ORIGINAL ARTICLE

Molecular characterisation of Haemoglobin Constant Spring and Haemoglobin Quong Sze with a Combine-Amplification Refractory Mutation System

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Abstract

***Background*: The interaction of the non-deletional α⁺-thalassaemia mutations Haemoglobin Constant Spring and Haemoglobin Quong Sze with the Southeast Asian double α-globin gene deletion results in non-deletional Haemoglobin H disease. Accurate detection of non-deletional Haemoglobin H disease, which is associated with severe phenotypes, is necessary as these mutations have been confirmed in the Malaysian population.**

***Methods*: DNA from two families with Haemoglobin H disease was extracted from EDTA-anticoagulated whole blood and subjected to molecular analysis for α-thalassaemia. A duplex polymerase chain reaction was used to detect the Southeast Asian α-globin gene deletion. Polymerase chain reaction-restriction fragment length polymorphism analysis was then carried out to determine the presence of Haemoglobin Constant Spring and Haemoglobin Quong Sze. A combine- amplification refractory mutation system protocol was optimised and implemented for the rapid and specific molecular characterisation of Haemoglobin Constant Spring and Haemoglobin Quong Sze in a single polymerase chain reaction.**

***Results and Conclusions*: The combine- amplification refractory mutation system for Haemoglobin Constant Spring and Haemoglobin Quong Sze, together with the duplex polymerase chain reaction, provides accurate pre- and postnatal diagnosis of non-deletional Haemoglobin H disease and allows detailed genotype analyses using minimal quantities of DNA.**

***Keywords*:** *Combine-ARMS, Hb Constant Spring, Hb Quong Sze*

Introduction

Thalassaemia is a public health problem in Malaysia, with about 4.5% of Chinese and 2.5% of Malays being carriers of α^0-thalassaemia (Southeast Asian (SEA) α-globin gene deletion, $--^{SEA}/\alpha\alpha$) (1). The loss of two α-globin genes in *cis* (--/αα) results in mild anaemia with microcytosis and hypochromic red blood cells. The loss of three α-globin genes causes deletional Haemoglobin H (HbH) disease (--/-α), whose presentation ranges from moderate anaemia to thalassaemia intermedia. Inheritance of α^0-thalassaemia with an α-globin structural variant results in non-deletional HbH disease ($--/\alpha^T\alpha$), a disorder with a more severe phenotype than deletional HbH disease. Patients with non-deletional HbH disease are more likely to have splenomegaly and need blood transfusions (2,3).

Non-deletional α^+-thalassaemia mutations give rise to α-globin structural variants (e.g., Hb Constant Spring) in addition to variants with structurally normal α-globin chains but that are expressed at a decreased level. More than 30 α-globin structural variants have been listed in the human globin gene mutation database (http://globin.cse.psu.edu). Haemoglobin Constant Spring (HbCS) involves a TAA→CAA base pair substitution in the termination codon of the α2-globin gene (HBA2 c.427T>C). The end product is an elongated α-globin chain with additional 31 amino acid residues (4). It was first observed in Constant Spring, Jamaica, in a Chinese family

with haemoglobin H disease (5). HbCS is the most common α-globin structural variant in Malaysia and in other Southeast Asian countries (6,7). In Malaysia, HbCS has been reported in Malay, Chinese and Indian populations at frequencies of 2.24%, 0.66% and 0.16% respectively (8,9). HbCS has also been observed among Aborigines ("Orang Asli" population) in East and West Malaysia (10). Haemoglobin Quong Sze (HbQS) is another non-deletional α-globin gene defect. It results from a gene mutation in the α2-globin gene whereby the amino acid leucine is substituted by proline (CTG→CCG, codon 125) (11,12). HbQS (HBA2 c.377T>C) is a rare and highly unstable haemoglobin variant reported in the Chinese population and in Thailand (13,14).

Both deletional (--/-α) and non-deletional (--/$\alpha^T\alpha$) HbH disease have been observed in the different ethnic groups in Malaysia, particularly among Malays and Chinese (15). However, non-deletional HbH disease caused by HbQS has not been previously reported, as the typical α-globin structural variant encountered in Malaysia is HbCS.

The detection of HbCS and HbQS is carried out using DNA amplification techniques and restriction enzyme digestion of amplified PCR products (16–19). In Malaysia, HbCS is detected by molecular analysis, Hb electrophoresis, high-performance liquid chromatography (HPLC) and isoelectric focusing. However, this abnormal α-globin, which comprised of only 1–2% of the total haemoglobin, is unstable and the fraction of slow-moving haemoglobin can be missed when non-molecular techniques are used.

This study highlights the presence of two α-globin structural variants, HbCS and HbQS, in Malaysia, as well as their molecular characterisation using a sensitive and specific Combine-ARMS technique developed in-house.

Materials and Methods

Family study

Two families (A and B), each with a child with HbH disease, were referred for molecular characterisation for α-thalassaemia at the University Malaya Medical Centre (UMMC). Patient A was four years of age when she was admitted with jaundice, severe anaemia and a 4-cm hepatomegaly. Family B consists of a Chinese couple and their 10-month-old daughter who was referred for pallor and lethargy. Based on clinical and haematological investigations, the accompanying diagnosis from the consultants involved in both cases suggested HbH disease with probable involvement of HbCS. Molecular characterisation of α-thalassaemia was carried out for both families. The haematological and Hb analysis data of patients A (carried out at UMMC) and B (carried out at Singapore General Hospital) are shown in Table 1.

DNA extraction

Ethical and institutional approval to carry out studies on α-thalassaemia was obtained from the Ethics Committee of the University Malaya Medical Centre (UMMC) in accordance with the Declaration of Helsinki. Informed and signed consent was also obtained from the parents of both families. Blood (5 mL) was collected from patients and family members in sodium-EDTA tubes and DNA was extracted using proteinase K and sodium dodecyl sulphate. Extracted DNA was purified using phenol-chloroform-isoamyl alcohol and precipitated with 4 M sodium acetate and ethanol.

Duplex PCR for the detection of the SEA deletion

The presence of the SEA α-globin gene deletion was detected using a duplex PCR protocol (20) in which the SEA deletion was amplified as a 730-bp fragment and the normal α-globin gene sequence between the ψα-α2-globin genes was amplified as a 136-bp fragment. DNA from α-thalassaemia carriers will amplify both the 730-bp SEA deletion-specific sequence and the normal 136-bp ψα-α2-globin gene sequence. DNA from a Hb Bart's hydrops foetalis (--/--) will specifically amplify only the SEA deletion-specific fragment.

PCR-RFLP analysis for HbCS and HbQS

The polymerase chain reaction-restriction fragment length polymorphism technique was used to detect HbCS in the families' genomes. A 339-bp sequence spanning the 3'-termination codon of the α2-globin gene that contains the HbCS mutation was amplified and then subjected to restriction enzyme analysis using *Mse* I (17). The detection of HbQS was carried out by amplifying the same 339-bp sequence spanning the 3'-termination codon of the α2-globin gene, followed by restriction enzyme digestion with *Msp* I (19).

Combine-ARMS for the detection of Hb CS and Hb QS

The C-ARMS primers for the detection of HbCS and HbQS were synthesised in two forms: the normal gene sequences and the mutant α-globin gene sequences, which contain one nucleotide substitution at the 3' end and an additional one-base pair mismatch at the third or fourth base from the

Table 1: Haematological data and haemoglobin analysis of patients A and B with non-deletional HbH disease

Parameters	Patient A HbH disease with HbCS	Patient B HbH disease with HbQS	Normal range
Sex-Age	Female - 4 years	Female -10 months	Patient A, UMMC[a] Patient B, SGH[b]
RBC (x10^{12}/L)	2.57	5.88	4.0–5.4 3.8–5.5
Hb (g/dL)	5.86	9.2	11.5–14.5 10.0–15.0
MCV (fL)	74	53.5	77–91 76–96
MCH (pg)	22.8	15.7	24–30 27–32
MCHC (g/dL)	31	29.3	30–35 32–36
RDW (%)	19.2	21.6	12–15 10.9–15.7
Hb electrophoresis	Hb A, Hb A2 and weak, Hb H bands present	HbA, HbA2 and Hb H bands present	-
Hb A2	2.8 %	1.8 %	2.2–3.3 2.1-3.1
Hb F	-	0.2 %	0.1–1.3 0.1–5.0
HbH inclusion bodies	Positive (numerous)	Not in report	-
Blood film	Severe anisopoikilocytosis, target cells and some polychromatic cells present	Red cells are hypochromic, microcytic, moderately severe anisopoikilocytosis and numerous target cells present	-

[a] University Malaya Medical Centre, UMMC; [b]Singapore General Hospital, SGH
Hb, haemoglobin; RBC, red blood cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width; -, not available

3' end. In each reaction, amplification of a 323-bp region between the $^{G}\gamma$- and ε-globin genes of the β-globin gene complex in chromosome 11 (primer E, 5'-AGTGCTGCAAGAAGAACAACTACC-3' and primer F, 5'-CTCTGCATCATGGGCAGTGAGCTC-3') was included as an internal control to check amplification efficiency (21).

DNA amplification was carried out in a PCR mixture that contained 10X buffer (750 mM Tris-HCl, 200 mM (NH4)2SO4, 0.1% Tween 20), 0.2 mM of each dNTP, 1 mM MgCl2, 1 U Taq DNA polymerase and 1 μg of DNA, in a 25-μl reaction. First, 50 pmol of forward primer CS-1 (5'-CCTGGGCCGCACTGACCCTATT-3') was added to 20 pmol each of reverse primers CS-M (5'-AGGAGGAACGGCTACCGAGGCTCCAGATTG-3') and QS-M (5'-CGGTGCTCACAGAAGCCAGGAACTTGGCCG-3') to amplify either a 183-bp HbCS mutant band and/or a 138-bp HbQS mutant band. The mixture was denatured at 95°C for 5 min, followed by 30 cycles of 93°C for 1 min, 65°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 3 min. A similar C-ARMS protocol was carried out for the amplification of the normal α-globin gene sequence; in this reaction, forward primer CS-1 was added to 20 pmol each of reverse primers CS-N (5'-AGGAGGAACGGCTACCGAGGCTCCAGATTA-3') and QS-N (5' CGGTGCTCACAGAAGCCAGGAACTTGGCCA-3') to amplify the 183-bp HbCS and 138-bp HbQS normal bands. Amplified DNA was visualised after electrophoresis in a 1.5% agarose gel and staining with ethidium bromide.

Results

Figure 1 shows the gel electrophoresis results of the duplex PCR (upper gel) for the detection of α^0-thalassaemia and the C-ARMS for HbCS and HbQS (lower gel). In the duplex PCR (upper gel), the 730-bp SEA deletion-specific band was observed in DNA from patient A and her mother (lanes 3 and 4) and in DNA from patient B (lane 7) and her father (lane 6). DNA from all members of families A and B amplified the 136-bp normal ψα-α2-globin genes. In C-ARMS (lower gel), the 183-bp HbCS-specific mutant band was amplified in DNA from patient A (lane 3) and her father (lane 2). The 138-bp HbQS specific mutant band was amplified in DNA from patient B and her mother (lanes 7 and 8). The 323-bp internal control band was amplified in DNA from all the family members.

The family pedigree with complete genotypes of both families is shown above the gels in Figure 1. The two patients referred for molecular analysis were confirmed to have HbH disease; patient A is compound heterozygous for the SEA deletion and HbCS ($--^{SEA}/\alpha^{CS}\alpha$) and patient B is compound heterozygous for the SEA deletion and HbQS ($--^{SEA}/\alpha^{QS}\alpha$).

The sensitivity of the in-house-developed C-ARMS was evaluated with HbCS (50 patients) and HbQS (10 patients) control DNA. The respective 183-bp HbCS and 138-bp HbQS sequences were amplified from the control DNA. In addition, the specificity of the C-ARMS technique was evaluated using DNA from normal individuals (n=100); non-specific amplification was not observed.

Discussion

A rapid and simple molecular method for the detection of α-globin structural variants is especially useful in regions where non-deletional HbH disease is present. Accurate detection of non-deletional HbH disease is necessary as these disorders have been associated with more severe phenotypes, including neonatal death, compared to the deletional forms of HbH disease (2, 22–24). HbH hydrops foetalis is usually associated with non-deletion mutations that give rise to hyper-unstable α-globin chains (25). A HbH hydrops foetalis case due to the association of the $--^{SEA}$ deletion with the HbQS mutation has been reported in a Chinese woman (26). Affected foetuses have severe anaemia and symptoms consistent with the classic presentation of Hb Bart's hydrops foetalis. HbH disease is considered a public health problem in California, USA, and neonatal screening programs for HbH and HbCS have been incorporated into the universal newborn screening program for haemoglobinopathies in that state (27,28). The haematological data show a relatively low red blood cell count for patient A. The patient is undergoing regular blood transfusion at three-weekly intervals; her pretransfusion haemoglobin ranges between 8–10 g/dL, whereas her post-transfusion haemoglobin is between 12–14 g/dL.

HbCS has been reported in the Malaysian population, with the highest frequency among Malays, followed by the Chinese and Indian populations (7,29). The presence of HbQS in Malaysia has not been previously documented and this study presents the first report of HbH disease associated with HbQS in Malaysia. In HbCS, the mRNA of the mutant gene is unstable and the small quantities of abnormal α-globins in the peripheral blood of HbCS patients make detection difficult, especially in heterozygotes (30). The non-deletional α-globin gene mutation in HbQS results in highly unstable α-globin chains (3). The detection of HbCS by gel electrophoresis requires fresh blood samples, and the highly unstable α-globin chains in HbQS make this Hb variant undetectable by routine electrophoresis (14).

Accurate detection of both HbCS and HbQS requires molecular techniques. In Malaysia, where both disorders are likely to be involved in non-deletional HbH disease, C-ARMS offers rapid and simple confirmation of diagnosis. PCR-RFLP also yields accurate diagnosis, but it is more expensive due to the higher cost of restriction enzymes in Southeast Asian countries. C-ARMS utilises five oligonucleotide primers that can be stored at -70°C. The primers E and F used for amplification of the 323-bp internal control are the same standard internal control primers commonly used in single ARMS. Using a standard PCR kit that includes Taq polymerase, 10X buffer and magnesium chloride, C-ARMS is cost-effective.

ARMS has been widely used for molecular characterisation and prenatal diagnosis of β-thalassaemia worldwide. Using ARMS, characterisation of β-thalassaemia was achieved in 98.7% of Malaysian Chinese patients (31), over 97% of patients in Pakistan (32), 84.2% of Egyptian patients (33) and 75% of patients in the Khuzestan provinces of Iran (34). A disadvantage usually associated with ARMS is false-positive and false-negative amplifications. In our laboratory, careful evaluation of primer concentrations and PCR conditions during the development of the ARMS method has resulted in sensitive and specific amplification. False-positive results due to maternal contamination can be further checked with DNA amplification of the VNTR locus DIS80

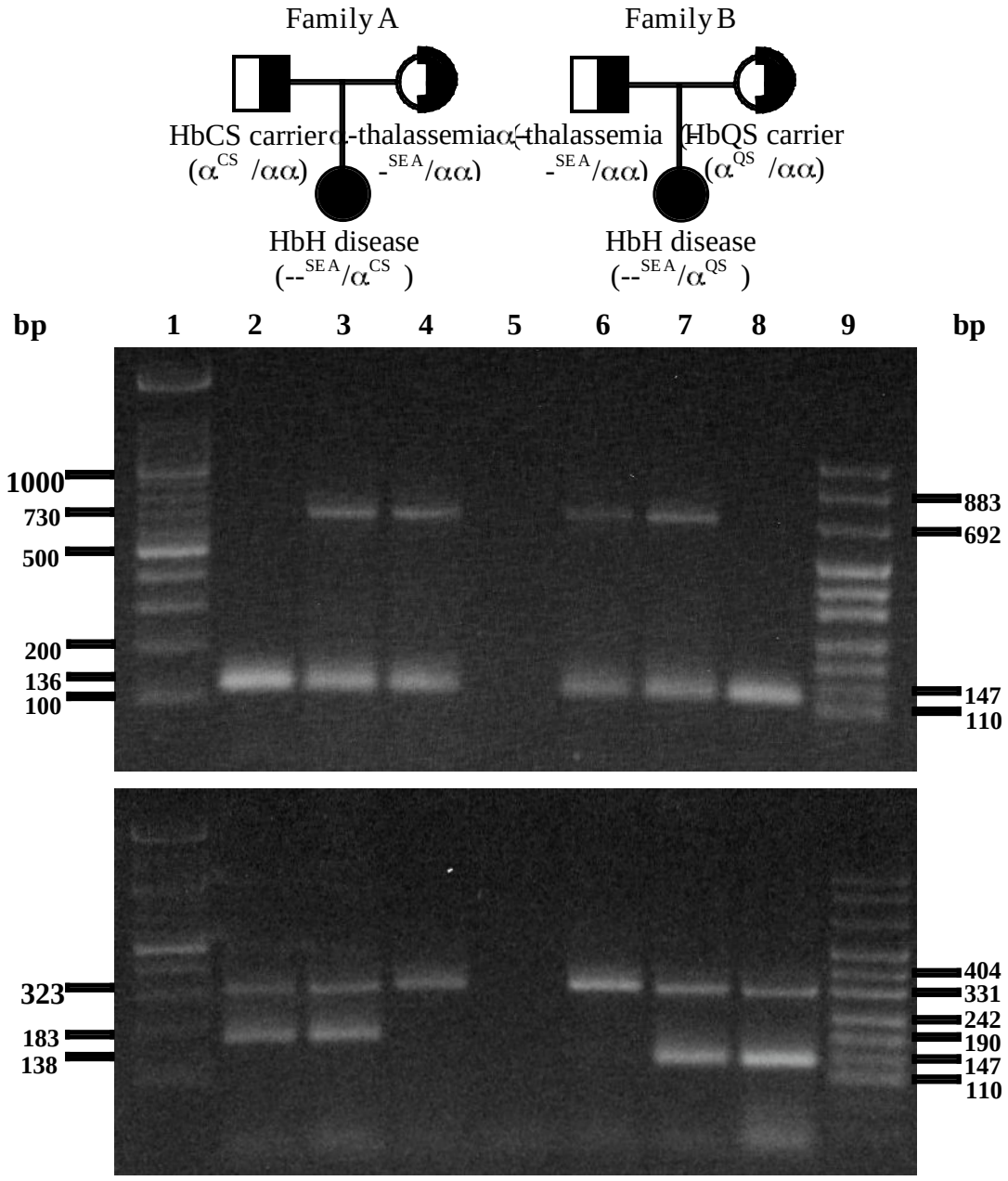

Figure 1: Gel electrophoresis of the amplified SEA deletion-specific sequence and the normal ψα-α2-globin genes in a duplex-PCR (upper gel) and the detection of HbCS and HbQS using C-ARMS (lower gel). The affected daughter in family A was admitted with hepatomegaly of 4cm, jaundice and severe anaemia at four years of age. The family was molecularly characterised with the SEA deletion and HbCS. Patient B was referred for pallor and lethargy, and she was characterised with the SEA deletion and HbQS

Upper gel. Duplex-PCR for the detection of SEA deletion
Lane 1: 100 bp DNA ladder; lane 2: father of patient A, 136 bp normal ψα-α2-globin genes region band; lane 3: patient A, 730 bp deletion-specific band and 136 bp normal band; lane 4: mother of patient A, 730 bp deletion-specific band and 136 bp normal band; lane 5: water control (no DNA added); lane 6: father of patient B, 730 bp deletion-specific band and 136 bp normal band; Lane 7: patient B, 730 bp deletion-specific band and 136 bp normal band; Lane 8: mother of patient B, 136 bp normal ψα-α2-globin genes region band; Lane 9: pUC Mix molecular weight marker.

Lower gel: C-ARMS for detection of HbCS and Hb QS
Lane 1: 100 bp DNA ladder; lane 2: father of patient A, 323 bp normal Gγ- ε-globin gene region internal control band and 183 bp HbCS mutant band; lane 3: patient A, 323 bp internal control band and 183 bp HbCS mutant band; lane 4: mother of patient A, 323 bp internal control band; lane 5: water control (no DNA added); lane 6: father of patient B, 323 bp internal control band; Lane 7: patient B, 323 bp internal control band and 138 bp HbQS mutant band; Lane 8: mother of patient B, 323 bp internal control band and 138 bp HbQS mutant band; Lane 9: pUC Mix molecular weight marker.

(31) and haplotype construction at six polymorphic restriction sites along the β-globin gene cluster (35). The most common α-chain variant in Southeast Asia is HbCS, followed by the rarer HbQS in Malaysia and Singapore. The frequency of HbQS in Malaysia has not been reported, except for the fact that it is reported as a rare mutation. Hb Pakse is another rare α-chain variant and has been reported more commonly in Thailand (36). The Combine-ARMS for HbCS and HbQS was specifically established for rapid detection of the two main non-deletional α^+-thalassaemia mutations in Malaysia. The sensitivity and specificity of this technique will allow confirmation of these two α-globin structural variants and thus yield valuable updated data on the actual frequencies of HbCS and HbQS in the Malaysian population.

Studies on the co-inheritance of β-thalassaemia with HbH disease have also shown variable clinical heterogeneity depending on whether deletional or non-deletional HbH disease is involved. Clinicians and genetic counsellors must take into account the role of HbCS and HbQS in HbH patients in whom α- and β-thalassaemias and Hb variants co-exist, particularly in Malaysia and other countries in Southeast Asia.

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