

Novel compound heterozygous mutations in the *SPTA1* gene, causing hereditary spherocytosis in a neonate with Coombs-negative hemolytic jaundice

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Abstract. Hereditary spherocytosis (HS) is a common heterogeneous type of inherited hemolytic anemia characterized by jaundice and splenomegaly. Diagnosis of HS in neonates is considered unreliable, and is generally based on positive family history, spherocytes in peripheral smears, increased osmotic fragility, and jaundice. In the present study, routine laboratory tests, next-generation sequencing, and Sanger sequencing were applied to diagnose a neonatal patient with Coombs-negative hemolytic jaundice. The neonate had no family history of HS; however, spherocytes were observed in peripheral smears, and the patient exhibited Coombs-negative and severe hemolytic jaundice, normal mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV), normal glucose-6-phosphate dehydrogenase activity, negative thalassemia genetic mutation screening results, and negative autoimmune antibody tests. Novel compound heterozygous mutations in the spectrin- α , erythrocytic 1 (*SPTA1*) gene (c.3897-1G>C and c.5029G>A) were identified. The *SPTA1* c.3897-1G>C mutation in intron 27-1, which disrupted the consensus splice site, was inherited from his asymptomatic mother, and the *SPTA1* c.5029G>A (p.Gly1677Arg) mutation in *trans* with the *SPTA1* c.3897-1G>C mutation was inherited from his asymptomatic father. Sanger sequencing of mRNA

reverse transcribed into cDNA identified a deletion of the first 10 nucleotides of exon 28, confirming the splicing mutation. In conclusion, the present study reports a rare case of autosomal-recessive HS with a severe clinical phenotype, but normal MCHC and MCV.

Introduction

Hereditary spherocytosis (HS) is a group of heterogeneously inherited hemolytic anemic disorders, characterized by the presence of spherical-shaped spherocytes on peripheral blood film, jaundice and splenomegaly (1). HS is the most frequent cause of inherited chronic hemolysis in North America and northern Europe, with prevalence rates of 1/5,000 and 1/2,000, respectively. HS is usually associated with quantitative or qualitative abnormalities in red blood cell membrane proteins, including ankyrin, band 3, β -spectrin, spectrin- α , erythrocytic 1 and protein 4.2, encoded by *ANK1*, *SLC4A1*, *SPTB*, *SPTA1* and *EPB42*, respectively (2). Splenectomy is widely considered as the standard surgical treatment in moderate and severe forms of HS (3). Newborn infants who have HS may develop anemia, extreme hyperbilirubinemia, bilirubin-induced neurologic dysfunction and kernicterus (4,5). Exact diagnosis of HS is considered unreliable around birth. However, if the early diagnosis of HS is recognized, appropriate treatment provided at the beginning of congenital hemolysis may prevent adverse outcomes in neonates with HS, caused by either *de novo* or compound heterozygous mutations, particularly for those with no family history of HS (6,7).

Deficiencies in ankyrin, band 3, spectrins or protein 4.2 account for 50-60, 15-20, 20 and <5% of patients with HS, respectively (8). Ethnic differences may exist: For instance in Japan, ankyrin defects account for only 5-10% of patients with HS, whereas protein 4.2 defects account for 45-50% (9). The major structural component of the skeletal network is the spectrin tetramer, which is formed by α -spectrin and β -spectrin (10). Spectrins are the central components of a complex spectrin-actin scaffold at the inner surface of the erythrocyte membrane. The C-terminal of α -spectrin and the N-terminal of β -spectrin associate to form heterodimers ($\alpha\beta$), and two heterodimers associate at the N-terminal of α -spectrin and the C-terminal of β -spectrin to form functional tetramers.

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The tetramers constitute the filaments of this network, and attach to the cellular membrane via interactions with various transmembrane proteins (11). Deficiency of α -spectrin is only clinically apparent in the homozygous or compound heterozygous state, and these patients have severe clinical manifestations. *SPTA1* mutations cause HS in association with recessive forms of inheritance, and the presence of two null *SPTA1* alleles is speculated to be lethal (12,13).

In the present study, a neonate with severe Coombs-negative hemolytic jaundice but no family history of HS was observed. The neonate was further clinically diagnosed with HS based on the following features: Spherocytes on the blood film, increased osmotic fragility, normal G6PD activity, normal hemoglobin in electrophoresis, negative thalassemia genetic mutation screening results and negative autoimmune antibody tests. Normal mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were observed. The molecular basis of HS was elucidated via next-generation sequencing (NGS) of relevant genes. Novel compound heterozygous mutations in the *SPTA1* gene (c.3897-1G>C and c.5029G>A), which were inherited from his asymptomatic mother and father, respectively, were identified to be responsible for his clinical phenotype.

Materials and methods

Subjects. A one-month old neonate with suspected HS and his asymptomatic parents (age, 30 years) were asked to participate in the study in February 2017. Written informed consent to use their blood samples and consent for publication were obtained from the parents, and this study was formally approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All procedures were performed in accordance with the approved guidelines.

The available clinical characteristics of the neonate, along with hematological data, osmotic fragility, glucose-6-phosphate dehydrogenase (G6PD) activity, thalassemia genetic mutation screening and autoimmune antibody tests are summarized in Table I.

Ampliseq NGS panel design. A custom NGS panel was designed on the Ion AmpliSeq Designer website (<http://www.ampliseq.com>; Ion AmpliSeq Designer; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to sequence all exons and adjacent introns of the *ANK1*, *SLC4A1*, *SPTB*, *SPTA1* and *EPB42* genes, with coverage of 96.71, 96.53, 99.54, 100 and 100%, respectively. The uncovered region was amplified and sequenced by Sanger sequencing.

NGS. Ion Torrent adapter-ligated DNA libraries were built according to the AmpliSeq™ Library Preparation kit 2.0 96Lv (Thermo Fisher Scientific, Inc.) using 10 ng of input DNA from peripheral blood mononuclear cells (PBMCs), quantitated by Qubit 2.0 (Invitrogen; Thermo Fisher Scientific, Inc.) in two primer-pools with 118 and 115 amplicons, respectively. The polymerase chain reaction (PCR) products of the two pools were mixed together, with samples barcoded using Ion Xpress Barcodes 1-16 (Thermo Fisher Scientific, Inc.), followed by purification using AMPure XP beads (Beckman Coulter, Inc.,

Brea, CA, USA) and quantification by Ion Library TaqMan™ Quantitation kit (Thermo Fisher Scientific, Inc.). Prepared libraries were pooled in equal amount and diluted to 100 pM. Subsequently, 2 μ l of pooled library were enriched using a Ion One-Touch Two (OT2) system with the Ion Personal Genome Machine™ (PGM) Hi-Q™ OT2 kit (Thermo Fisher Scientific, Inc.), followed by enrichment of Ion Sphere Particles on the Ion OneTouch Enrichment System apparatus (Thermo Fisher Scientific, Inc.). Enriched products were sequenced using the Ion Torrent PGM Hi-Q™ Sequencing kit using the Ion 316™ chip v2 (Thermo Fisher Scientific, Inc.).

Bioinformatics analysis. Raw data, following removal of adapter sequences, were aligned to the hg19 human reference genome. Coverage analysis and variant identification were performed on the Ion Torrent Server software version 4.4.2 (Thermo Fisher Scientific, Inc.), with default parameters. Variant annotation was performed with the Ion Reporter 5.0 software (Thermo Fisher Scientific, Inc.), according to the nomenclature recommended by the Human Genome Variation Society (<http://www.hgvs.org>), to classify variants as single nucleotide variants, multiple nucleotide variants or indels. All identified variants and regions with <20X coverage depth were visually verified with the Integrative Genomics Viewer v2.3.8 (Broad Institute, Cambridge, MA, USA). The clinical significance of annotated variants was assessed according to The 1000 Genomes Project (1000G; <http://www.internationalgenome.org/>), the Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk/ac/index.php>), the Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org/>), the Exome Variant Server (EVS; <http://evs.gs.washington.edu/EVS/>), ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/variation>) and InterVar (<http://wintervar.wglab.org/>) databases (14). Functional prediction of missense mutations was performed using multiple software packages, including Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org/www/SIFT_BLink_submit.html), MutationTaster (<http://www.mutationtaster.org/>) and Polymorphism Phenotyping (PolyPhen)-2 (<http://genetics.bwh.harvard.edu/pph2/>) (15-17).

Sanger sequencing. Sanger sequencing was performed as previously described (18). Briefly, genomic DNA was extracted from PBMCs with the QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany). Coding exons and splice junctions of relevant genes were amplified for identified mutations, failed amplicons or uncovered regions. RNA was extracted from PBMCs with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA at 42°C for 1 h for sequencing. Sanger sequencing was performed bi-directionally on an ABI 3500 Dx Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). NM_003126.2 was used as the reference transcript of the *SPTA1* gene. The following primers were used in the study: *SPTA1* c.3897-1G>C: Forward, 5'-CAAGGGATTGTTATCATAGG TG-3' and reverse, 5'-AAGGGGAAGAAATCAGTGA-3'; *SPTA1* c.5029G>A: Forward, 5'-CTACCTCTATCGCTCCCA CAT-3' and reverse, 5'-AACTGCTCTTCACTTCTCTCCA-3'; RT PCR primers: Forward, 5'-GTTTCAGGCTCTTCAGCGA CG-3' and reverse, 5'-CAAATCGTCCCGTTTCTTCAT-3'. PCR was performed using I-5™ 2X High-Fidelity Master Mix

Table I. Blood test results.

Hematological parameters	Result	Reference
RBC	$3.77 \times 10^{12}/l^b$	$4.3-5.8 \times 10^{12}/l$
MCV	99.7 fl	82-100 fl
MCH	33.4 pg	27-34 pg
MCHC	33.5 g/dl	31.6-35.4 g/dl
TBIL	$114.5 \mu\text{mol}/l^a$	$3.4-20.5 \mu\text{mol}/l$
IBIL	$104.7 \mu\text{mol}/l^a$	$\leq 13.3 \mu\text{mol}/l$
BRD	$9.8 \mu\text{mol}/l^a$	$0-6.8 \mu\text{mol}/l$
G6PD	4,415 U/l	$>1,100 \text{ U}/l$

^aElevated; ^bDecreased. RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; TBIL, total bilirubin; IBIL, indirect bilirubin; BRD, bilirubin direct; G6PD, glucose-6-phosphate dehydrogenase.

(MCLAB, South San Francisco, CA, USA) in a 50 μl volume including 19 μl water, 25 μl master mix, 2 μl forward primer, 2 μl reverse primer and 2 μl DNA or cDNA. The PCR was performed as following: Initial denaturation at 98°C for 2 min, followed by 35 cycles of 98°C for 10 sec, 62°C for 30 sec, 72°C for 3 sec, and a final extension at 72°C for 1 min.

Protein structure analysis. Protein structure analysis of the *SPTA1* c.5029G>A (p.Gly1677Arg) mutation was performed using the HOPE software (version 1.1.1; <http://www.cmbi.ru.nl/hope/>) (19). The sequence relative to *SPTA1* was downloaded from UniProtKB (<https://www.uniprot.org/uniprot/P02549>).

Results

Clinical features of the neonate with HS. This male neonate was delivered vaginally at 35 weeks of gestation (G2P2G35+4) following a normal pregnancy. No family members had a history of anemia or jaundice. The neonate weighed 5,100 g, and his Apgar scores were 9 and 10 (1 and 10 min, respectively), with no dysmorphic features. At 30 h, jaundice was observed, with a total serum bilirubin of $304.4 \mu\text{mol}/l$ (transcutaneous jaundice) and hemoglobin (Hb) of 118 g/l; phototherapy was initiated. On day 8, the neonate was transferred to the affiliated hospital of the present study due to jaundice and neonatal anemia, with an Hb of 85 g/l. The blood type of the parents was A Rh(+). The clinical characteristics of the neonate on day 8 are presented in Table I. Spherocytes were observed in peripheral smears, Coombs test was negative, G6PD activity was normal, no abnormal Hb was observed in electrophoresis, α - and β -thalassemia genetic mutation screen results were negative and autoimmune antibody tests were negative. Osmotic fragility was also increased. Discrepancies occurred in the patient between alterations in MCHC, MCV and clinical phenotype. The neonate had normal MCHC, MCV, and MCHC/MCV ratio, but received various blood transfusions in the three subsequent months due to severe anemia. The 31-year old father and 32-year old mother were asymptomatic.

NGS output and coverage. The sequencing of coding exons and adjacent introns of five HS-associated genes on the PGM achieved an average output of 425,997 mapped reads (98.9% on target). In summary, 100% of targeted amplicons were covered at least once, 99.14% amplicons were covered at least 20 times, and 97.42% amplicons were covered at least 100 times. The mean uniformity of base coverage was 95.09% in this panel. The average read depth was 1,453 fold.

Mutation detection and sanger sequencing validation. A total of 51 variants in the neonate were found and processed to discover pathogenic mutations. Following annotation and filtration, novel compound heterozygous mutations in the *SPTA1* gene (c.3897-1G>C and c.5029G>A) were identified. The *SPTA1* c.3897-1G>C (chr1:158615385, hg19) in intron 27-1, which may disrupt the consensus splice site, was inherited from his asymptomatic mother. The *SPTA1* c.5029G>A (p.Gly1677Arg, chr1:158607983, hg19) in *trans* with the *SPTA1* c.3897-1G>C mutation was inherited from his asymptomatic father (Fig. 1).

Functional prediction of the mutation. Sanger sequencing of mRNA reverse transcribed into cDNA identified a deletion of the first 10 nucleotides of exon 28, and confirmed that the *SPTA1* c.3897-1G>C in intron 27-1 disrupted the consensus splice site (Fig. 2). This mutation was not found in the 1000G, EVS, ExAC or HGMD databases.

The *SPTA1* c.5029G>A (p.Gly1677Arg) mutation was not found in the 1000G, EVS or HGMD databases, whereas four carriers (one in a South Asian and three in Non-Finnish Europeans) were found in the ExAC database, with an allele frequency of 3.328×10^{-05} , indicating a very low frequency in the population. This variant was predicted to be 'probably damaging' in PolyPhen, 'deleterious' in SIFT, 'disease causing' in MutationTaster, and 'likely pathogenic' in InterVar, with evidence of PM2 [absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium (20-22)], PM3 (detected in *trans* with a pathogenic variant), PP2 (missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease) and PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product) (23). The *SPTA1* c.5029G>A (p.Gly1677Arg) mutation may function in *trans* with *SPTA1* c.3897-1G>C in intron 27-1, causing HS in the neonate.

Protein structure analysis of the *SPTA1* c.5029G>A mutation. Protein structure analysis of the *SPTA1* c.5029G>A (p.Gly1677Arg) mutation was performed using the HOPE approach (<http://www.cmbi.ru.nl/hope/>) (19). The mutant residue was larger compared with the wild-type residue. The wild-type residue charge was neutral, and the mutant residue charge was positive. The wild-type residue was more hydrophobic compared with the mutant residue (Fig. 3A). The mutation was located within a stretch of residues that was repeated in the protein; this repeat was termed Spectrin 16. The wild-type residue was a glycine, which is the most flexible of all residues (19). This flexibility may be necessary for the function of the protein, and mutation of this glycine may abolish this function (Fig. 3B). The mutated residue was

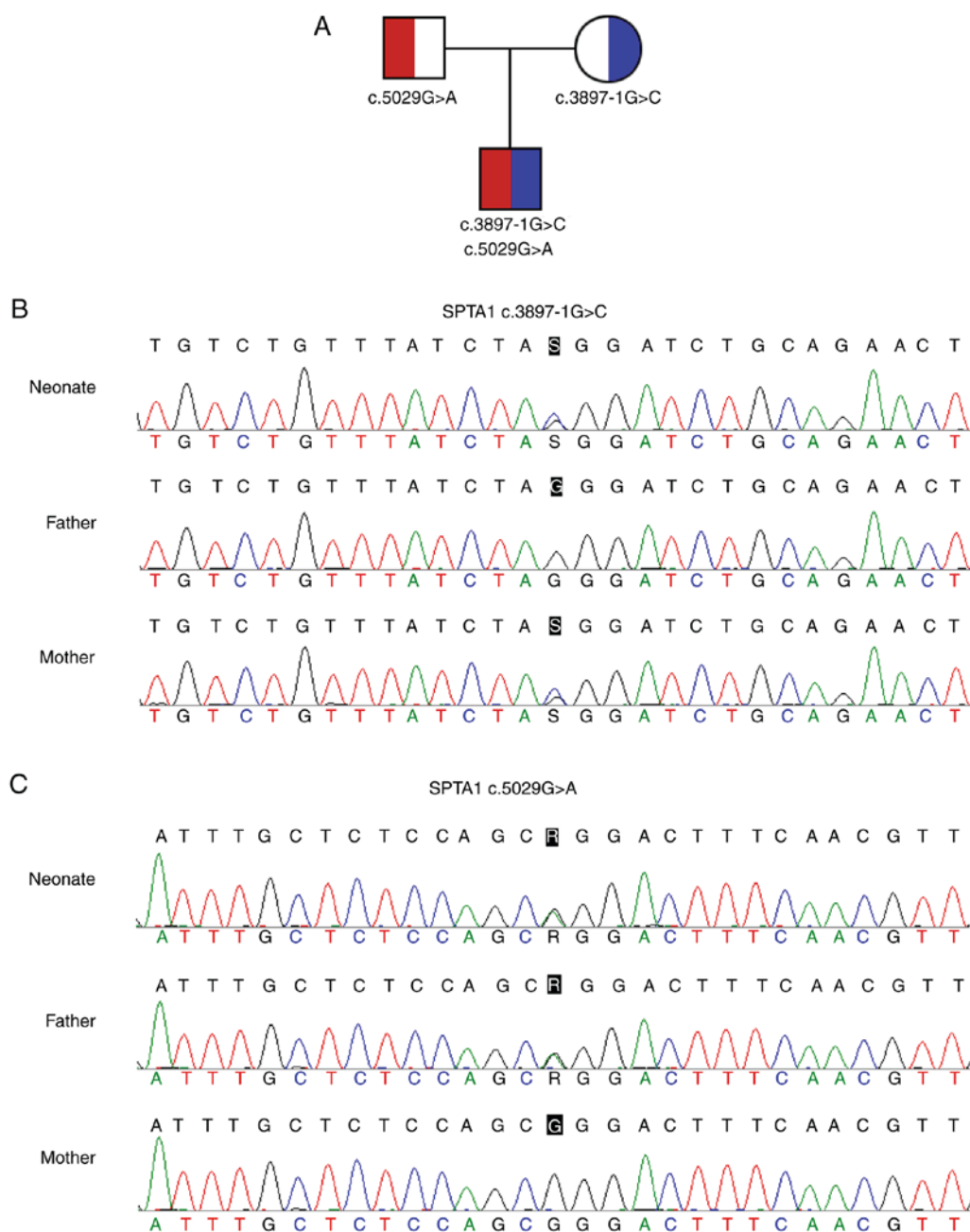


Figure 1. Compound heterozygous mutations in the *SPTA1* gene. (A) Family tree of a Chinese family with a neonate with hereditary spherocytosis. Square and circle denote male and female, respectively. (B) *SPTA1* c.3897-1G>C maternally transmitted mutation. (C) *SPTA1* c.5029G>A paternally transmitted mutation. SPTA1, spectrin- α , erythrocytic 1.

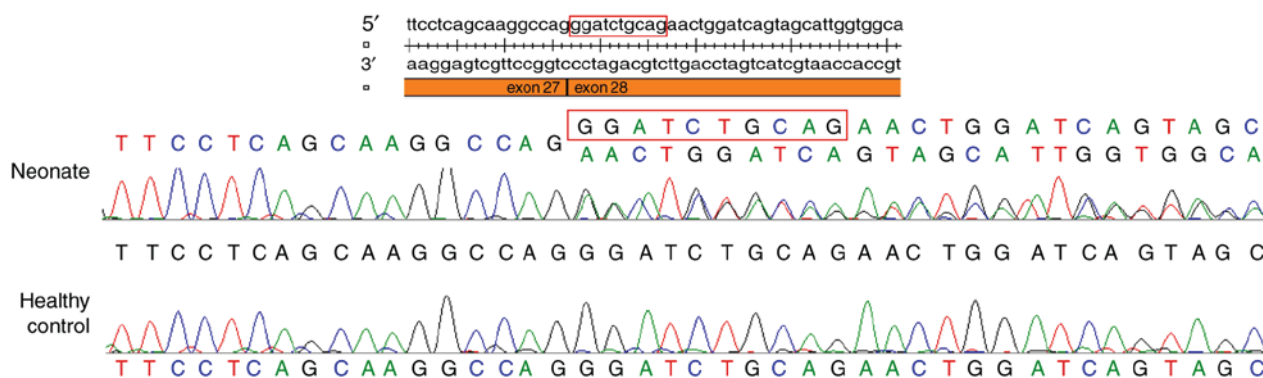


Figure 2. Sanger sequencing of mRNA reverse transcribed into cDNA from peripheral blood mononuclear cells. A deletion of the first 10 nucleotides of exon 28 was observed in the neonate.

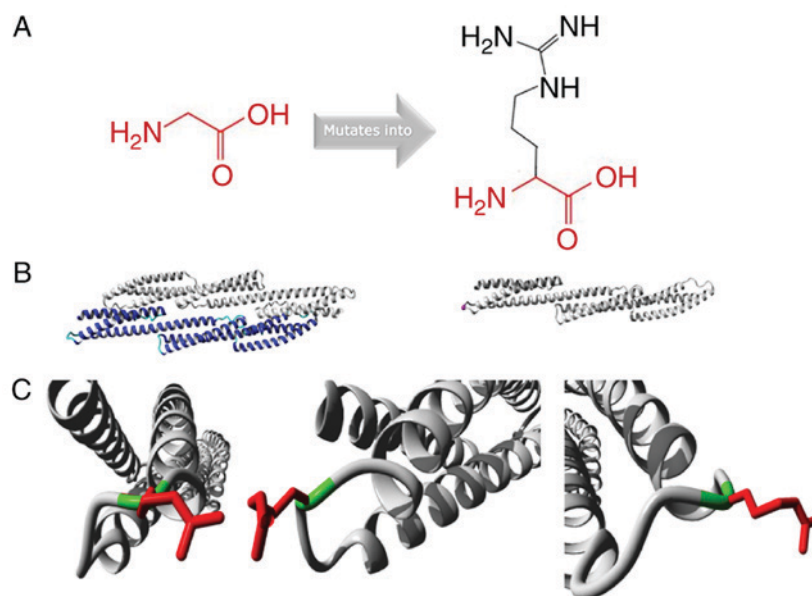


Figure 3. Protein structure analysis of *SPTA1* c.5029G>A (p.Gly1677Arg). (A) Schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is colored red. The side chain, unique for each amino acid, is colored black. (B) Left is an overview of the protein in ribbon-presentation. The protein is colored by element: α -helix, blue; β -strand, red; turn, green; 3/10 helix, yellow; and random coil, cyan. Other molecules in the complex are colored grey when present. Right is an overview of the mutant protein in ribbon-presentation. The protein is colored grey and the side chain of the mutated residue is colored magenta (pink spheres). (C) Close-up of the mutation. The protein is colored grey, the side chains of the wild-type and the mutant residue are presented and colored green and red, respectively. The structure is presented in three different angles.

located in a domain that was important for binding of other molecules, and was in contact with residues in a domain that was also important for binding. The mutation may disturb the interaction between these two domains and negatively affect the function of the protein. Moreover, the torsion angles for this residue were unusual. Only glycine was flexible enough to generate these torsion angles, and mutation into another residue may force the local backbone into an incorrect conformation and disrupt the local structure (Fig. 3C).

Discussion

HS is a heterogeneous disorder, and the majority of cases are inherited in an autosomal-dominant fashion with an unambiguous family history. The phenotype of HS in neonates ranges from asymptomatic to hydrops fetalis (6,13). HS is the third most common underlying hemolytic anemia, following G6PD and ABO hemolytic disease, among neonates listed in the USA Kernicterus Registry (6). Moreover, HS is the leading cause of Coombs-negative hemolytic anemia, requiring blood transfusion in the first months of life (6). Neonates with HS may present with extreme hyperbilirubinemia, thus a timely diagnosis may prevent adverse outcomes in neonates with HS (24).

For neonates with Coombs-negative hemolytic jaundice and a negative family history, the presence of spherocytes on blood film, an increased MCHC and a decreased MCV suggest a diagnosis of HS (25). Christensen *et al* (26), and Christensen and Sheffield (27), speculated that an MCHC of 36.0 g/dl or more could alert caregivers to the possibility of HS. However, the neonate described in the present study exhibited normal MCHC, MCV and MCHC/MCV ratio. Hemolytic jaundice and anemia were the principal signs of HS, and blood transfusions were performed almost monthly to maintain the Hb concentration

in the blood. Neonates with normal MCHC and MCV have also been reported previously. For example, Yaish *et al* (25) described a neonate with α -spectrin deficient HS confirmed by SDS-PAGE, and the neonate had Coombs-negative spherocytic hemolytic jaundice in addition to normal MCHC and MCV. These results suggested that for patients with normal MCHC, MCV or MCHC/MCV ratio, additional tests may be required. NGS is widely used in the diagnosis of hereditary hemolytic anemia, including HS (28-31). Moreover, the mutation spectrum and genetic characteristics in the Chinese population have been identified by exome sequencing (31). Additionally, the eosin-5'-maleimide binding test, performed using flow cytometry, has been also recommended for HS diagnosis (32).

The *SPTA1* gene is located at 1q22-23, and α -spectrin is an integral part of the erythrocyte cytoskeleton, which is linked to the surface of the plasma membrane through ankyrin and band 3. Homozygous or compound heterozygous mutations in the *SPTA1* gene may be involved in the pathogenesis of recessive spectrin-deficient HS, and heterozygous individuals may still produce sufficient α -spectrin to balance β -spectrin and maintain the erythrocyte cytoskeleton (33,34). Missense and splicing mutations account for the majority of cases.

In this case, novel compound heterozygous mutations in the *SPTA1* gene (c.3897-1G>C and c.5029G>A) were identified in the neonate. The *SPTA1* c.3897-1G>C mutation was not found in the 1000G, EVS, ExAC or HGMD databases. Sanger sequencing of the mRNA reverse transcribed into cDNA identified a deletion of the first 10 nucleotides of exon 28, and the ninth and tenth nucleotides of exon 28 were AG. This result indicated that the *SPTA1* c.3897-1G>C mutation in the consensus splice site led to a new AG splice acceptor site at the ninth and tenth nucleotides of exon 28, resulting in a deletion of the first 10 nucleotides of exon 28.

The *SPTA1* c.5029G>A mutation resulted in a glycine-to-arginine substitution at amino acid position 1,677 of α -spectrin. Various types of software predicted that this missense mutation would have deleterious effects. Moreover, protein structure analysis implied that this mutation may negatively affect the function of α -spectrin as follows. First, the wild-type residue charge was neutral, whereas the mutant residue was positive. Additionally, the wild-type residue was more hydrophobic compared with the mutant residue, and the mutated residue was located in a domain important for the binding of other molecules and in contact with residues in a domain important for binding. The mutation may disrupt the interaction between these two domains. Only glycine is flexible enough to generate these torsion angles, and mutation into arginine may force the local backbone into an incorrect conformation and disrupt the local structure. Thus, *SPTA1* c.5029G>A (p.Gly1677Arg) in *trans* with the *SPTA1* c.3897-1G>C mutation may have caused HS in this neonate.

In summary, novel compound heterozygous mutations in the *SPTA1* gene (c.3897-1G>C and c.5029G>A) may be responsible for the clinical manifestations of the described neonatal patient with HS. In the present study, NGS provided a rapid and powerful approach for the genetic diagnosis of HS.

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Availability of data and materials

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

Authors' contributions

AL, YL and QH designed the study. XW and YL performed the experiments and drafted the manuscript. AL and QH cared for the patient. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent to use blood samples from the subjects was obtained from the parents. The present study was formally approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All procedures were performed in accordance with the approved guidelines.

Patient consent for publication

Written consent for publication from all subjects was obtained.

Competing interests

The authors declare that they have no competing interests.

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