

Thalassemia intermedia in HbH-CS disease with compound heterozygosity for β -thalassemia: Challenges in hemoglobin analysis and clinical diagnosis

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Co-inheritance of α -thalassemia with homozygosity or compound heterozygosity for β -thalassemia may ameliorate β -thalassemia major. A wide range of clinical phenotypes is produced depending on the number of α -thalassemia alleles ($-\alpha/\alpha$, $-/\alpha$, $-/-$). The co-inheritance of β -thalassemia with α -thalassemia with a single gene deletion ($-\alpha/\alpha$) is usually associated with thalassemia major. In contrast, the co-inheritance of β -thalassemia with two α -genes deleted in *cis* or *trans* ($-/\alpha$ or $-\alpha/-$) generally produces β -thalassemia intermedia. In Southeast Asia, the most common defect responsible for α -thalassemia is the Southeast Asian (SEA) deletion of 20.5 kilobases. The presence of the SEA deletion with Hb Constant Spring (HbCS) produces HbH-CS disease. Co-inheritance of HbH-CS with compound heterozygosity for β -thalassemia is very rare. This study presents a Malay patient with HbH-CS disorder and β^0/β^+ -thalassemia. The SEA deletion was confirmed in the patient using a duplex-PCR. A Combine-Amplification Refractory Mutation System (C-ARMS) technique to simultaneously detect HbCS and Hb Quong Sze confirmed HbCS in the patient. Compound heterozygosity for CD41/42 and Poly A was confirmed using the ARMS. This is a unique case as the SEA α -gene deletion in *cis* ($--^{SEA}/\alpha$) is generally not present in the Malays, who more commonly possess the two α -gene deletion in *trans* ($-\alpha/-$). In addition, the β -globin gene mutation at CD41/42 is a common mutation in the Chinese and not in the Malays. The presence of both the SEA deletion and CD41/42 in the mother of the patient suggests the possible introduction of these two defects into the family by marriage with a Chinese.

Key words: thalassemia intermedia, Hb Constant Spring, CD41/42, Poly A, Amplification Refractory Mutation System, Duplex-PCR

INTRODUCTION

The hemoglobinopathies which include the thalassemias and hemoglobin (Hb) variants are the most common autosomal recessive disorders in the world. Thalassemia results in reduced or absence in the production of globin chains that make up hemoglobin. Different globin chains can be affected and the most common are α - and β -thalassemia. The most severe form of α -thalassemia is Hb Bart's hydrops fetalis where all four α -

globin genes are deleted ($-/-$) and the fetus dies *in utero* or very soon after birth (Lie-Injo et al., 1962). The deletion of three α -globin genes ($-/-\alpha$) results in HbH disease. In contrast, patients with the most severe form of β -thalassemia require life-long blood transfusions.

Structural Hb variants are caused by amino acid substitutions in the globin chains that lead to alterations in the stability and functional properties of hemoglobin. These variants affect either the α - or β -globin chains, and patients in the heterozygous states are generally asymptomatic. The most common α -chain variant in Southeast Asia is Hb Constant Spring (HbCS) while the clinically important β -Hb variants are HbS, HbC and

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HbE (Clegg et al., 1971; Laig et al., 1990).

In Southeast Asia where both thalassemia and Hb variants are prevalent, co-inheritance of different forms of thalassemia with hemoglobin variants are common. Deletional HbH disease ($--/\alpha$) results from co-inheritance of the SEA deletion ($--^{SEA}$) with a single $-\alpha^{3.7}$ or $-\alpha^{4.2}$ deletion, and affected individuals show moderate hemolytic anemia. In contrast, non-deletional HbH disease ($--/\alpha^T\alpha$) show a more severe phenotype with thalassemia intermedia and splenomegaly (Chui et al., 2003). These individuals possess the SEA deletion ($--^{SEA}$) with an α -hemoglobin variant (generally HbCS) and affected individuals have been reported in Hong Kong, Thailand, Taiwan and Malaysia (Chan et al., 1988; George and Wong, 1993; Winichagoon et al., 1993; Liu et al., 1994).

The co-inheritance of α -thalassemia with β -thalassemia major results in patients with a milder condition - thalassemia intermedia. The interactions involve single ($-\alpha$), two ($--$) and three α -globin gene ($--/\alpha$) deletions that may ameliorate β -thalassemia major (Ma et al., 2002; Wee et al., 2008). However, the co-inheritance of HbH-CS disease ($--^{SEA}/\alpha^{CS}\alpha$) with compound heterozygosity for β -thalassemia is rare. This study presents a family where both husband and wife possess $\alpha\beta$ -thalassemia. Their

four-year old daughter showed hemolytic anemia since birth and molecular analysis confirmed thalassemia intermedia with HbH-CS and β^0/β^+ -thalassemia.

MATERIALS AND METHODS

Patient samples The patient presented since birth with hemolytic anemia and hepatosplenomegaly. She was on follow-up in the Pediatric Clinic and her hemoglobin at 2 months was 7 g/dL. She was given her first blood transfusion at 16 months when her hemoglobin dropped to 4.9 g/dL. Detail molecular studies for Hb disorders were carried out when the patient was four years old. Routine peripheral blood counts, red cell indices, Hb electrophoresis and HbH inclusion bodies were determined according to standard laboratory procedures. Verbal and written consent was obtained from the parents prior to blood collection. This study was approved by the Medical Ethics Committee of UMMC in accordance with the Declaration of Helsinki.

DNA extraction DNA was extracted in Tris-EDTA (pH 8) using sodium-dodecyl sulphate and proteinase-K. Extracted DNA was purified and precipitated DNA

Table 1. Hematological, Hb electrophoresis and genotypic results for the patient and her parents

	Patient HbH disease/ β -thalassaemia major ($--^{SEA}/\alpha^{CS}\alpha$ & CD41/42- Poly A)	Normal range (paediatrics)	Father $\alpha\beta$ - thalassaemia ($\alpha^{CS}\alpha/\alpha\alpha$ & Poly A)	Mother $\alpha\beta$ - thalassaemia ($--^{SEA}/\alpha$ & CD41/42)	Normal range (adult)
Hb (g/dl)	6.9	10–14	14.4	9.9	11.5–16.5
Reticulocyte ($\times 10^{12}/L$)	4.39	3.80–5.00	Not done	Not done	0.2–2.00
PCV	0.2	0.30–0.42	Not done	Not done	0.37–0.47
MCV (fl)	46	73–90	84.9	70.4	76–96
MCH (pg)	16	24–34	27.2	23.7	27–32
MCHC (g/dl)	343	300–360	Not done	Not done	300–350
RDW(%)	32.6	11.0–15.0	Not done	Not done	12–15
Peripheral blood film	Marked anisocytosis, hypochromia, microcytosis, moderate polychromasia, numerous target cells	–	No abnormality observed	Beta- thalassaemia trait	–
Hb A (%)	53%	–	86.9	83.9	–
Hb A ₂ (%)	9.7	1.5–3.3	3.8	6.2	2.2–3.3
HbF (%)	35.7	< 1.1	0.5	0.6	0.1–1.3
HbH inclusion bodies	Not detected	–	Not detected	Not detected	–

was solubilized in double distilled water.

Duplex-PCR for detection of the Southeast Asian deletion DNA amplification to confirm the Southeast Asian (SEA) double α -globin gene deletion ($--^{SEA}$) was carried out using a Duplex-PCR. Primers for the Duplex-PCR amplify the 730 bp SEA deletion-specific sequence and the 136 bp normal sequence between the $\psi\alpha$ - α 2-globin genes (Wee et al., 2005a).

Gap-PCR for detection of the single $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions The $-\alpha^{3.7}$ deletion sequence was amplified as a 1.8 kb fragment and the $-\alpha^{4.2}$ deletion sequence as a 2.1 kb fragment (Proudfoot and Maniatis, 1980; Baysal and Huisman, 1994; Wee et al., 2005b). Heterozygosity or homozygosity for the single α -globin gene deletions were confirmed by amplification of the normal α -globin gene sequence between the $\psi\alpha$ - α 2-globin genes and the normal α 2-globin gene sequence.

Amplification Refractory Mutation System (ARMS) for detection of β -globin gene mutations β -thalassemia in the family was evaluated using ARMS. The β -globin gene mutations determined using ARMS are at the initiation codon for translation (T-G), -29 (A-G), -28 (A-G), CAP +1 (A-C), CD8/9 (+G), CD15 (G-A), CD17 (A-T), CD19 (A-G), HbE (G-A), IVS1-1 (G-T), IVS1-5 (G-C), CD41/42 (-TTCT), CD71/72 (+A), IVS2-654 (C-T) and poly A (A-G). Primer sequences were selected to amplify the β -globin mutations as specific molecular weight fragments with the absence of non-specific amplifications (Old et al., 2001; Tan et al., 1998). The β -globin gene mutations detected were confirmed by genomic sequencing.

Combine-ARMS for confirmation of Hb Constant Spring and Hb Quong Sze An in-house protocol was developed for the simultaneous amplification of two α -hemoglobin variants, Hb CS and Hb Quong Sze (HbQS). Amplification of the 183 bp HbCS mutant sequence was carried out using primers CS-1: 5'-CCTGGCCGCACTGACCCTATT-3' and CS-M: 5'-AGGAGGAAC-GGCTACCGAGGCTCCAGATTG-3'. Amplification of the 138 bp HbQS mutant sequence was carried out using CS-1 with a different reverse primer QS-M: 5'-CGGTGCTCACAGAAGCCA-GGAACTTGGCCG-3'. The 183 bp HbCS normal fragment was amplified using CS-1 with CS-N: 5'-AGGAGGAACGGCTACCGAGGCTCCAGATTA-3' and the 138 bp normal HbQS fragment was amplified using CS-1 with primer QS-N: 5'-CGGTGCTCACAGAAGCCAGGAAGCTTGGCCA-3'. In each reaction, amplification of a 323 bp region between the ϵ -globin and γ -globin genes in the β -globin gene complex was included as internal control (Tan et al., 1998; Tan et al., 2006). Amplified DNA was visualized after gel electrophoresis in 1.5% agarose and ethidium bromide staining.

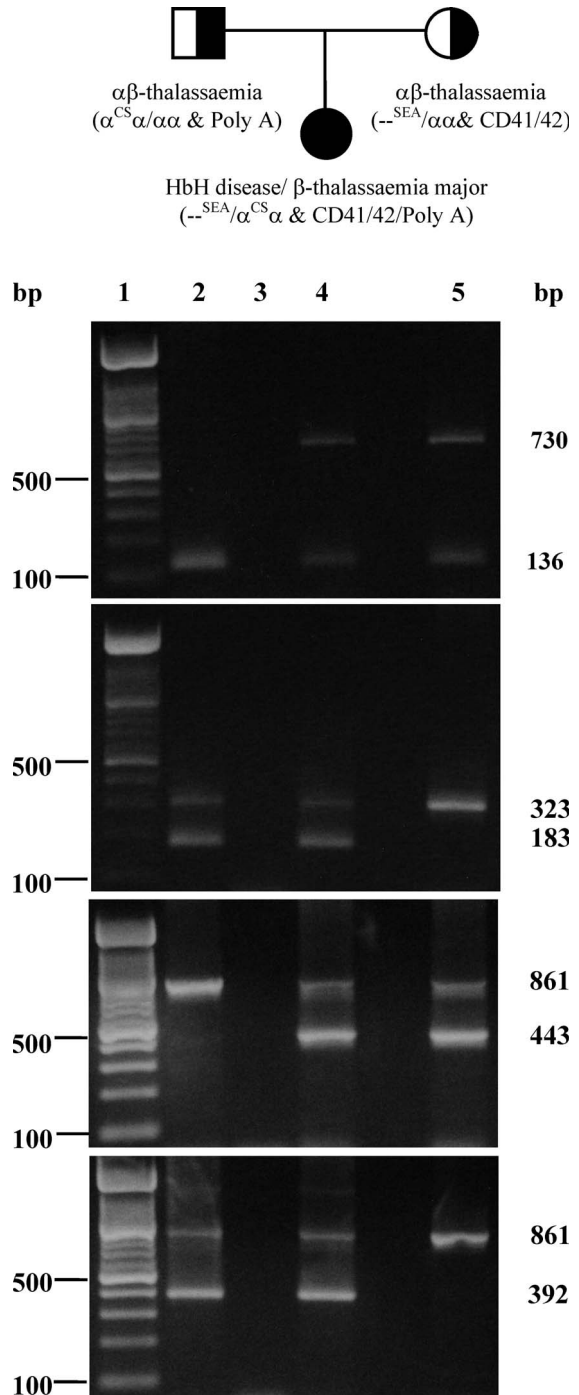


Fig. 1. Gel electrophoresis of the amplified PCR products after molecular characterization using Duplex-PCR, Combine-ARMS and ARMS. In all gels 1, 2, 3 and 4, lane 1: 100 bp molecular weight marker; lane 2: DNA from the father; lane 3: PCR control where no DNA was added; lane 4: DNA from the patient; lane 5: DNA from the mother. gel 1: lane 2: 136 bp normal $\psi\alpha$ - α 2 fragment; lanes 4 and 5: 730 bp SEA deletion-specific fragment and 136 bp normal fragment. gel 2: lanes 2 and 4: 183 bp HbCS and 323 bp internal control band; lane 5: 323 bp internal control band. gel 3: lane 2: 861 bp internal control band only; lanes 4 and 5: 861 bp internal control band and 443 bp Cd41/42 mutant band. gel 4: lanes 2 and 4: 861 bp internal control band and 392 bp Poly A mutant band; lane 5: 861 bp internal control band.

RESULTS

Table 1 shows the thalassemia results carried out. The patient had low Hb, MCV, MCH and her peripheral blood film showed markedly hypochromic and microcytic red cells. A preliminary diagnosis of thalassemia intermedia was made in view of the Hb analysis and normal ferritin.

HbH inclusion bodies were not detected in the family. Molecular studies for the family were recommended to check for a more severe hemoglobinopathy.

Figure 1 shows the gel electrophoresis results after molecular characterization of DNA. Lane 1 in all the four gels contains the 100 bp molecular weight marker, lane 3 is the PCR control where no DNA was added. Using a duplex-PCR to detect the SEA deletion, patient and mother's DNA amplified the 730 bp SEA-deletion-specific fragment and 136 bp normal sequence (gel 1, lanes 4, 5). The father's DNA amplified only the 136 bp normal sequence indicating the absence of the SEA deletion (gel 1, lane 2). Using gap-PCR, the single α -globin gene deletions ($-\alpha^{3.7}$ and $-\alpha^{4.2}$) were not detected in the family (gel results not shown). Using Combine-ARMS to detect the presence of both HbCS and HbQS, patient and father's DNA amplified the 183 bp HbCS-specific fragment and the 323 bp internal control (gel 2, lanes 2, 4) while the mother's DNA amplified only the 323 bp internal control band (gel 2, lane 5). Using ARMS, the β -globin mutation at CD41/42 was amplified as a 443 bp fragment in patient and mother's DNA (gel 3, lanes 4, 5) together with the 861 bp internal control. The CD41/42 mutant fragment was absent in the father's DNA, only the internal control fragment was observed (gel 3, lane 2). The β -globin gene mutation at Poly A was amplified as a 392 bp fragment in patient and father's DNA (gel 4, lanes 2, 4) together with the internal control. The Poly A mutant fragment was not amplified in DNA from the mother (gel 4, lane 5). Molecular analysis confirmed the patient's genotype as $^{-SEA}/\alpha^{CS}\alpha$, β°/β^{+} (CD41/42-PolyA); father's genotype as $\alpha^{CS}\alpha/\alpha\alpha$, β^{N}/β^{+} (Poly A) and mother's genotype as $^{-SEA}/\alpha\alpha$, β^{N}/β° (Cd41/42). Thus, the patient was confirmed with HbH-CS disease with compound heterozygosity for β -thalassemia, her father was confirmed with heterozygous HbCS and β -thalassemia and her mother with α/β -thalassemia.

DISCUSSION

Preliminary diagnosis of Hb disorders are carried out using hemoglobin analysis and electrophoresis that include hemoglobin values, MCV, MCH and HbA₂ and HbF percentages. The reliance on Hb analysis alone may not be accurate especially in populations with a high frequency of different globin gene mutations. Interactions between α - and β -thalassemias may produce moder-

ate to severe phenotypes depending on the molecular defects involved.

The co-inheritance of heterozygous β -thalassemia with homozygous α^{+} -thalassemia ($-\alpha/\alpha$) or heterozygous α° -thalassemia ($--/\alpha\alpha$) can present a near-normal hematological picture (Kanavakis et al., 1982). Heterozygous β° -thalassemia with co-inheritance of heterozygous α° -thalassemia ($--/\alpha\alpha$) show normal MCV and MCH values (Rosatelli et al., 1984). In contrast, patients with co-inheritance of deletional HbH ($---/\alpha$) disease with heterozygous β -thalassemia show anemia with markedly hypochromic and microcytic red cells and very low MCV and MCH indices (Knox-Macaulay et al., 1972; Ma et al., 2001). In a study of co-inheritance of β -thalassemia major (IVS1-6, T→C/IVS1-1, G→A) with deletional HbH disease ($^{-Med}/\alpha^{3.7}$), the patient showed marked anemia with low MCV of 48.7 but was never transfused as his hemoglobin maintained at 8.5–9.5 g/l (Kanavakis et al., 2004). His α /non- α -globin chain biosynthesis was balanced and in this interaction, a mild clinical status was manifested.

The frequency of co-inheritance of β -thalassemia with non-deletional HbH disease (HbH with an α -globin structural variant) is very rare compared with co-inheritance of β -thalassemia with deletional HbH disease. Concurrent heterozygous β -thalassemia with HbH-Quong Sze was reported in a 33 year-old patient with infrequent blood transfusions and splenomegaly (Ma et al., 2001). Her Hb analysis showed detectable HbH inclusion bodies, slightly increased HbA₂ levels of 4% and normal HbF. In this study, our patient with compound heterozygous β -thalassemia and HbH-CS showed a thalassemia intermedia phenotype at seven months with low MCV and MCH, increased HbA₂ of 9.7% and hepatosplenomegaly. However, HbH inclusion bodies were not detected. The higher levels of HbA₂ in these patients may be a result of the survival of red-cell populations with increased production of δ -chains (Gibbons et al., 2001).

The spectrum of mutations responsible for Hb disorders is population specific. Studies on co-inheritance of β -thalassemia with HbH disease have showed variable clinical heterogeneity depending on whether deletional or non-deletional HbH disease is involved. In conclusion, although co-inheritance of β -thalassemia with HbH disease may result in at most, an intermedia thalassemia phenotype, confirmation of the defects should include detail molecular analysis with family studies. The varied genotypes confirmed will provide valuable information that will assist greatly in genetic counseling of affected families.

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