

Genetic diagnosis and pathogenic analysis of an atypical hereditary spherocytosis combined with UGT1A1 partial deficiency: A case report

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Received April 17, 2017; Accepted August 31, 2017

DOI: 10.3892/mmr.2017.7867

Abstract. Patients with combined hereditary spherocytosis (HS) and uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) deficiency have been reported sporadically. A discrepancy between the level of elevated serum bilirubin concentration and the degree of anemia may suggest the possibility of a coexistence of these conditions. In the present case report, a 20-year-old female presented with congenital jaundice and anemia, but did not present with the discrepancy between hyperbilirubinemia and anemia in the patient's childhood, and was not previously diagnosed with either HS or UGT1A1 deficiency. During a follow-up of >10 years, the patient's hyperbilirubinemia accumulated progressively, whereas the patient's anemia became relatively mild. Upon further genetic analysis, it was determined that the patient had HS combined with UGT1A1 partial deficiency. Next generation sequencing combined with direct sequencing was used to identify a novel heterozygous mutation (c.G828T; p.Y276X) in the spectrin β gene, which is causative for HS. Sequence analysis of the patients' *UGT1A1* gene revealed a compound heterozygote with c.G211A (p.G71R) and T3279G mutations, which reduced UGT1A1 activity to 30-60% of the normal level. Genetic analysis was crucial for determining the diagnosis and pathogenesis of this unusual case.

Introduction

Hereditary spherocytosis (HS) and uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) deficiency are common inherited conditions characterized by unconjugated

hyperbilirubinemia. HS is an inherited hemolytic anemia caused by constitutional erythrocyte membrane defects, and characterized clinically by anemia with variable severity, jaundice and splenomegaly (1). Diagnosis is usually established based on the presence of spherocytes, increased erythrocyte osmotic fragility and the absence of any other cause of the hemolytic anemia (1). UGT1A1 is responsible for bilirubin glucuronidation, and reduced activity of UGT1A1 is associated with an increased level of unconjugated bilirubin (2). UGT1A1 deficiency is a common hereditary condition of bilirubin metabolism in the general population, particularly in Asian communities (2,3).

As the prevalence of HS is 1 in 2,000 (4), and that of UGT1A1 caused by G211A mutation deficiency is much higher at 13-23% in Asian newborns (2,3), diagnoses of their coexistence should not be rare; however, the number of documented cases is low (5,6). This underdiagnosis may be attributed to one condition masking another (7), or to a lack of conclusive evidence for their coexistence. In the present case report, atypical HS combined with UGT1A1 partial deficiency was diagnosed by pathogenic gene analysis. A novel c.G828T (Y276X) mutation in spectrin β (*SPTB*), which is causative for HS, along with compound heterozygous mutations in *UGT1A1*, T3279G and G211A, which are pathogenic for UGT1A1 partial deficiency, were identified. In addition, the individual pathogenesis of hyperbilirubinemia was investigated for this patient.

Materials and methods

Patient. The present study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (Changsha, China), and written informed consent was obtained from the patient and the patient's mother. On July 12, 2016, a 20-year-old female with congenital jaundice and anemia for ~20 years was re-hospitalized to confirm the patient's condition. The proband was diagnosed with neonatal jaundice several days following birth. In 2003, when the patient was 7 years old, splenomegaly, anemia and jaundice were detected. Between 2004 and 2005, hyperplastic anemia (hemoglobin, 96 g/l; reticulocytes, 0.05), splenomegaly, elevated serum bilirubin [total bilirubin (TBIL), 36.9 μ mol/l;

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Key words: hereditary spherocytosis, uridine diphosphate glucuronosyltransferase 1A1, genetic diagnosis, pathogenesis, next generation sequencing, spectrin β gene

direct bilirubin, 11.3 $\mu\text{mol/l}$] and detectable spherocytes in blood smear were identified, and HS was suspected. However, the erythrocyte osmotic fragility test, which is a crucial test for HS diagnosis, was repeatedly normal. Furthermore, the elevated serum bilirubin concentration decreased to normal level following phenobarbitone treatment, which suggested UGT1A1 deficiency, which would not contribute to hyperplastic anemia and splenomegaly. Subsequently, the patient has been interviewed regularly. Upon infection or overexertion, the patient's serum bilirubin increased significantly, mainly by unconjugated bilirubin. Notably, bilirubin levels gradually increased over time, whereas the anemia was mild. In 2013, when the patient was 17 years old, the patient's serum TBIL rose to $>80 \mu\text{mol/l}$ and hemoglobin was $>100 \text{ g/l}$. There was no history of alcoholism, hepatitis, drug ingestion or drug abuse. The proband's father had a history of cholelithiasis, and succumbed to cirrhosis in his twenties. The patient's father's brother also died early, and presented with jaundice at his death.

Clinical analysis. The proband received a detailed clinical evaluation at the Second Xiangya Hospital of Central South University. Extensive laboratory testing was performed, including complete blood count, liver functional tests, lactate dehydrogenase, vitamin B12, folate, iron studies, rheumatoid factor and anti-nuclear antibodies testing, erythrocyte osmotic fragility test, hemoglobin electrophoresis, Ham test, glucose-6-phosphate dehydrogenase activity, Coombs' direct and indirect tests, flow cytometric test using eosin-5'-maleimide (EMA) labeling of red blood cells (1,8) as well as abdominal computed tomography (CT) scans. Additionally, bone marrow and blood smears of the patients were stained by Wright-Giemsa staining for 30 min at room temperature. Then the smears were observed using a light microscope, erythrocyte morphology was evaluated, and the proportion of spherocytes was counted among 500 erythrocytes.

Genetic analysis. A total of 5 ml peripheral vein blood was drawn from the proband and the proband's mother. The genomic DNA was extracted using QIAamp DNA Blood Mini kit (cat. no. 51104; Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol, quantified using an Eppendorf BioPhotometer® D30 (Eppendorf, Hamburg, Germany) and stored at -20°C until use. Then the genomic DNA library was constructed according to the Agilent's protocol for Preparing Samples for Sequencing Genomic DNA. Using the SureSelectXT Reagent kits (cat. no. G9611B; Agilent Technologies, Inc., Santa Clara, CA, USA), 3 μg high-quality gDNA was sheared by the Bioruptor® Plus sonication device (Diagenode S.A., Seraing, Belgium) with 175 peak incident power, end-repaired at 20°C (30 min), adenylated at 37°C (30 min) and ligated with an adaptor at 20°C (15 min) using a next-generation high throughput sequencer (HiSeq 2000 v3; Illumina, Inc., San Diego, CA, USA). The DNA fragments were purified at each step with AMPure XP beads DNA Purification kit (cat. no. A63880; Beckman Coulter, Inc., Brea, CA, USA) according to the manufacturer's protocol.

The 5 known genes associated with inherited spherocytosis include ankyrin 1, *SPTB*, spectrin α erythrocytic 11, solute

carrier family 4 member 1 and erythrocyte membrane protein band 4.2 gene. To amplify and capture the 5 known genes a customized Agilent Comparative Genomic Hybridization Array for the exons and adjacent intron regions (50 bp) of the 5 genes and SureSelect Focused Exome kit (cat. no. 5190-7788, Agilent Technologies, Inc.) were used according to the manufacturer's protocol. The adaptor-ligated library was amplified in 25 μl reaction using 5X Herculanase II Reaction Buffer (5 μl), SureSelect Primer and SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (0.625 μl each), 100 mM dNTP mix (0.25 μl), Herculanase II Fusion DNA Polymerase (0.5 μl) and adaptor-ligated DNA (18 μl). The thermal cycler conditions were as follows: Initial denaturation at 98°C for 2 min, 4-6 cycles of denaturation at 98°C (30 sec), annealing at 65°C (30 sec) and extension at 72°C (1 min), and final extension at 72°C (10 min).

To capture the target DNA fragments, 500 ng total library, which was concentrated by a vacuum concentrator (Concentrator Plus; Eppendorf), was hybridized with streptavidin-coated beads, at 65°C for 24 h. Then the captured gene fragments were sequenced by the next-generation high throughput sequencer. The results demonstrated that 88.61% of target bases were covered to a total depth of $>20\times$ with high quality (Q20) reads. Reads were aligned to the reference sequence University of California Santa Cruz, human genome assembly 19 (UCSC.hg19; genome.ucsc.edu/). Variants, including single-nucleotide polymorphisms (SNPs) and indels, were identified and called with the VCFtools program of the SAMTools software, version 0.1.16 (samtools.sourceforge.net/). Amino acid substitutions that affected protein function were annotated with polymorphism phenotyping v2 (PolyPhen-2; genetics.bwh.harvard.edu/pph2) and MutationTaster2 (mutationtaster.org). Pathogenicity of the variants was interpreted according to the American College of Medical Genetics (ACMG) guidelines (9).

The detected mutation in *SPTB* was confirmed by direct sequencing of the polymerase chain reaction (PCR) products amplified from the genomic DNA samples of the patient and her mother, which were extracted from their blood as mentioned above. Genome DNA samples were amplified in 25 μl reactions using 2X Power Taq PCR MasterMix (12.5 μl ; BioTeke Corporation, Beijing, China), nuclease-free water (11 μl), 10 pmol/ μl forward and reverse primers (0.5 μl each), and 100 ng/ μl template (0.5 μl). The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C (30 sec), annealing at 57°C (30 sec) and extension at 72°C (1 min), and final extension at 72°C (7 min). Primers for PCR were designed using primer3 software (primer3.ut.ee): *SPTB* forward, 5'-TGCTCTGTTGGTTGTCACTTG-3' and reverse, 5'-AGCCATCAATGTGCCAAGG-3'. PCR products were examined on 1% agarose gels containing 0.5 $\mu\text{g/ml}$ ethidium bromide and were subsequently sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sequence comparisons and analysis were performed using Phred-Phrap-Consed version 12.0 software (phrap.org/phredphrapconsed.html). Sequencing results were compared with the reference sequence for *SPTB* (GenBank accession no. NM_001024858) in the UCSC database to confirm potential mutation. Then the detected mutation in

Table I. Primers sequences of *uridine diphosphate glucuronosyltransferase 1A1* gene.

<i>UGT1A1</i> exon	Primer sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)
Exon 1-1	F: TATAAGTAGGAGAGGGCGAACC R: TCAAATTCAGGCTGCATG	588	57
Exon 1-2	F: GGCCTCCCTGGCAGAAAG R: ATGCCAAAGACAGACTCAAACC	617	60
Exon 2	F: AGGAACCCTTCCTCCTTTAGA R: GAAGCTGGAAGTCTGGGATTAG	402	59
Exon 3	F: CCTCAGAAGCCTTCACAGTTAC R: ATCCAATCCGCCCAACATAC	255	59
Exon 4	F: GTGTCCAGCTGTGAAACTCA R: TGAATGCCATGACCAAAGTATTC	323	55
Exon 5	F: CAACAGGGCAAGACTCTGTATC R: CCTTATTTCCCACCCACTTCTC	489	60
Promoter	F: ACAGGTTTCCATGGCGAAAG R: TGTTTTGATCACACGCTGCA	782	56
PBREM	F: GGTCACTCAATTCCAAGGGG R: GCATCCAAGCCAGCAAGTAA	598	61

PBREM, phenobarbital responsive enhancer module; *UGT1A1*, uridine diphosphate glucuronosyltransferase 1A1; F, forward; R, reverse; bp, base pairs.

SPTB (G828T) was absent in the 1,000 Genomes Project database and Exome Aggregation (10).

Amplification of the exons, the promoter region and the enhancer region [that is, the phenobarbital responsive enhancer module (PBREM)] of *UGT1A1* was performed by PCR. Genome DNA samples were amplified in reactions as mentioned above. Thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C (30 sec), annealing temperatures as shown in Table I (30 sec), extension at 72°C (1 min), and final extension at 72°C (7 min). Primer sequences are listed in Table I. Amplified DNA fragments were sequenced as aforementioned. Mutations in *UGT1A1* were identified by comparing the sequencing results with the reported reference sequence (GenBank accession no. NM_000463).

Results

Clinical diagnosis. Physical examination revealed cutaneous and icteric sclera; spleen was palpable 4 cm below costal margin, and was firm and non-tender. TBIL was 106.2 $\mu\text{mol/l}$ and direct bilirubin was 7.5 $\mu\text{mol/l}$. The complete blood count revealed hemoglobin 103 g/l, reticulocytes 0.358x10¹²/l, mean corpuscular volume 84.3 fl, mean corpuscular hemoglobin 30.6 pg and mean corpuscular hemoglobin concentration 363 g/l, and white blood cell and platelet counts were normal.

Extensive laboratory evaluation revealed normal levels of alanine aminotransferase, aspartate transaminase, lactate dehydrogenase, vitamin B12, folate, iron studies, rheumatoid factor and anti-nuclear antibodies. A series of diagnostic tests for hemolytic anemia, including erythrocyte osmotic fragility test, hemoglobin electrophoresis, Ham's test, glucose-6-phosphate dehydrogenase activity, Coombs' direct and indirect tests were normal. Bone marrow smear indicated hypercellular

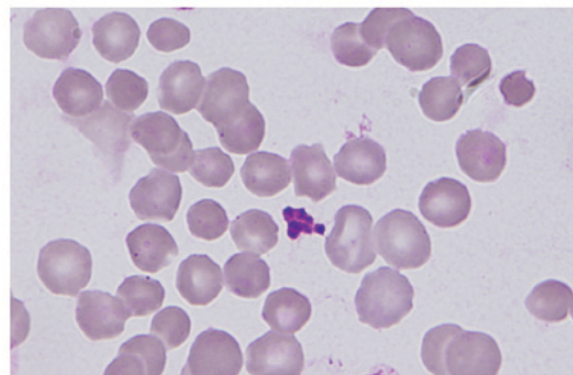


Figure 1. Peripheral blood smear from the patient. Some red cells presented with dense, spheroidal shape using the Wright-Giemsa stain, whereas other cells retained a central concavity. Magnification, x400.

marrow with myeloid-to-erythroid precursor ratio of 0.4:1. Blood smear results demonstrated that spherocytes accounted for 62 out of 500 red blood cells (Fig. 1). EMA test of red blood for the diagnosis of HS was positive. An abdominal CT scan revealed splenomegaly without the combination of gallstone or cholangiectasis.

HS was diagnosed clinically based on the presence of hyperplastic anemia, splenomegaly and spherocytes, and particularly, the positive EMA test. Although the erythrocyte osmotic fragility test was repeatedly normal, the positive EMA test supported the diagnosis of HS.

Pathogenic mutation analysis of *SPTB*. Sequencing analysis revealed that the proband was heterozygous for the c.G828T (p.Y276X) mutation within exon 7 of *SPTB*, which was not present in the mother (Fig. 2). This mutation introduced a premature stop codon at amino acid residue 276, which created

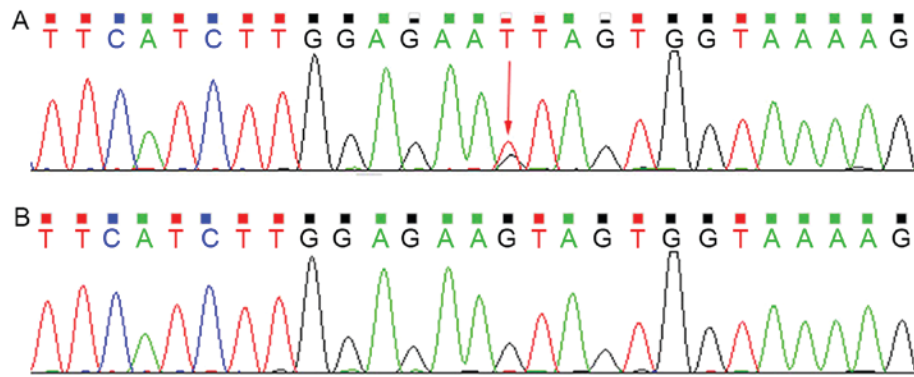


Figure 2. Nonsense mutation of c.G828T (p.Y276X) within exon 7 of the spectrin β gene. The sequencing results of (A) the patient (mutant) and (B) the patient's mother (wild-type). The red arrow indicates the mutation.

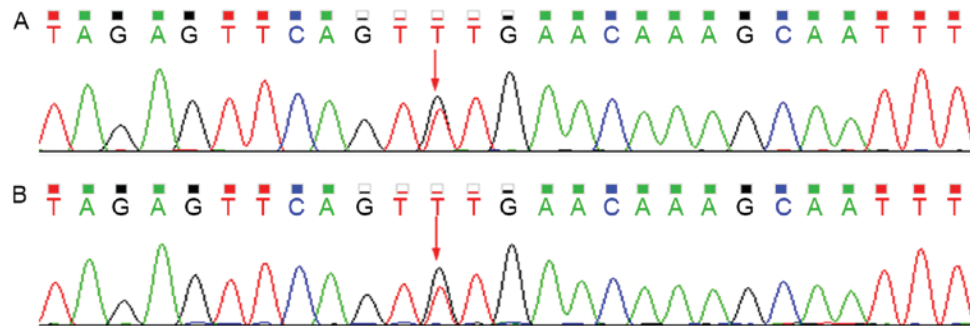


Figure 3. Heterozygous mutation c.T3279G within the phenobarbital-responsive enhancer module of the uridine diphosphate glucuronosyltransferase 1A1 gene. Sequencing results of (A) the patient and (B) the patient's mother, suggested that the c.T3279G in the patient may have been inherited from the patient's mother. The red arrow indicates the mutation.

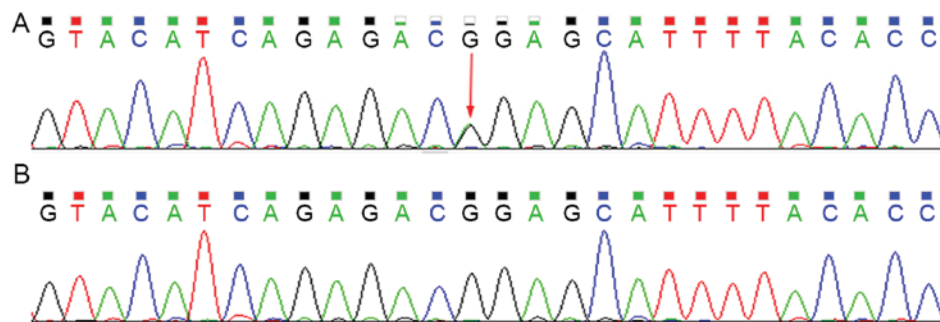


Figure 4. Missense mutation of c.G211A (p.G71R) within exon 1 of the uridine diphosphate glucuronosyltransferase 1A1 gene. The sequencing results of (A) the patient (mutant) and (B) the patient's mother (wild-type). The red arrow indicates the mutation.

a truncated protein and was predicted to be disease-causing by the analytical software used. In addition, it had not been reported previously and is a novel mutation.

Although *SPTB* c.G828T may be a novel mutation, several nonsense mutations located downstream of it have been previously identified as pathogenic mutations (11,12). Therefore, combined with the patient's clinical diagnosis of HS, the c.G828T SNP was identified as a pathogenic mutation, and was probably causative for this HS development in this patient, according to the ACMG guidelines. Notably, although previously reported cases of HS that had nonsense mutations in *SPTB* displayed a conspicuous spherocytosis with frequently encountered dense spiculated red blood cells (11), the spherocytes of the present case had not exhibited prominent surface projections (Fig. 1).

Pathogenic mutation analysis of *UGT1A1*. Sequencing analysis revealed that the patient harbored the c.G211A (p.G71R) heterozygous mutation within exon 1 of *UGT1A1* and the T3279G heterozygous mutation within the PBREM of *UGT1A1*. The heterozygous T3279G mutation was also detected in the proband's mother, whereas the G211A mutation was not (Figs. 3 and 4).

In a previous study, the heterozygous G211A mutation was reported to reduce UGT1A1 transferase activity to 60% of the normal level, and the homozygous G211A mutation reduced the activity to 30%, whereas the homozygous T3279G mutation reduced the activity to approximately 60% (13). Therefore, the compound heterozygous mutations may result in 30-60% activity of the normal UGT1A1 level.

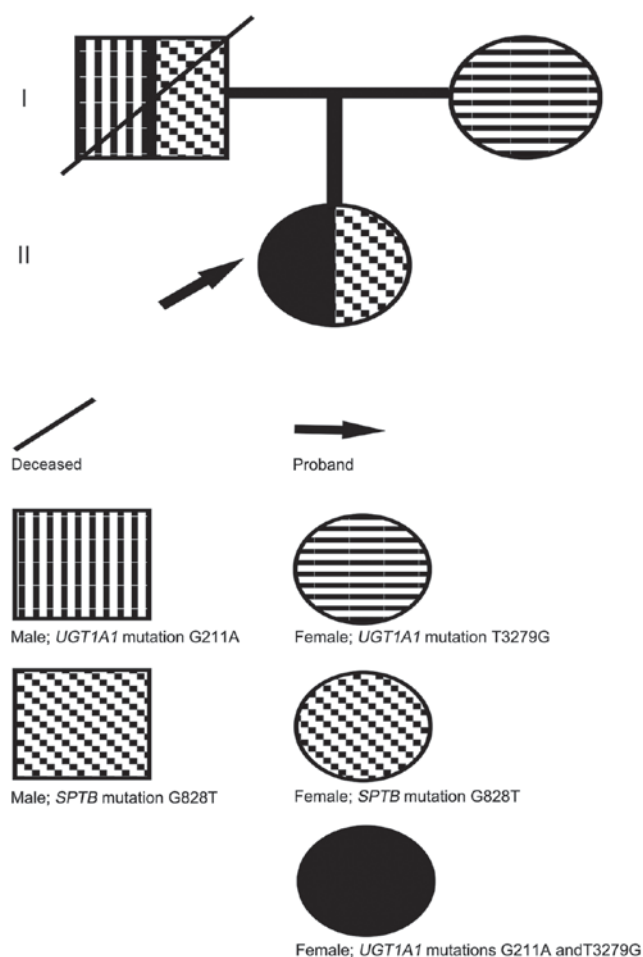


Figure 5. Pedigree of the case study patient's family with hereditary spherocytosis and *UGT1A1* deficiency. *SPTB*, spectrin β ; *UGT1A1*, uridine diphosphate glucuronosyltransferase 1A1.

Based on the present results, *UGT1A1* partial deficiency was diagnosed.

Discussion

HS and *UGT1A1* deficiency are relatively common causes of unconjugated hyperbilirubinemia, and their coexistence may interfere with the proper diagnosis (2-4). The present study described a female Chinese patient presenting with congenital jaundice and anemia that was eventually diagnosed as having HS combined with *UGT1A1* deficiency following follow-up of >10 years.

In the present case, anemia, splenomegaly, reticulocytosis, spherocytes, hypercellular bone marrow with remarkable erythroid hyperplasia and positive family history had led to the presumptive diagnosis of HS. However, the erythrocyte osmotic fragility test was repeatedly normal, and there was no cut-off value of spherocytes for the diagnosis of HS. Therefore, confirmation of the diagnosis relied on the positive EMA test and genetic analysis.

As the patient's clinical findings were not typical for both HS and *UGT1A1* deficiency, the diagnoses had been pending for >10 years. Notably, the serum bilirubin levels increased over time; while the patient's anemia became milder. Then, up to puberty, the discrepancy in the severity of hyperbilirubinemia

and anemia became significant, which may be related to the increased hemoglobin turnover around puberty (5). This discrepancy suggests that the patient's HS coexists with other conditions associated with unconjugated hyperbilirubinemia, especially *UGT1A1* partial deficiency. Therefore, for patients with similar discrepancies (inappropriately high serum bilirubin level compared with the degree of hemolysis), the possibility of the coexistence of *UGT1A1* partial deficiency and HS should be considered.

HS is normally inherited in an autosomal dominant manner, which may be induced by pathogenic mutations in ankyrin 1 on chromosome 8p11, *SPTB* on chromosome 14q23, spectrin α erythrocytic 1 on chromosome 1q21, solute carrier family 4 member 1 on chromosome 17q21, or erythrocyte membrane protein band 4.2 on chromosome 15q15 (14). As HS may develop from multiple pathogenic genes, genetic diagnosis was not applicable prior to the clinical application of NGS. In previously reported cases of HS combined with *UGT1A1* deficiency, the diagnosis of HS was based on typical clinical findings, such as spherocytes with increased erythrocyte osmotic fragility (5,6). Thus, atypical cases with normal erythrocyte osmotic fragility may have been underdiagnosed. The present study used NGS combined with direct sequencing to identify a novel pathogenic mutation, *SPTB* G828T, and the diagnosis of HS was confirmed in this case study. The *SPTB* G828T mutation was not present in the proband's mother, and was probably inherited from the deceased father (Fig. 5).

UGT1A1 deficiency results from the causative mutations in *UGT1A1* gene, which is located on chromosome 2q37 (15). A number mutations or SNPs of *UGT1A1* that influence bilirubin glucuronidation have been identified, which may cause Crigler-Najjar syndrome, Gilbert's syndrome, neonatal hyperbilirubinemia or non-symptom based on the severity of the decrease in enzymatic activity (16,17). Therefore, genetic analysis was crucial to clarify the diagnosis and severity of *UGT1A1* deficiency. In the present case report, compound heterozygous mutations (T3279G and G211A) probably led to the reduced *UGT1A1* activity, to 30-60% of normal activity; patients with 30-60% *UGT1A1* activity are typically asymptomatic (13), other than experiencing jaundice if not in combination with other conditions such as HS. The T3279G mutation of *UGT1A1* in the proband was also detected in the mother, but the G211A mutation was not, which suggested that the patient inherited the T3279G mutation from the patient's mother and the G211A mutation from the patient's deceased father (Fig. 5). Additionally, although the proband did not develop gallstones, which is common in patients with both HS and *UGT1A1* deficiency (18), the patient was considered to be at-risk for cholelithiasis and was advised to accept periodical abdominal ultrasonic examination.

In conclusion, both HS and *UGT1A1* deficiency are associated with hyperbilirubinemia, and their coexistence is often underdiagnosed. The discrepancy between the level of elevated serum bilirubin and the degree of hemolysis suggested the possibility of the coexistence. However, when the clinical or laboratory findings were inconclusive, the diagnosis was questioned; thus, genetic analysis was crucial to confirm the diagnosis and to avoid underdiagnosis. Early diagnosis saves time-consuming clinical reasoning and observation. Comprehensive NGS, as a new diagnostic tool, may

not only identify HS specifically and efficiently, but also may contribute to expanding the mutation spectrum of associated genes. In addition, genetic analysis combined with evaluation of UGT1A1 activity may clarify the pathogenesis of the associated hyperbilirubinemia and may explain the clinical variability, which may aid patients in making informed medical and personal decisions.

Acknowledgements

The present study was supported in part by the National Natural Sciences Foundation of China (grant no. 81100360).

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