SPANDIDOS PUBLICATIONS
EXPERIMENTAL AND THERAPEUTIC MEDICINE 31: 168, 2026

Detection of overlooked rare thalassemia genotypes: A case series

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Received October 10, 2025; Accepted February 17, 2026

DOI: 10.3892/etm.2026.13163

Abstract. Thalassemia is the most prevalent type of monogenic disorder, requiring multiple laboratory tests for diagnosis. The complex structure and mutations of hemoglobin (Hb) genes pose notable obstacles to genetic counselling and prenatal diagnosis. Consequently, detecting and confirming rare thalassemia genotypes that are easily overlooked holds considerable importance in these fields. The present study describes three cases of incidentally discovered rare thalassemia genotypes. It analyzes their detection and diagnostic pathways to investigate causes of misdiagnosis and missed diagnosis in rare thalassemia, with the aim of enhancing sensitivity in identifying rare thalassemia and improving diagnostic standards. The findings revealed that all subjects carried rare thalassemia genotypes: Case 1 exhibited a normal phenotype as an α-thalassemia heterozygote ($\alpha\alpha/\alpha\alpha^{3'UTR\ +71\ G>C}$, β^{N}/β^{N}); case 2 presented with a family history of hereditary diseases and a history of iron deficiency anemia, with a compound αβ-thalassemia triple genotype [$-^{Southeast\ Asian}/\alpha\alpha^{Quong\ Sze}$ (HBA2: c.377T>C), $\beta^{intron\ variant\ site\ (IVS)II\text{-}672\ A>C}/\beta^{N}$]; and case 3 exhibited a normal phenotype and was a compound carrier of rare αβ-double genotypes ($\alpha\alpha/\alpha\alpha^{CD\ 27\ GAG>AAG}$, $\beta^{IVSII\text{-}180\ T>C}/\beta^{N}$). To the best of our knowledge, the $\alpha\alpha/\alpha\alpha^{3'UTR\ +71\ G>C}$ and $\beta^{IVSII\text{-}180\ T>C}/\beta^{N}$ genotypes are reported for the first time in the Chinese population; case 2 is the first reported rare instance of a triple thalassemia gene carrier; and case 3 is the first documented case of a compound double rare thalassemia gene carrier. The present report on rare thalassemia genes, particularly the two cases of rare thalassemia gene combinations, enrich the spectrum of thalassemia gene mutations in the Chinese population. Moreover, the present study provides valuable molecular information and case studies for thalassemia diagnosis and counselling, and offers clinical experience for the identification and diagnosis of rare thalassemia gene cases that are often overlooked.

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Key words: thalassemia, misdiagnosis, rare genotypes, gene mutations, gene combinations

Introduction

In laboratories, hemoglobin (Hb) electrophoresis is commonly employed as a primary screening method for the approximate analysis of thalassemia types, although it cannot definitively determine genotypes (1,2). Furthermore, PCR-Reverse Dot Blot technology (3-5) and multicolor melting curve analysis based on quantitative PCR (6,7) have become core methods for secondary screening due to their low cost and high throughput. However, these techniques are limited by probe coverage and their inability to detect rare thalassemia genotypes (8).

Sanger sequencing is considered the gold standard for thalassemia genetic testing (9-12), it is suitable for point mutation validation but challenging for the detection of complex variations (9,13). Next-generation sequencing (9,13-15) and third-generation sequencing (16-21) are increasingly employed to detect novel thalassemia mutations. Nevertheless, given the complexity of Hb gene structural alterations and the diversity of variations, no infallible method exists to detect all thalassemia types despite notable advances in these techniques (22).

Beyond technical considerations, the high cost of testing represents a marked barrier (11,13,23,24). Moreover, the phenotypic and genetic diversity of certain rare cases of thalassemia (25-27), the absence of characteristic clinical manifestations in patients (28-32), alterations in conventional diagnostic criteria (31,32) or compound cases masked by common thalassemia phenotypes (33) readily lead to missed or misdiagnoses. Therefore, despite substantial advances in current testing technologies and platforms, the ability to employ optimal diagnostic methods and techniques for every patient, ultimately confirming their condition, remains unrealistic across diverse nations, regions and populations, particularly for rare cases. Additionally, timely identification and confirmation of rare disease cases persistently pose clinical challenges. Consequently, investigating and documenting the discovery process for overlooked rare thalassemia genotypes is essential and clinically notable.

The present study describes the cases of three rare thalassemia genotypes unexpectedly identified during a research project on rare thalassemia cases at The First People's Hospital of Zhaoqing (Zhaoqing, China). The study discusses how rare thalassemia genes are often overlooked, leading to missed or misdiagnosed cases, and offers novel insights for detecting and diagnosing rare thalassemia gene variants.

Case report

Patients. All subjects were men from Zhaoqing, Guangdong (China) and constituted three participants recruited for the research project entitled 'Study of Rare Thalassemia Gene Mutations in the Zhaoqing Population'. Case 1 was a patient aged 32 years, with no family history of anemia. Prior to family planning, the patient underwent thalassemia screening at The First People's Hospital of Zhaoqing in January 2021. Routine hematological phenotyping revealed normal red blood cell (RBC), Hb, mean corpuscular volume (MCV) and mean corpuscular Hb (MCH) levels; Hb electrophoresis analysis demonstrated Hb Constant Spring (HbCS) bands. Routine genetic testing showed no abnormalities in thalassemia-related genes. Case 2 was a patient aged 56 years, who presented with a history of iron deficiency anemia and familial anemia. In July 2022, during a period of hospitalization at The First People's Hospital of Zhaoqing, thalassemia-related testing revealed reduced RBC, Hb, MCV and MCH levels in hematological phenotyping. Hb electrophoresis analysis indicated HbH disease and HbCS bands. Routine thalassemia gene testing revealed [$-^{\text{Southeast Asian (SEA)}}/\alpha\alpha^{\text{Quong Sze (QS)}}$ (HBA2: c.377T>C)], β^N/β^N genotypes. Case 3 was a patient aged 31 years, with no family history of anemia. Routine hematological phenotyping conducted in March 2023 showed normal RBC, Hb, MCV and MCH levels. Hb electrophoresis and routine thalassemia gene testing were requested for fertility reasons. Hb electrophoresis alone revealed an HbX + HbA_2 abnormality.

The present study was approved by the Ethics Committee of Zhaoqing First People's Hospital (approval no. B2024-02-04) and was performed in accordance with the ethical principles for medical research involving human subjects as outlined in The Declaration of Helsinki. Written informed consent was obtained from all subjects prior to inclusion in this study, authorizing the publication of their clinical data and imaging materials.

Reagents and instruments. The XN-1000 hematology analyzer and its accompanying reagents were purchased from Sysmex Corporation. The CAPILLARYS2 fully automated capillary electrophoresis system and its associated reagents, purchased from Sebia S.A., were used for Hb analysis. The Whole Blood Genomic DNA Extraction Kit (cat. no. 20240501), and α- and β-Thalassemia Gene Detection and Hybridization kits (cat. no. 20240501) were procured for thalassemia gene testing, and the HB-2012A medical nucleic acid molecular hybridization instrument (product no. YYC1801024) and HBNP-4801A automated extraction system (product no. YYD20201114) were purchased from Guangdong Kaipu Technology Co., Ltd. The Applied Biosystems Veriti™ Dx Thermal Cycler and 3730xl DNA Analyzer were purchased from Thermo Fisher Scientific, Inc. All relevant testing procedures were strictly performed in accordance with the instrument and kit manuals.

Specimen collection. A total of four tubes of EDTA-K2-anticoagulated venous blood (2 ml/tube) were collected from each patient for use in complete blood count, Hb electrophoresis, thalassemia gene detection and sequencing.

Hematological parameter analysis. RBC parameters were analyzed using the fully automated modular blood and body fluid analyzer XN-1000 and its corresponding reagents. Reference values for hematological parameters in adult men are listed in Table I.

Hb analysis. Peripheral blood Hb electrophoresis was performed using the CAPILLARYS 2 fully automated capillary electrophoresis system. Electrophoresis patterns were divided into zones Z1-Z15 to identify and analyze the proportion of each band, with primary focus on fetal Hb bands and HbA_2 bands. The presence of abnormal Hb and its potential type were determined based on the region where the Hb peak pattern was located. Analyses of abnormal Hb peaks were performed with reference to the abnormal Hb distribution spectrum provided by Sebia S.A. The reference ranges for Hb electrophoresis components are listed in Table I.

Nucleic acid extraction. Human genomic DNA was extracted from 200 μl whole blood samples using an automated extraction system and matching extraction reagents. The concentration of the extracted DNA was then determined by measuring the absorbance at 260 nm using an ultraviolet spectrophotometer.

Common thalassemia gene testing. Thalassemia gene detection employs gap PCR and multiplex PCR amplification techniques, using the α- and β-thalassemia gene detection and hybridization kits. The primer sequences, including probes on the hybridization membrane strips (Tables SI and SII), and reaction systems used in the present study are described in a previous study (34). Through the flow-through hybridization method, three common deletion-type α-thalassemia classifications prevalent in the Chinese population were simultaneously detected: $-\alpha^{3.7}$ (rightward), $-\alpha^{4.2}$ (leftward) and $-^{SEA}$, and three mutation-type α-thalassemia classifications were detected: HbCS (HbCS or HBA2: c.427T>C), Hb QS and Hb Westmead (Hb WS or HBA2: c.369C>G). In addition, 15 common β-thalassemia classifications in the Chinese population were detected: -29 (A>G) (HBB: c.-79A>G), -28 (A>G) (HBB: c.-78A>G), CAPM (-AAAC) (HBB: c.-50A>C), InitM (ATG>AGG) (HBB: c.2T>G), codons 14/15 (+G) (HBB: c.45_46insG), codon 17 (AAG>TAG) (HBB: c.52A>T), codon 26 (GAG>AAG) (HBB: c.79G>A), codons 27/28 (+C) (HBB: c.84_85insC), codon 31 (-C) (HBB: c.94delC), intron variant site (IVS)I-1 (G>A, G>T) [HBB: c.92+1(G>T)], IVSI-5 (G>C) (HBB: c.92+5G>C), 41/42M (-TTCT) (HBB: c.126_129delCTTT), codon 43 (GAG>TAG) (HBB: c.130G>T), codons 71/72 (+A) (HBB: c.216_217insA) and IVSII-654 (C>T) (HBB: c.316-197C>T). The probe arrangement on the hybridization membrane strips is shown in Table II.

Briefly, after nucleic acid extraction, the detection process requires multiplex PCR amplification and flow cytometric hybridization to be completed. First, multiplex PCR amplification was performed, which involves two reaction systems. α-Thalassemia PCR (Reaction System 1) comprised a 50-μl reaction volume containing 100 ng DNA template, 25 μl 2X GC buffer, 4 mmol/l $MgCl_2$ and 0.2 mmol/l each primer (34), 0.2 mmol each dNTP and 2.5 units HotStart DNA polymerase. This was performed on an Applied Biosystems Veriti™ Dx Thermal Cycler (Applied Biosystems; Thermo Fisher

Table I. Characteristics and hematological parameters of three patients with thalassemia.

Characteristic	Case 1	Case 2	Case 3	Reference range
Sex	Male	Male	Male	-
Age, years	32	56	31	-
RBC, 10^{12}/l	4.85	4.07	5.15	4.5-5.5
Hb, g/l	153	55	147	120-160
MCV, fl	97.1	72.0	87.9	82.0-100.0
MCH, pg	31.5	22.8	28.6	27.0-34.0
HbA, %	97.3	92.9	85.5	94.5-97.5
HbA_2, %	2.2	1.8	0.0	2.5-3.5
HbH, %	0.0	4.6	0.0	0.0
HbCS, %	0.5	0.7	0.0	0.0
HbX + HbA_2	0.0	0.0	14.5	0.0
α genotype	$\alpha\alpha/\alpha\alpha^{3'UTR\ +71\ G>C}$	$-^{SEA}/\alpha\alpha^{QS}$	$\alpha\alpha/\alpha\alpha^{CD27\ GAG>AAG}$	αα/αα
β genotype	β^N/β^N	$\beta^{IVSII\text{-}672\ A>C}/\beta^N$	$\beta^{IVSII\text{-}180\ T>C}/\beta^N$	β^N/β^N

RBC, red blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; HbCS, Hb Constant Spring; ^{QS}, Quong Sze; ^{SEA}, Southeast Asian; ^{IVS}, intron variant site.

Table II. Sequence of probe arrangement on hybridized membrane strips.

	Hp					
Vp	1	2	3	4	5	6
1(N)	41-41/-43	14-15/-17	654	71-71	-28/29	βE
2(M)	41-42	17	654	71-72	-28	βE
3(M)	43	14-15	IVSI-1	IVSI-5	-29	CAP
4(N/M)	NP	CSN	QSN	α3.7	WSM	InitM
5(M)	SEA	CS	QS	α4.2	31	27-28

Hp, horizontal position; Vp, vertical position; N, normal control; M, muation; βE, β enhancer; IVSI, intron variant site I; CAP, cap site (or 5'-untranslated region); NP, α-globin gene normal control; CS, Constant Spring; QS, Quong Sze; WS, Westmead; Init, initiation codon; SEA, Southeast Asia.

Scientific, Inc) under the following program: Denaturation at 95˚C for 15 min, followed by 35 cycles at 98˚C for 40 sec, 64˚C for 70 sec and 72˚C for 150 sec, and a final extension step at 72˚C for 5 min. β-Thalassemia PCR (Reaction System 2) was performed in a 50-µl reaction mixture containing 100 ng DNA template, 5 µl 10X buffer, 3.5 mmol/l $MgCl_2$, 0.2 mmol/l each primer (34), 0.2 mmol/l each dNTP, 2.5 units DNA Taq polymerase and 1 unit uracil DNA glycosidase. The PCR conditions were as follows: 5 min at 37˚C, 3 min pre-denaturation at 94˚C, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C at 30 sec, with a final 5-min extension at 72˚C. Amplification products from both reaction systems were subsequently denatured and subjected to hybridization analysis. Flow hybridization was performed using the α- and β-thalassemia gene hybridization kit (Guangdong Kaipu Technology Co., Ltd.), strictly adhering to the manufacturer's protocol. This assay employs Hybribio's proprietary flow hybridization technology [U.S. Patents 5,741,647 (35) and 6,020,187 (36)], which actively guides target molecules toward membrane fibers containing immobilized probes, trapping complementary molecules after double-strand formation. Following rigorous washing steps, hybridization products are detected by adding streptavidin-horseradish peroxidase conjugate. This conjugate binds to biotin-labeled PCR products, generating a blue-violet precipitate at probe locations via the substrate (nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate). Results are interpreted by direct visual inspection.

Rare thalassemia gene mutation detection. Peripheral blood DNA was extracted using a whole blood genomic extraction kit. Amplification was then performed using Gap-PCR and multiplex PCR. The amplified products underwent agarose gel electrophoresis on 1.0% (w/v) gels, which were developed using 0.5 µg/ml ethidium bromide solution, to confirm the target fragment, which was subsequently sent to Guangdong Kaipu Technology Co., Ltd. for sequencing analysis of rare thalassemia genes using the Sanger double-deoxy chain

termination method. The amplification primers (Table SIII) and reaction conditions used in the present study are described in previous studies (37,38). Detected gene sequences were annotated against the databases HbVar (http://globin.bx.psu.edu/hbvar) and Ithanet (https://www.ithanet.eu/) to determine the type of mutation detected. All experimental procedures were strictly performed in accordance with standard operating procedures.

Hematological analysis findings. Patient hemolytic anemia characteristics and hematological parameters are presented in Table I. Case 1 exhibited normal RBC, Hb, MCV and MCH levels, but reduced HbA_2 and elevated HbCS levels (Fig. 1A). The routine hemolytic anemia gene testing results were as follows: Undetected, but a faintly stained circle appeared at the position indicated by the arrow on the hybridization membrane strip (Fig. 1B). Case 2 exhibited reduced levels of RBC, Hb, MCV and MCH. Hb electrophoresis revealed decreased HbA and HbA_2, with abnormal bands HbH and HbCS visible (Fig. 1C). The routine thalassemia gene testing results were as follows: $-^{SEA}/\alpha\alpha^{QS}$, β^N/β^N (Fig. 1D), confirming HbH disease. Case 3 exhibited normal RBC, Hb, MCV and MCH levels, but elevated HbX + HbA_2 levels, due to inherent methodological limitations of the instruments and reagents employed during the study design phase, it was not possible to separate HbA_2 from HbX. Consequently, it was not feasible to calculate the HbA_2 value independently (Fig. 1E). The routine thalassemia gene testing results were as follows: Not detected (Fig. 1F).

Rare thalassemia gene mutation detection analysis. Sequencing of the α1 globin gene in case 1 revealed no rare genotypes. Sequencing of the α2 globin gene identified a heterozygous mutation at the HBA2:c.*71 G>C (3'UTR +71 G>C) locus, representing a rare genotype reported for the first time in the Chinese population, to the best of our knowledge. Sequencing of the β globin gene detected no abnormal genotypes. Consequently, this patient exhibited α-thalassemia with the rare α-gene genotype $\alpha\alpha/\alpha\alpha^{3'UTR\ +71\ G>C}$ heterozygosity (Fig. 2A).

The routine thalassemia gene testing of case 2 yielded results of $-^{SEA}/\alpha\alpha^{QS}$, with no rare thalassemia mutation types detected in either the α1 or α2 globin gene; however, the β globin gene sequencing revealed a rare heterozygous mutation: $^{\beta IVSII\text{-}672\ A>C/\beta N}$ (HBB:c.316-179 A>C*)*. Therefore, this patient exhibited compound α,β-thalassemia (Fig. 2B), characterized by the genotype $-^{SEA/\alpha\alpha QS}$ *c*ombined with $^{\beta IVSII\text{-}672\ A>C/\beta N}$. This combination of thalassemia genotypes represents the first reported case in the Chinese population, to the best of our knowledge.

No rare genotypes were detected in the α1 globin gene sequencing of case 3; however, sequencing of the α2 globin gene revealed a heterozygous mutation at the HBA2:c.82 G>A (CD 27 GAG>AAG) site (Fig. 2C). The β globin gene sequencing revealed a heterozygous mutation at the HBB:c.315+180 T>C (IVSII-180 T>C) locus (Fig. 2D), representing a previously unreported rare genotype, to the best of our knowledge. Consequently, this patient exhibited a heterozygous double rare genotype for $\alpha\alpha/\alpha\alpha^{CD\ 27\ GAG>AAG}$ heterozygous compound $\beta^{IVSII\text{-}180\ T>C}/\beta^N$ double rare thalassemia genotype. This combination represents the first reported case in the Chinese population, to the best of our knowledge.

Discussion

Accurate diagnosis of thalassemia genotypes in carriers is crucial for identifying couples at risk of producing offspring with Hb disorders, particularly those with rare genotypes. With the application and promotion of new testing methods and technologies, alongside the nationwide prevalence of premarital and prenatal genetic testing (39,40), cases of thalassemia with unknown and rare mutation types have been increasingly identified in the Chinese population over the years (5,13,15,18-22,37,41-43). However, due to several factors, clinical attention to these cases remains low, potentially leading to oversight and increased risk of misdiagnosis or missed diagnosis (43-45). Moreover, Zhaoqing, located in southern China, is a high-prevalence area for thalassemia (46,47); in 2014, Li *et al* (47) reported a thalassemia prevalence rate of 19.45% in this region. Therefore, the discovery of three rare thalassemia genotypes in the present study was unexpected, representing conditions that are susceptible to being overlooked, and thus are prone to misdiagnosis or missed diagnosis.

Compared with α-thalassemia major, the clinical manifestations in α-thalassemia minor are more diverse and exhibit a more severe phenotype (48); however, in the present study, case 1 exhibited essentially normal clinical and hematological characteristics, with only mildly reduced HbA_2 and mildly elevated HbCS. Routine thalassemia gene testing revealed no abnormalities, but a faintly stained band appeared on the hybridization membrane ($\alpha^{4.2}$ locus). Further sequencing revealed non-deletion α-thalassemia due to a heterozygous HBA2:c.*71 G>C (3'UTR +71 G>C) mutation. This mutation corresponded to a G-to-C substitution at position 71 in the 3'untranslated region of the α2 globin gene (https://www.ithanet.eu/db/ithagenes?ithaID=3753), indicating that it is most likely a silencing mutation. As a heterozygous case, this patient would have been easily overlooked and misdiagnosed, and now represents the first reported instance in the Chinese population, to the best of our knowledge. Given that non-deletion defects may cause more severe atypical HbH disease states compared with deletion-type defects (48), establishing this diagnosis holds notable implications for genetic counselling and prenatal diagnosis in affected individuals. Reviewing the discovery and diagnostic process of the present case suggests that analyzing any suspicious details during testing may aid in identifying and diagnosing rare thalassemia cases.

HbH disease, also known as intermediate α-thalassemia, belongs to the non-transfusion-dependent thalassemia group (49). It is primarily found in tropical and subtropical regions, such as southern China, Southeast Asia, India and the Middle East (50). Preliminary Hb electrophoresis in case 2 revealed HbH bands. The routine genetic testing results of $-^{SEA}/\alpha\alpha^{QS}$ led to a provisional diagnosis of HbH disease. Due to the presence of atypical HbCS bands on Hb electrophoresis and persistent iron deficiency anemia, further sequencing revealed concomitant rare $^{\beta IVSII\text{-}672\ A>C/\beta N}$ heterozygous mutations. This mutation has only been reported in Guangxi (51) and Fujian (52) in China. Moreover, the Guangxi case was a double heterozygote for β-thalassemia, presenting clinically without anemia but with slightly reduced MCV (80 fl) and elevated HbA_2 (3.8%); the Fujian case lacked detailed characterization.

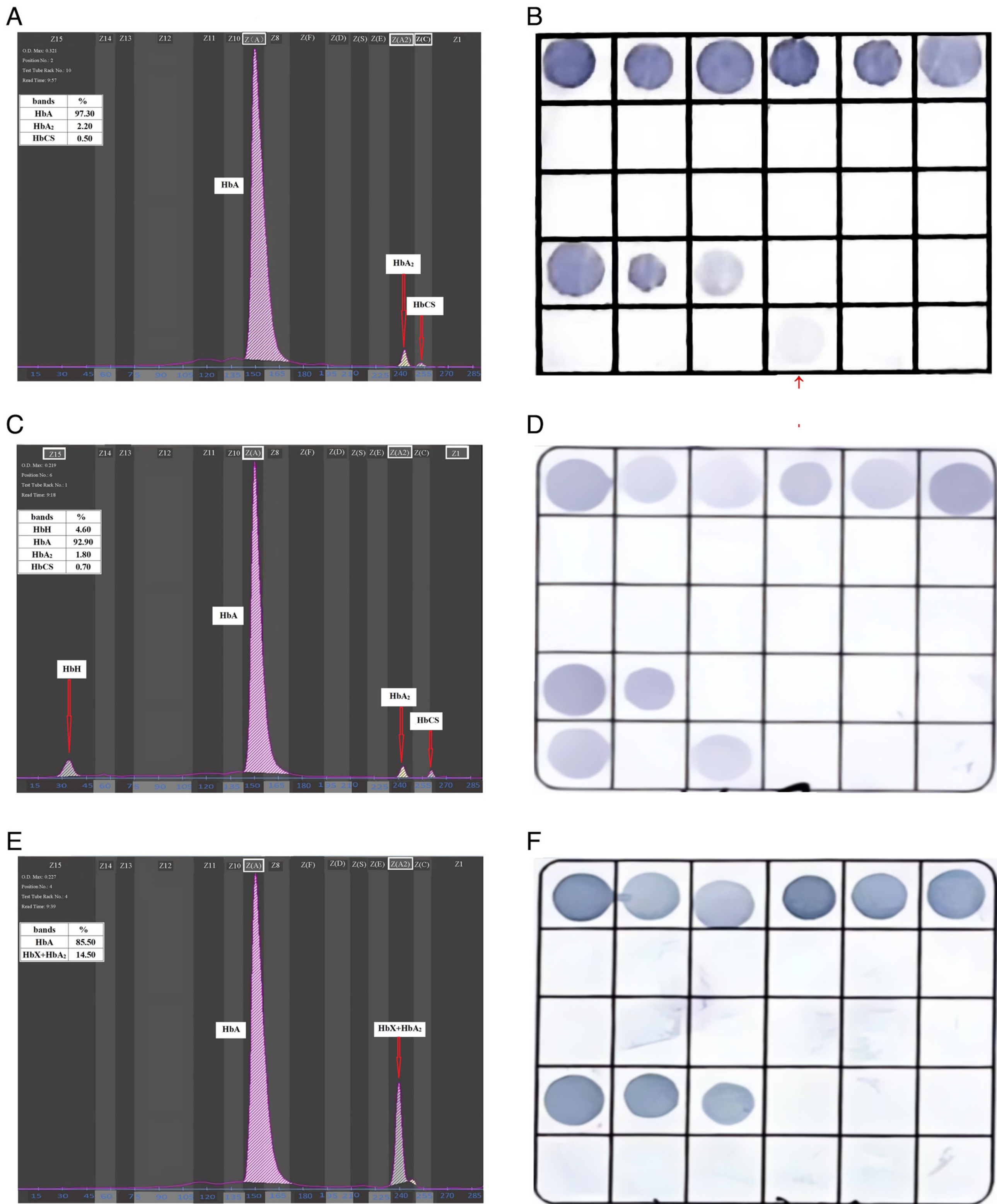

Figure 1. Hemoglobin electrophoretic profiles and hybridization profiles of common thalassemia genes in three subjects. (A) Electropherogram of case 1 showing reduced HbA_2 levels and increased HbCS levels. (B) Results of routine thalassemia gene testing in case 1; no positive signal was detected, but a faintly stained circle was visible at the position indicated by the red arrow in the membrane strip. (C) Electropherogram of case 2 showing reduced HbA and HbA_2, with abnormal bands HbH and HbCS visible. (D) Case 2 routine thalassemia gene test results: $-^{SEA}/\alpha\alpha^{QS}$, β^N/β^N. (E) Case 3 electropherogram showing elevated levels of HbX + HbA_2. (F) Case 3 routine thalassemia gene test result: Not detected. Hb, hemoglobin; HbCS, Hb Constant Spring; ^{QS}, Quong Sze; ^{SEA}, Southeast Asian.

Elevated HbA_2 is the most prominent feature of β-thalassemia carriers (53,54). Clinically, β-thalassemia heterozygotes are typically asymptomatic, presenting with a phenotypic profile of microcytic anemia and hypochromia accompanied by increased HbA_2 levels. However, in rare instances, HbA_2 levels in β-thalassemia carriers

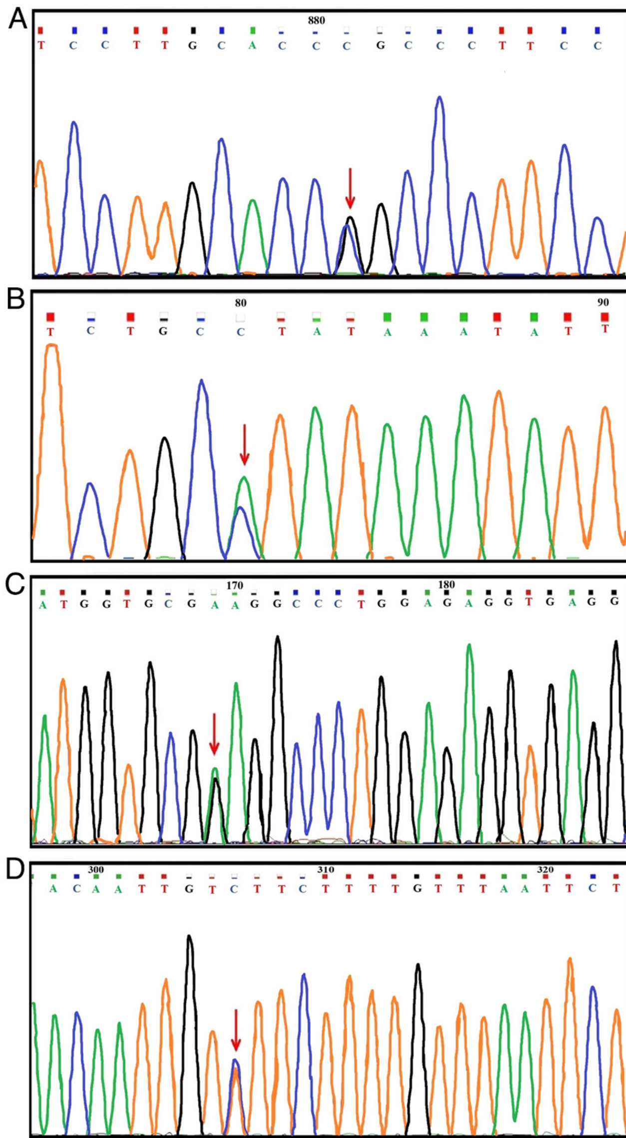

Figure 2. DNA sequencing profiles of the thalassemia gene in three subjects. (A) Case 1 sequencing trace with an arrow labeling the HBA2:c.*71 G>C (3'UTR +71 G>C) locus mutation. (B) Case 2 sequencing trace with an arrow labeling the HBB:c.316-179 A>C (IVSII-672 A>C) locus mutation. (C) First DNA sequencing heterozygous mutation profile of case 3 with an arrow labeling the HBA2:c.82 G>A (CD27 GAG>AAG) site mutation. (D) Second DNA sequencing mutation profile of case 3 with an arrow labeling the HBB:c.315+180 T>C (IVSII-180 T>C) site mutation. IVS, intron variant site.

may be normal (55) or even reduced. Factors such as mild β-thalassemia mutations, concomitant α- or δ-thalassemia, or α-gene triploidy may contribute to decreased HbA_2 levels in β-thalassemia heterozygotes (56,57). It has also been reported that prolonged iron deficiency may cause reduced HbA_2 levels (54). The decreased HbA_2 observed in case 2 of the present study is hypothesized to be associated with these factors. Cases of rare β-thalassemia genotypes without elevated HbA_2 are easily overlooked and may be missed, particularly in patients with concomitant iron deficiency anemia, warranting heightened vigilance. The present subject represents the first reported triple compound case of HbH disease with compound β-thalassemia in the Chinese population, to the best of our knowledge.

The pathological mechanism of thalassemia lies in the imbalance of Hb chain synthesis, leading to ineffective erythropoiesis, increased hemolysis and iron metabolism disorders. Clinical manifestations exhibit heterogeneity, ranging from near-normal to severe, and from asymptomatic to requiring lifelong blood transfusion support (58). A genetic study of haemoglobinopathies performed by Yu *et al* (59) among a large cohort of asymptomatic individuals in Sichuan (southwestern China) revealed a high carrier frequency for β-thalassemia. Case 3 was phenotypically normal, but requested Hb electrophoresis and routine thalassemia gene testing for reproductive reasons. This identified a HbX + HbA_2 band abnormality. Suspecting the patient may harbor a rare thalassemia gene, further Sanger sequencing was performed, which identified a rare α,β-thalassemia compound genotype: HBA2:c.82 G>A (CD27 GAG>AAG) heterozygous mutation combined with an HBB:c.315+180 T>C (IVSII-180 T>C) heterozygous mutation. This α,β-thalassemia compound genotype case remains unreported in the Chinese population, to the best of our knowledge. Its normal phenotype may be associated with re-established synthesis equilibrium between α and β globin chains, as well as the male sex and age of the patient. HBA2:c.82 G>A (CD27 GAG>AAG) was first documented in the Chinese population (60); the mutation involves a G to A substitution at nucleotide position 82 in the α^2 globin gene coding sequence, resulting in a substitution of glutamic acid for lysine in the corresponding encoded amino acid. This leads to Hb Shuangfeng, with clinical manifestations being pathogenic or likely pathogenic (Ithanet database reference, https://www.ithanet.eu/db/ithagenes?ithaID=495%20%20//). HBB:c.315+180 T>C represents a mutation from T to C at nucleotide position 180 within the second intron of the β gene, with an undefined clinical phenotype (Ithanet database reference, https://www.ithanet.eu/db/ithagenes?ithaID=3682). This genotype has not been previously reported in the Chinese population, to the best of our knowledge. Moreover, this case of a compound rare genotype presents with an apparently normal phenotype. Without Hb electrophoresis analysis and subsequent sequencing, it may have been overlooked and misdiagnosed. This could have led to an erroneous prenatal diagnosis and genetic counselling, potentially resulting in offspring with severe haemoglobinopathies; therefore, caution is warranted.

In conclusion, the present study describes three cases of thalassemia, two of which exhibited silent genetic expression. Case 2 was identified as a carrier of a triple thalassemia compound, characterized by low HbA2 levels and clinical manifestations masked by HbH disease. These cases were identified through sensitivity to uncommon abnormalities observed during Hb electrophoresis and routine thalassemia gene testing, followed by further sequencing after patient consultation. Giambona *et al* (61) proposed that reducing or eliminating errors in haemoglobinopathy screening programs requires comprehensive consideration of genetic factors related or unrelated to β and α globin gene clusters, iron metabolism abnormalities, endocrine disorders, certain anemia types, and even inter- and intra-laboratory variability in HbA2 assays. Therefore, the present study concurs with these proposals, particularly regarding the identification and confirmation of rare cases of silent gene expression.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Guangdong Province Zhaoqing City Science and Technology Innovation Guidance Project Fund (grant no. 2023040316008).

Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CL designed the study and wrote the manuscript. CL, SC, GX and LL performed the human specimen testing. LL and ZL supplemented and analyzed the data, produced the graphs and revised the paper. CL and LL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study received approval from the Ethics Committee of The First People's Hospital of Zhaoqing (Zhaoqing, China; ethics approval no. B2024-02-04). Participants provided written informed consent to take part in the present study.

Patient consent for publication

Written informed consent was obtained from all subjects prior to inclusion in the present for the publication of clinical data and images.

Competing interests

The authors declare that they have no competing interests.

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