations of SMN1 in suspected SMA family

A total of 6 single nucleotide variants (SNVs) of SMN1 were identified in the 83 samples, and they were showed in Table 1. 3 SNVs located in exon, 1 in UTR5 and 2 in intron; c.[84C>T], c.[271C>T], c.[−39A>G] and g.[70240639G>C] were firstly reported here; c.[84C>T] and c.[462A>G] were synonymous mutations, and c.[271C>T] were stopgaine; c.[271C>T] caused changes in encoded amino acids. Furthermore, c.[271C>T] was found in Proband–3 and her father, and c.[462A>G] was occurred in 17 SMA patients, 2 carrier and 2 normal individuals. In addition, 1 carrier was with c.[84C>T], 1 SMA patient with c.[−39A>G], and 1 SMA patient with g.[70240639G>C].

Novel mutations occurred in only SMA patients

Compared with control group, a total of 9102 mutation were selected out in SMA patients with homozygous deletion of *SMN1* exon7. They were located in the exon region, and occurred only in SMA patients not in carriers and normal individuals. Among them, 2415 genes and some indefinite genes were included, and 8619 SNVs, 267 deletions and 216 inserts were contained. Here, the indefinite genes were removed, and the 30 most frequent mutations were showed in Table 2 (Frequency ≥ 50%). It was obvious from Table 2 that only the *MCCC2* missense mutation c.[1001A>G] located on chromosome 5q13, which was the same location as *SMN1* and *SMN2.* The others were unlinked to 5q13.

Discussion

With the development of bioinformatics, more and more mutations have been discovered in *SMN1,* and some of them possess significant clinical implications. Ganji et al (14) reported conducted mutation screening of *SMN1* in 4 patients with 1 copy of *SMN1,* and identified 2 novel mutations including a single nucleotide insertion in exon 7 (c.861_862insT/p.R288X) and a deletion of nucleotide G in exon 3 (c.286delG/p.D96Tfs*53). Yamamoto et al (15) revealed 4 intragenic mutations (p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys), and location of the mutations were associated with the clinical severity of SMA. Ronchi et al (16) described a novel *SMN1* mutation that affected the donor splice site of exon 7 and resulted in an unusually severe SMA phenotype with rapid fatal outcome in an Italian infant. In this article, we found 6 *SMN1* SNVs in 28 core families of suspected SMA patients, including 4 novel mutations c.[84C>T], c.[271C>T], c.[−39A>G] and g.[70240639G>C], which had never been previously reported. At present, MLPA is the gold standard for clinical diagnosis of SMA. However, MLPA can only detect the deletion of *SMN1* according to the gene copy number, not detect point mutations of *SMN1.* It is well known that about 4% of SMA patients bear one *SMN1* copy with an intragenic mutation. Therefore, some

SMA patients are inevitably misdiagnosed as carriers. In this study, Proband–3 was with one *SMN1* copy and the *SMN1* stopgain mutation c.[271C>T], and the heterozygous deletion of *SMN1* exon 7 was from her mother, and the *SMN1* stopgain mutation c.[271C>T] from her father. The *SMN1* stopgain mutation c.[271C>T] was never reported before, and it led to a amino acid change. Although MLPA results showed Proband–3 to be carrier, some SMA-related clinical features were occurred on her. Here, we suspected that Proband–3 might be a SMA patient caused by the heterozygous deletion of *SMN1* exon 7 combined with the *SMN1* stopgain mutation c.[271C>T]. Simultaneously, c.[271C>T] might be involved in the onset of SMA. In addition, [2+0] genotype carriers are two *SMN1* copies on one chromosome and with deletion of *SMN1* on the other chromosome (17). In this article, we found 2 patients (Proband–1 and Proband–2) whose one parent was carriers and the other parent with 3 *SMN1* exon7 copies (Proband–1's mother and Proband–2's father). Based on our results we suspected that Proband–1's mother and Proband–2's father might be [2+0] genotype carriers.

SMN1 and *SMN2* present on chromosome 5q13, and of the 5q13-linked SMA patients, 96.4% show homozygous absence of *SMN1* exons 7 and 8 or exon 7 only, whereas 3.6% present a compound heterozygosity with a subtle mutation on one chromosome and a deletion/gene conversion on the other chromosome (6). Here, we identified more mutations combined with homozygous absence of *SMN1* exons7 (Table 2). The 3 most frequent mutations were the insertion mutation c.[−41_−40insCTCT] in *SPTA1* exon1 (rs111674514), the SNV c.[1001A>G] in *FUT5* exon2 (rs778984), and the SNV c.[−117A>G] in *MCCC2* exon1 (rs11746722). *SPTA1* encodes the human erythroid alpha-spectrin, which is an actin crosslinking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton, and functions in the determination of cell shape, arrangement of transmembrane proteins, and organization of organelles (18, 19). Mutations in *SPTA1* can lead to a variety of hereditary red blood cell disorders, including elliptocytosis type 2, pyropoikilocytosis, and spherocytic hemolytic anemia (20, 21). *FUT5* encodes alpha1,3-fucosyltransferase in human (22). The down-regulation of *FUT5* reduces the expression of sialyl-Lewis antigens and the adhesion and binding capacities of gastric cancer cells (23). Gene transfer of alpha1,3-fucosyltransferase increased tumor growth of the PC−3 human prostate cancer cell line through enhanced adhesion to prostatic stromal cells (24). Methylcrotonyl CoA carboxylase β (MCCβ) is encoded by *MCCC2,* and point mutations and deletion events in *MCC2* can lead to MCC deficiency (25, 26). MCC deficiency is a rare autosomal recessive genetic disorder whose clinical presentations range from benign to profound metabolic acidosis and death in infancy, which is has something in common with SMA in some ways. *MCCC2* locates on chromosome 5q13, which was the same as *SMN1.* Some studies indicated that *SMN1* was the causative gene, and other genes on 5q13 region acted as modifier gene (such as *SMN2, NAIP* and *GTF2H2),*, which were associated with disease severity (27). The mutations rs111674514 in *SPTA1,* rs778984 in *FUT5* and rs11746722 in *MCCC2* have been identified previously, but the clinical significance remains uncertain. In this article, we found they were widely prevalent in SMA patients, and almost nonexistent in non-patients. Therefore, it suggested they might be involved in the morbidity of SMA.

Conclusions

We found more mutations in both *SMN1* and other genes, and some of them were associated with the onset of SMA, such as the *SMN1* stopgain mutation c.[271C>T], the *SPTA1* insertion mutation c.[−41_−40insCTCT], the *FUT5* SNV c.[1001A>G], and the *MCCC2* SNV c.[−117A>G].

Abbreviations

SMA: spinal muscular atrophy; MLPA: multiplex ligation probe amplification; SNVs: single nucleotide variants; *SMN:* survival of motor neuron; *NAIP:* neuronal apoptosis inhibitory protein; GATK: genome analysis toolkit; MCCβ: Methylcrotonyl CoA carboxylase β

Declarations

Acknowledgements

We would like to thank all the members of our research group for their enthusiastic participation in this study.

Authors' contributions

CC designed the study and drafted the initial manuscript, and revised the manuscript. YM participated in the design of study and critically reviewed the manuscript. JS and YF collected samples and critically reviewed the manuscript. JW, ZX and XW analyzed and interpreted data and reviewed the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Program of Tianjin Science and Technology Plan (No. 18ZXDBSY00170), the Key Project of Tianjin Health Care Professionals (Grant No.16KG166) and the National Natural Science Foundation of China (Grant No.81771589). The authors declare that they have no conflict of interest with the organizations that sponsored the research, and the funding bodys were not involved in study design, data collection, analysis and writing of the study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the subjects signed the informed consent forms for genetic testing routinely, and the study was approved by the medical ethics committee of Tianjin Children's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Mutations in *SMN1*

Position	*MT*	*Ex-In*	*Function*	*REF>ALT*	*dbSNP*	*cHGVS*	*AAChange*	*Frequency*
70234668	*snv*	*exon2*	*synonymous*	*C>T*	*NA*	*c.[84C>T]*	*NA*	*3.61% (3/83)*
70220892	*snv*	*exon3*	*stopgain*	*C>T*	*NA*	*c.[271C>T]*	*p.[Gln91X]*	*2.41% (2/83)*
70238373	*snv*	*exon4*	*synonymous*	*A>G*	*rs4915*	*c.[462A>G]*	*NA*	*83.13% (69/83)*
70220892	*snv*	*UTR5*	*unknown*	*A>G*	*NA*	*c.[-39A>G]*	*NA*	*4.82% (4/83)*
70240639	*snv*	*intron*	*unknown*	*G>C*	*NA*	*g.[70240639G>C]*	*NA*	*8.43% (7/83)*
70247937	*snv*	*intron*	*unknown*	*A>C*	*rs200563560*	*g.[70247937A>C]*	*NA*	*1.20% (1/83)*

MT, mutation type; Ex-In, exon or intron; snv, single nucleotide variant; NA, no report or no change; X, unknown amino acids; Frequency, frequency in all samples.

Due to technical limitations, table 2 is only available as a download in the supplemental files section

Supplementary Files

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- *Table2.docx*