β-thalassemia caused by compound heterozygous mutations and cured by bone marrow transplantation: A case report

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Abstract. In the present study, a rare familial case of severe thalassemia with compound spontaneous mutations is reported. A 2.5-year-old boy, who suffered from severe anemia with yellowish skin, enlarged liver and spleen, was provided with a blood transfusion every 20 days to maintain hemoglobin levels between 90 and 100 g/l. Sanger sequencing combined with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Gap-PCR revealed that the proband was a carrier of 4 compound heterozygous mutations: Hemoglobin subunit β (*HBB*):IVS-II-654(C>T)β⁺; Southeast Asian-type-hereditary persistence of fetal hemoglobin (SEA-*HPFH*); *HBB*:c316-148G>T; hemoglobin subunit α2 (*HBA2*):c.46G>A. The father of the proband was identified as a carrier of the heterozygous SEA-*HPFH* mutation, the mother was a carrier of compound heterozygous mutations of *HBB*:IVS-II-654(C>T) and *HBA2*:c.46G>A, and the elder sister was heterozygous for *HBB*:IVS-II-654(C>T)β⁺. Based on these genetic results, it was determined that the proband had both of heavy β-thalassemia and α-thalassemia. Upon human leukocyte antigen matching, bone marrow transplantation (BMT) was successfully performed on the proband by selecting his HLA-compatible sister as a donor. Following treatment, the proband was revealed to only carry the IVS-II-654(C>T)β⁺ heterozygous mutation, and further regular blood transfusions have been avoided; BMT results remained normal at six months follow-up.

Introduction

Thalassemia is an inherited hemoglobinopathy that is caused by an imbalance in the ratio of α-globin and β-globin synthesis, which results in hemolytic anemia owing to the shortened lifespan of red blood cells (1). Clinically, α- and β-thalassemias occur as a result of genetic defects (2). β-thalassemia is characterized by a defect in the synthesis of β-globin chains of the hemoglobin tetramer. Mutations in the hemoglobin subunit β(*HBB*) gene lead to an abnormal formation of hemoglobin, which results in improper oxygen transportation and the destruction of red blood cells. These mutations either partially or completely terminate β-globin chain synthesis, which are classified as β⁺ and β⁰ mutations, respectively. Every year, ~60,000 newborns are diagnosed with β-thalassemia worldwide (3,4). The type of genetic mutation and distribution frequency of β-thalassemia exhibits obvious regional differences and ethnic characteristics. High incidence rates of β-thalassemia have been reported in the southern China (3,4), and the carrier rates of the populations of those regions were 3-24%. Currently, >200 types of β-globin gene mutations have been identified around the world, and >30 mutations have been recorded in China (5). A previous study demonstrated that compound mutations may lead to severe thalassemia or thalassemia intermedia (6). Many of these compound mutations are familial alterations, and spontaneous somatic mutations are quite rare. The present study reported a case of compound heavy β-thalassemia plus α-thalassemia caused by both familial and somatic mutations. To treat the proband, all family members of the patient were subjected to human leukocyte antigen (HLA) typing, and bone marrow transplantation (BMT) was successfully performed between the proband and his HLA-identical sister.

Case report

Written informed consent was obtained from all participants in the present study. The proband was a boy (age, 2 years

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6 months) with severe anemia (hemoglobin level, 62 g/l) accompanied by yellowish skin, enlarged liver and spleen, among other symptoms present since he was 6 months old. Based on these clinical and hematological characteristics (Table I), the patient was diagnosed with heavy β-thalassemia (2). Sanger sequencing was performed to identify any mutations in the proband and other family members. To conduct Sanger sequencing, three pairs of polymerase chain reaction (PCR)/sequencing primers were designed for hemoglobin subunit α1 (HBA1), HBA2 and hemoglobin subunit β (HBB; PCR primer sequences are patented and withheld by the Beijing Genomics Institute-Shenzhen, Shenzhen, Guangdong, China). Genomic DNA was extracted from peripheral blood using the PureLink™ Genomic DNA Mini kit (cat. no. K182000; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The PCR mixture was prepared in a 25 μl reaction solution containing 0.4 μM of each primer, 25 ng genomic DNA and 12.5 μl 2X GoldStarTaq Master Mix (CWBiotech, Beijing, China). PCR was performed in a ABI 9700 PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: An initial hot-start step at 95°C for 10 min, followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 90 sec at 72°C, and a final extension of 10 min at 72°C. The PCR products were purified using Centricon®-100columns (cat. no. P/NN930-2119; EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol, and then sequenced using the ABI 3730xl DNA Analyzer (Applied BioSystems; Thermo Fisher Scientific, Inc.). The sequencing results were analyzed using Lasergene software v7.1 (DNASTAR, Inc., Madison, WI, USA). These analyses demonstrated that the proband was a carrier of the thalassemia gene mutation IVS-II-654(C>T)β^+. To further diagnose the proband and their family members, additional blood tests with Sysmex XE-5000 Automatic Hematology Analyzer and Sebia automatic electrophoresis were performed. Peripheral blood (~2 ml) was collected from each individual, maintained in tubes with EDTA anticoagulant, subjected to blood cell examination using the Sysmex XE-5000 analyzer (Sysmex Corporation, Kobe, Japan) and then analyzed using Laboman EasyAccess v4.2 software (Sysmex Corporation). For Sebia automatic electrophoresis, 2 ml peripheral blood was collected and centrifuged at 3,000 x g for 5 min at room temperature. The plasma was then removed and the samples were subjected to Sebia automatic electrophoresis using the Sebia MINICAP (Sebia, Lisses, France) and analyzed with Phoresis CORE v7.4.7 software (Sebia). The results revealed that the proband had β-thalassemia, whereas his mother and sister suffered from microcytic hypochromic anemia (Table I); the father was phenotypically normal.

To determine the genetic background of this case, peripheral blood DNA was obtained from all available family members for full clinical genetic testing for thalassemia. Control peripheral blood DNA was collected from two disease-free volunteers (34-year-old male; 25-year-old female; classed as disease-free based on normal hemoglobin levels identified via routine blood tests and Sebia automatic electrophoresis) who were recruited from within the Department of Pediatrics (Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou). Sanger sequencing combined with reverse

Table I. Results of routine blood test and hemoglobin electrophoresis.

Variable	Normal range	Proband	Father	Mother	Sister
RBC (x10^{12})	4.0-5.5	2.85	3.9	2.74	2.67
MCV (f1)	80-100	69.4	82	75	74
MCH (pg/cell)	26-38	21.9	29.5	23.7	23.5
MCHC (g/l)	300-360	316	378	340	334
Hb (g/l)	120-160	62.0	112	105	90
HbA (%)	>95	78.2	97.3	95.9	95.2
HbA2 (%)	1-3	3.8	2.0	3.5	3.6
HbF (%)	<2	18.0	0.1	0.2	0.5

Hb, hemoglobin; HbF, fetal hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cells.

Table II. Thalassemia genotypes of the proband and family members.

Individual	Genotype
Proband	$\beta^{\text{IVS-II-654}}/\beta^{\text{N}}$; *HBA2*:c.46G>A; *HBB*:c.316-148G>T; SEA-*HPFH*
Father	SEA-*HPFH*/β^{N}
Mother	$\beta^{\text{IVS-II-654}}/\beta^{\text{N}}$; *HBA2*:c.46G>A
Sister	$\beta^{\text{IVS-II-654}}/\beta^{\text{N}}$

HBA2, hemoglobin subunit α2; HBB, hemoglobin subunit β; N, normal; SEA-*HPFH*, Southeast Asian-type-hereditary persistence of fetal hemoglobin.

transcription-quantitative PCR (RT-qPCR) were used to detect the 338 known types of globin gene sequence mutations that lead to thalassemia. RT-qPCR was performed to detect the unknown α- and β-globin genes, and was performed as described previously (7,8). Densitometry was performed using StepOne™ v2.0 software (Thermo Fisher Scientific, Inc.). The results were normalized to those of GAPDH and the $2^{-\Delta\Delta Cq}$ method was used (9). For statistical analysis, a one-way analysis of variance was performed on SPSS v17.0 software (SPSS, Inc., Chicago, IL, USA), and a Dunnett's post hoc test was used for multiple comparison; $P<0.05$ was considered to indicate a statistically significant difference. The data indicated that the proband had compound mutations of the heterozygous IVS-II-654(C>T)β^+, Southeast Asian (SEA)-type-hereditary persistence of fetal hemoglobin (SEA-*HPFH*) deletion (NC-000011.9:g.5222878-5250288del) (10), *HBA2*:c.46G>A heterozygous, and *HBB*:c.316-148G>T heterozygous (Fig. 1). Although Sanger sequencing indicated that the father had a normal IVS-II-654 gene, results from RT-qPCR (Fig. 1E) indicated that he carried a SEA-*HPFH* mutation (a deletion of the β chain), which was verified by Gap-PCR. Gap-PCR was applied to detect 5α-thalassemia deletions including the SEA, α-3.7, α-4.2, Filipino and Thailand deletions, and
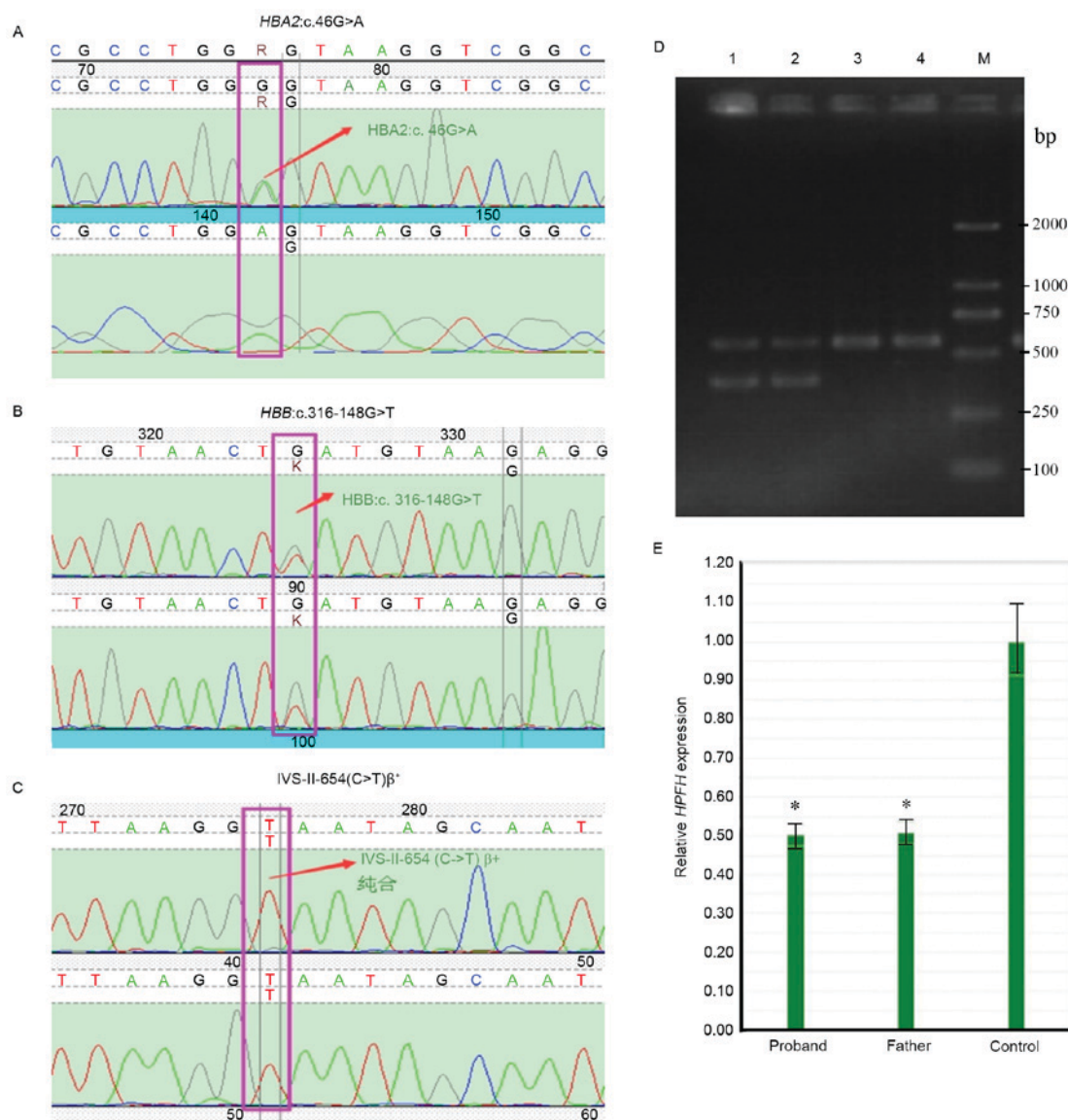
Figure 1. Identification of our mutated genes in the proband by DNA sequencing and gel electrophoresis. Sanger sequencing of the mutations: (A) *HBA2*:c.46G>A; (B) *HBB*:c.316-148G>T; and (C) *HBB*:IVS-II-654(C>T)β⁺. Mutated nucleotides are outlined with a magenta box. (D) Identification of the *HPFH* deletion by gel electrophoresis; the lower bands indicated DNA fragment deletion in the proband (lane 1) and father (lane 2), whereas the normal controls showed one regular band (lanes 3 and 4). (E) Reverse transcription-quantitative polymerase chain reaction demonstrated that lower expression of the *HPFH* gene in proband and father compared to the normal control. *P<0.05 vs. control. *HBA2*, hemoglobin subunit α2; *HBB*, hemoglobin subunit β; *HPFH*, hereditary persistence of fetal hemoglobin; Normal controls, blood DNA samples collected from disease-free volunteers.

was performed as described previously (11). The mother of the proband was a carrier of the compound heterozygous mutations IVS-II-654(C>T)β⁺ and *HBA2*:c.46G>A, and his sister inherited the IVS-II-654(C>T)β⁺ heterozygous mutation from the mother (Figs. 1 and 2; Table II). Based on the above data, the proband was identified as having a somatic mutation *HBB*:c.316-148G>T, and inherited the other thalassemia mutations from his parents: That is, the SEA-*HPFH* mutation from the father and the IVS-II-654(C>T)β⁺ allele and *HBA2*:c.46G>A mutation from the mother (Fig. 2). Thus, the proband clinically displayed heavy thalassemia, whereas his mother and sister exhibited weaker manifestations of the disease.

Based on previous reports (12,13), it was determined that *HBA2*:c.46G>A and *HBB*:c.316-148G>T were novel alterations located near the known mutations in the thalassemia mutation
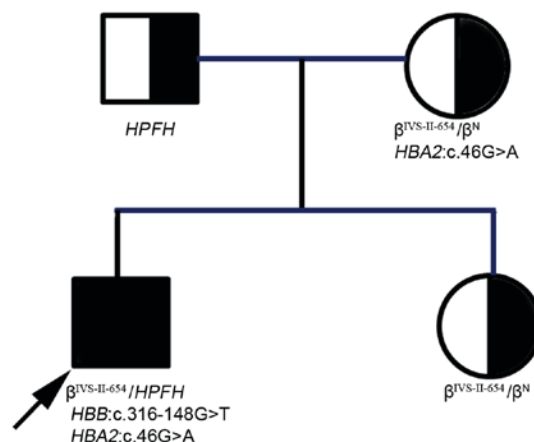

Figure 2. Pedigree and genetic mutations of thalassemia in the family of the proband (arrow).

Table III. Adjacent positions of novel mutations in the thalassemia mutation database.

Novel mutations	Known adjacent mutations
IVS-II-654(C>T)	AAGGCAATA→AAG^GTAATA, *HBB*:c.316-197C>T
IVS-II-705(T>G)	GATGTAAGA→GAG^GTAAGA, *HBB*:c.316-146T>G
IVS-II-726(A>G)	(adjacent sequence unavailable) *HBB*:c.316-125A>G
IVS-II-745(C>G)	CAGCTACCAT→CAG^G', *HBB*:c.316-106C>G
IVS-II-661(A>G)	(adjacent sequence unavailable) *HBB*:c.316-90A>G
Hb Ottawa, α2 or α1	15(A13)Gly>Arg, *HBA2*:c.46G>C

Underlined bases indicate mutated nucleotides. *HBA2*, hemoglobin subunit α2; *HBB*, hemoglobin subunit β;', indicates a truncation, the sequence behind was omitted.

Table IV. HLA type matching results of the proband with his parents and sister.

Case	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1
Proband	0301,1101	2705,4001	0202,0702	1001,1202	0501,0301
Father	0301.3303	2705,5801	0202,0302	0301,1001	0501,0201
Sister	0301,1101	2705,4001	0202,0702	1001,1202	0501,0301
Mother	1101,1102	4001,4601	0102,0702	0901,1202	0301,0303

HLA, human leukocyte antigen.

databases (12) (www.globin.bx.psu.edu/hbvar; www.ncbi.nlm. nih.gov/clinvar/?term=HBB[gene] AND single_gene[prop]) (Table III). *HBA2*:c.46G>A leads to an amino acid replacement (p.Gly16Ser) that causes a hemoglobin disease, whereas *HBB*:c.316-148G>T is a mutation in intron 2 of *HBB*, the pathogenic effect of which is unclear.

The HbF level of the proband was considerably high (18%; Table I), which was consistent with previous reports (1,2,14-16). Small-scale deletions or single-base mutations in the *HBB* gene may result in low HbF levels (0.5-6.0%), whereas large-scale deletions may cause high levels of HbF (6.0-15.0%) (16,17). HbF levels >15% may result from compound mutations, such as a large-scale deletion combined with other mutations (16,17). Upon determination of the genetic background of the proband, it was decided that a BMT should be performed. Firstly, the HLA types of the proband and other family members were determined through high-resolution HLA testing by PCR-sequence based typing. Briefly, DNA was extracted from the proband and family members then amplified by PCR using HLA Class I (exons 1-8) and HLA Class II (exons 2-4) primers (PCR primer sequences were patented and withheld by the Beijing Genomics Institute-Shenzhen), and the PCR product was purified for sequencing via the aforementioned Sanger sequencing methodology. The sequencing results indicated that the HLA type of the proband was fully matched with their sister (Table IV), thus the sister was selected as the bone marrow donor, and BMT was conducted involving the proband and sister. To monitor the results of the BMT, follow-up testing was performed using fluorescence *in situ* hybridization (FISH). Peripheral blood (~2 ml) were
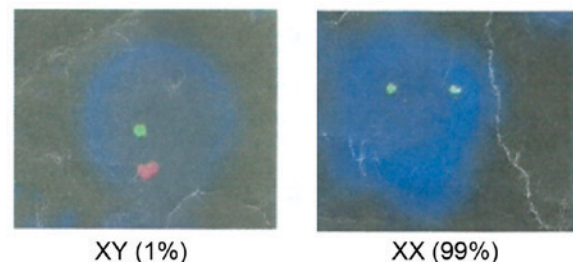

XY (1%)   XX (99%)

Figure 3. The CSPX/CSPY genetic testing by fluorescence *in situ* hybridization indicated that the donor cells accounted for 99% in the blood of the proband 30+ days post-bone marrow transplantation. The red signal identifies the Y chromosome and the green signal identifies the X chromosome. XY cells were from the proband, and XX cells were from the donor sister. CSPX, centromeric specific probe for the X chromosome.

collected from the proband and cultured in the presence of a mitogen [KaryoMAX®Phytohemagglutinin (M-Form; PHA); cat. no. 10576; Thermo Fisher Scientific, Inc.] for 68 h at 37°C, then 0.05-0.1 µg/ml KaryoMAXColcemid® Solution (mitotic inhibitor; cat. no. 15210; Thermo Fisher Scientific, Inc.) was added to the culture at room temperature for 20 min. Cells were subsequently treated with 5 ml hypotonic solution (0.068 M KCl) at room temperature for 15 min, then 1 ml fresh ice cold fixative (absolute methanol:glacial acetic acid, 3:1) was added, and the cells were spun down at 500 x g at room temperature for 7 min. The supernatant was then removed, and 5 ml of fresh, ice-cold fixative was added drop by drop (with continuous vortexing) at 4°C for 20 min. This fixation step was repeated until the supernatant became clear, then the cell pellet was resuspended in 1.5 ml

fixation solution and placed onto slides; the slides were left at 55°C overnight, then they were kept at 4°C for subsequent use. For FISH, the prepared slides were incubated at 50°C for 2.5 h, then denatured in 70% formamide at 70°C for 2 min, followed by dehydration in a series of ethanol solutions (70, 80, 90 and 100%) at room temperature for 2 min each. The slides were subjected to hybridization using a denatured CSPX/CSPY probe mixture (Beijing GP Medical Technologies, Ltd., Beijing, China; denatured at 75°C for 5 min then 0°C for 5-10 min) at 37°C for 15-17 h. Following hybridization, the slides were washed three times (10 min each) in 50% (v/v) formamide containing 2X SSC, then once in 2X SSC for 10 min and once in 2X SSC containing 0.1% NP-40 (Amresco LLC, Solon, OH, USA) for 5 min. The slides were then dehydrated in 70% ethanol for 3 min and allowed to air dry at room temperature. A total of 12 μl DAPI (Beijing GP Medical Technologies, Ltd.) was added to each slide and incubated at room temperature for 20 min in the dark followed by analysis using a fluorescence microscope (OLYMPUS-BX51; Olympus Corporation, Tokyo, Japan). The results demonstrated that the donor cells (karyotype: 46, XX) accounted for 99% of the blood cells in the proband 30 days post-treatment (Fig. 3), and the proband's blood was normal 60 days following BMT; four months post-BMT, the blood type of the proband was transformed from type O to type A, which was the same as the donor (the proband's sister). Six months following BMT, the proband exhibited a thalassemia genotype that was consistent with his sister; thus, no further blood transfusions were required.

Discussion

Thalassemia is an inherited, autosomal recessive blood disorder that is characterized by an abnormal formation of hemoglobin. Patients with thalassemia make less hemoglobin, and this hemoglobin is abnormal; patients also have fewer circulating red blood cells, which results in mild to severe microcytic anemia (1,2,15,18). Currently, the diagnosis of thalassemia relies on routine blood testing combined with blood hemoglobin electrophoresis and thalassemia gene detection (17). Conventional thalassemia gene screening methods only detect known point mutations. In the present study, the single-gene screening method was noted to miss some potential genetic changes; for example, Sanger sequencing failed to detect the SEA-*HPFH* deletion mutation of the father, and prior to RT-qPCR testing the proband was initially misdiagnosed as having a IVS-II-654(C>T)β⁺ homozygous mutant. These findings suggested that multiple thalassemia gene screening methods may be required for precise genotyping of the disease.

The mother of the proband carried the IVS-II-654(C>T) mutation located on chromosome 11, which is a commonly identified β-thalassemia mutation in Chinese people (14,19), and which results in a splicing error that produces abnormal mRNA and hemoglobin protein, eventually causing β-thalassemia (20). Although the IVS-II-654(C>T)β⁺ heterozygous mutation may cause 'light' β-thalassemia that does not require special treatment, homozygous and compound heterozygous mutations may lead to severe disease and the affected patient usually requires regular blood transfusions. In the present case, the proband inherited the IVS-II-654(C>T)β⁺ mutation from his mother and the SEA-*HPFH* deletion from his father, which constituted a compound heterozygous mutation that led to heavy β-thalassemia. The SEA-*HPFH* mutation is a rare β-thalassemia genotype in the Chinese population; it was first identified in Vietnamese and Cambodian patients (21,22). SEA-*HPFH* mutations involve a large DNA deletion that includes the β-globin gene cluster (18,21,23). The deletion range covers NC-000011.9:g. 5222878-5250288del, missing the entire β-globin gene and its 3'hyper sensitive site 1 (10,24,25), which may not be detected by the common β-thalassemia detection assays. The average SEA-*HPFH* carrier exhibits no clinical symptoms and their peripheral blood cells appear completely normal. However, when this mutation coincides with another β-gene mutation and/or constitute compound heterozygous mutations, it may lead to severe or heavy β-thalassemia, which is what occurred in the present case study.

To the best of our knowledge, the present study was the first to identify the *HBA2*:c.46G>A mutation in α chain on chromosome 16. This mutation leads to a Gly16Ser amino acid replacement; however, whether it is causative of thalassemia requires further analysis. The *HBB*:c.316-148G>T mutation is located in intron 2 of the β-globin gene, and was determined to be a novel somatic alteration identified in the proband. The alteration does not change the protein structure; however, whether it affects the generation of mRNA and causes the disease also merits further investigation. These novel mutations enriched the thalassemia mutation spectrum in the Chinese population, which may be helpful in future genetic counseling and clinical diagnosis.

Currently, hematopoietic stem cell transplantation (HSCT) is the only effective way to cure severe β-thalassemia in the clinical practice (15,26,27). The influence on transplant success rate of donor mainly depends on the HLA typing on chromosome 6 (28). In the present case, the HLA type of the sister completely matched that of the proband, which led to successful BMT and cured the patient of the disease.

Acknowledgements

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