Med & Health Jun 2020; 15(1): 106-118 https://doi.org/10.17576/MH.2020.1501.10

ORIGINAL ARTICLE

Detection of Haemoglobin S using Multiplex Ligation-Dependent Probe Amplification and Flow-through Hybridization Techniques: Experience in a Tertiary Hospital

WEE SY[1], HAFIZA A[1], AZMA RZ[1], NORUNALUWAR J[2], AZLIN I[1], MALISA MY[2], QISTINA WN[2], AINOON O[3]

[1]Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia.
[2]Department of Diagnostic Medical Laboratory, Univerisiti Kebangsaan Malaysia Medical Centre, 56000 Kuala Lumpur, Malaysia.
[3]Department of Medical Sciences II, Faculty of Medicine, Universiti Sains Islam Malaysia, 55100 Kuala Lumpur, Malaysia

ABSTRAK

Hemoglobin S (HbS, $\alpha_2\beta_2^{6Glu\rightarrow Val}$) merupakan variasi hemoglobin yang terbentuk hasil daripada mutasi GAG → GTG pada kodon 6 gen β-globin. Hemoglobinopati haemoglobin S (HbS) jarang ditemui di kalangan penduduk Malaysia tetapi selalunya dijumpai di kalangan pendatang asing dari Afrika. Walau bagaimanapun beberapa kes didapati dalam kaum India dan Melayu. Kajian ini meninjau keputusan makmal pesakit HbS dan penggunaan "multiplex ligation-dependent probe amplification" (MLPA) dan "flow-through hybridization" (FTH) dalam mengesan mutasi HbS. HbS dikenalpasti melalui kromatografi cecair prestasi tinggi (HPLC) dan/atau elektroforesis kapilari serta elektroforesis hemoglobin. Analisis molekul dijalankan menggunakan kaedah MLPA, FTH dan penjujukan Sanger. Dua warga Afrika, tiga Melayu dan dua India berusia antara 2-31 tahun telah dikenalpasti. Lima pesakit adalah HbS homozigot, seorang kompaun heterozigot HbS/β-talasemia dan seorang lagi pembawa HbS. Tahap hemoglobin (Hb) kes HbS homozigot adalah antara 7.4-10.2 g/dL dengan aras HbS dan HbF diantara 58.3-94.7% dan 1.5-35.5%. Hb untuk kes kompaun heterozigot HbS/β-talasemia adalah 5.8 g/dL dan normal pada pembawa HbS. Aras HbS, HbF dan HbA_2 untuk HbS/β-talasemia dan pembawa HbS adalah 67%, 27.2% dan 4.2%, dan 38.6%, 0.1% and 2.8% setiap satu. Kedua-dua kaedah MLPA dan FTH berjaya mengesan mutasi HbS dalam semua kes, manakala cuma FTH dapat menentukan zygositi mutasi HbS dan β-talasemia dalam satu ujian yang sama.

Address for correspondence and reprint requests: Dr. Hafiza Alauddin. Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel: +603-9145 9518 Email: drhafiza@ppukm.ukm.edu.my

Kata kunci: hemoglobin, reaksi rantaian multipleks polimerase, sabit

ABSTRACT

Haemoglobin S (HbS, $\alpha_2\beta_2^{6Glu\rightarrow Val}$) is a variant haemoglobin resulted from GAG→GTG mutation on codon 6 of HBB gene. HbS haemoglobinopathy is uncommon in Malaysia and mainly seen in immigrants. However, Malaysian Indians and Malays are rarely affected. This study reviewed the laboratory findings of patients with HbS and the utilization of multiplex ligation-dependent probe amplification (MLPA) and flow-through hybridization (FTH) in the detection of HbS mutation. HbS was identified and quantified by high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and cellulose acetate gel electrophoresis. Molecular analysis was performed using MLPA, FTH and Sanger Sequencing. Two Africans, three Malays and two Indian individuals aged between 2-31 years were identified from our laboratory. Five patients were homozygous HbS, one was compound heterozygous HbS/β-thalassemia and one was a carrier of HbS. The patients with homozygous HbS had their haemoglobin (Hb) ranging from 7.4-10.2 g/dL with HbS and HbF levels of 58.3-94.7% and 1.5-35.5%, respectively. The Hb of compound heterozygous HbS/β-thalassaemia patients was 5.8 g/dL and was normal in heterozygous HbS. HbS, HbF and HbA_2 levels for the HbS/β-thalassaemia and the carrier were 67%, 27.2% and 4.2%, and 38.6%, 0.1% and 2.8%, respectively. Both MLPA and FTH successfully detected HbS mutation in all cases but only FTH could identify the zygosity of the HbS mutation together with underlying concomitant β-thalassaemia in a single test.

Keywords: hemoglobin, multiplex polymerase chain reaction, sickle

INTRODUCTION

Sickle haemoglobin, HbS, a variant β-globin chain, is a product of a missense mutation (GAG→GTG) at position 6 of the HBB gene substituting glutamic acid for valine whose molecular alteration was first described in 1977 (Marotta et al. 1977). The sickle mutation can be inherited in heterozygous form as HbS trait, in homozygous form as sickle cell anemia or compound with another β-gene mutation or deletion. HbS has reduced solubility under deoxygenated conditions (Magdoff-Fairchild et al. 1976), especially in homozygous and some compound heterozygous states, that renders it to polymerise and lead to sickling of red blood cells.

Haemoglobin analysis via High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and cellulose acetate gel electrophoresis have been well established for the diagnosis of HbS (Wajcman & Moradkhani 2011). Measurements of HbS,

HbA, HbF and HbA2 levels and simultaneous detection of variant haemoglobin is useful in determining heterozygosity, homozygosity and possible compound heterozygosity of HbS. However, in instances where the clinical presentation and other laboratory findings are atypical, or in patients previously exposed to blood transfusions or hydroxyurea, molecular genotyping is needed for confirmation.

Definitive diagnosis of HbS haemoglobinopathy requires molecular analysis. Several molecular methods have been established to detect presence of the typical mutation. The common methods employed include polymerase chain reaction (PCR) based Restriction Fragment Length Polymorphism (RFLP) and Allele-Specific Amplification (ASA) (Hafiza et al. 2010). More recently, advanced technologies have paved ways for several other methods such Mutilple Ligation Probe Amplification (MLPA), a method which makes use of multiple probes to quantitatively amplify gene segments of interest depending on the test kits used. A fluorescent labelled universal tagged to each primer set with unique fragment length once amplified will give rise to quantifiable signals for detection (Harteveld et al. 2014). This method is able to screen for unknown mutations in beta gene as well as HbS mutation in a single test. However, confirmation by DNA sequencing must still follow. Another method, reversed dot blot (RDB) has been around for several years but more recently, improvement of this technique by way of hybridising several biotinylated primers on a single membrane has led to production of RDB-flow through hybridisation (FTH), able to amplify 25 known mutations, including two deletions, also in a single run (Lin et al. 2012)

We reviewed the laboratory features of heterozygous HbS, homozygous HbS and compound heterozygous HbS/β-thalassaemia patients diagnosed in our centre. We also reviewed the utilization of MLPA and FTH techniques in molecular diagnosis of HbS.

MATERIALS AND METHODS

Clinical Presentations, Haematological Parameters and Haemoglobin Analysis

This was a retrospective study of seven patients with HbS haemoglobinopathy presented to UKMMC from year 2008 to 2017. The index case of a family study of HbS published by Hafiza et al. (2010) was included in our current case series as Patient 6.

Demographic data and clinical manifestations of the patients were collected through review of patients' medical records. Full blood count (FBC) was performed using automated haematology analyzer, DxH800 (Beckman Coulter, CA, USA) or XN-3000 (Sysmex Corporation, Kobe, Japan). Haemoglobin analysis of six patients were performed at our laboratory. Haemolysates of the samples were analysed with HPLC and/or CE to measure haemoglobin subtypes. HPLC was carried out on VARIANT II β-Thalassemia Short Program (Bio-Rad Laboratories,

Hercules, CA, USA) and CE utilised Sebia Capillarys 2 System (Sebia, Paris, France). Haemoglobin electrophoresis was carried out on a Microtech648ISO (Interlab, Italy) at alkaline pH and on an Interlab G26 (Interlab, Italy) at acidic pH to confirm the presence of HbS. Results of haemoglobin analysis of patient 7 were obtained from his previous investigations in a private laboratory.

DNA Extraction

Genomic DNA was extracted from white blood cells (WBC) of the EDTA-anticoagulated peripheral blood samples using the Blood Mini DNA Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The DNA concentration and purity were determined spectrophotometrically at 260 and 280 nm and DNA samples were stored at -30^{0}C before use.

Multiplex Ligation-dependent Probe Amplification (MLPA)

SALSA MLPA KIT P102 HBB kit (MRC Holland, Amsterdam, the Netherlands) was used to analyse LCR, *HBE*, *HBG2*, *HBG1*, *HBD* and *HBB* regions according to the manufacturer's guidelines. Approximately 200 ng of genomic DNA were obtained from each MLPA sample. ABI 9600 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used for ligation and amplification process. PCR amplification was carried out for 33 cycles at 94^{o}C for 30 seconds, 60^{o}C for 30 seconds and 72^{o}C for 60 seconds. Final extension was carried out at 72^{o}C for 20 minutes. ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used to separate the amplified fragments. The results were analysed by My Coffylyser. Net (MRC Holland, Amsterdam, the Netherlands).

Flow-through Hybridization (FTH)

Four cases were further analysed via flow-through hybridization technology using Hybriobio Thalassemia Gene Diagnostic Kit Thal-b25MY (Hybribio Limited, Hong Kong, China). The diagnostic kit was designed to detect of the following 23 point mutations and 2 deletional β-globin gene mutations: IVS 1-5 (G>C), IVS 1-1 (G>T), CD 41/42 (-TCTT), CD 17 (A>T), TATA -28 (A>G), TATA -29 (A>G), IVS II-654 (C>T), CD 71/72 (+A), CD 43 (G>T), IVS 1-1 (G>A), CD 27/28 (+C), Initiation Codon (T>G), CD 26 (G>A) (HbE), CD 19 (A>G) (Hb Malay), CD 6 (A>T) (HbS), CD 6 (G>A) (HbC), Poly A (A>G), Cap Site +1 (A>C), -86 (C>G), -88 (C>T), CD 15 (G>A), CD 16 (-C), CD 35 (-C), 45 kb deletion (Filipino) dan 619 bp deletion (Norunaluwar et al. 2015). The hybridization process was performed following manufacturer's protocol.

Sanger's Sequencing

Polymerase chain reaction (PCR) amplification of 4 fragments (I-IV) which include the region of HBB gene was performed. Four sets of primers were used (First Base, Malaysia), the sequence of which are listed in Table 1. PCR was performed using My Cycler

Table 1: Primer sequences used for PCR reactions in DNA sequencing process

Fragments	Forward primer sequence	Reverse primer sequence
1	5′TGAAGTCCAACTCCTAAGCCA3′	5′CAACTTCATCCACGTTCACC
2	5′GGTGAACGTGGATGAAGTT3′	5′CTAAAACGATCCTGAGACTCCCA3′
3	5′GTGTACACATATTGACCAAA3′	5′AGCACACAGACCAGCACG TT3′
4	5′AACGTGCTGGTCTGTGTGCT3′	5′AAATGCACTGACCTCCCACAA3′

(Bio-Rad Laboratories, Hercules, CA, USA). The PCR reactions consisted of 0.4 M of forward and reverse primers, 1.0U of *Taq* DNA polymerase, 50 ng of template DNA, 2 mM of $MgCl_2$, 0.2 mM of dNTP's and 1.5x of PCR buffer (Promega, USA). For fragment 1 and 4, the PCR conditions involved initial denaturation at 95˚C for 15 minutes, 30 cycles of denaturation step at 95˚C for 1 minute, annealing temperature from 59˚C for 1 minute and extension at 72˚C for 1 minute and 30 seconds followed by final extension at 72˚C for 8 minutes. For fragment 2 and 3, the PCR conditions were similar to the above, but annealing was performed at 53˚C for 1 minute. DNA sequencing utilised ABI 3100 Genetic Analyzer (Applied Biosystem, USA). Purification of the PCR products were used commercially available kit (Qiagen, Germany). The sequencing reactions were run on Big Dye Terminator Kit V3.1 (Applied Biosystem, USA) following these reactions; 10 ng/µl of purified PCR products, 2 µl of Big Dye Terminator Kit V3.1, 3 µl of Sequencing Buffer, 3.2 pmol of primer in a total volume of 20 µl. Cycle sequencing was performed using 40 cycles and consisted of 96˚C at 1 minute, 96˚C at 10 seconds and 50˚C at 5 seconds and 60˚C for 4 minutes. Purification of the products of cycle sequencing were peformed using ethanol/sodium acetate precipitation technique. Finally, standard method was used to sequence the products using Applied Biosystem, USA. Analysis of the sequencing results was performed on Basic Local Alignment System Tools (BLAST).

RESULTS

Seven patients with HbS haemoglobinopathy have been identified. The patients ranged from two to 31 years at the time of diagnosis at Universiti Kebangsaan Malaysia Medical Centre (UKMMC). They were Africans, three Malays and two Malaysian Indians. Five of the patients were homozygous HbS, one was compound heterozygous HbS/β-thalassemia and one was heterozygous HbS. All patients were not on hydroxyurea and had no history of blood transfusion in the preceeding three months at the time of haemoglobin analysis was performed. Demographic data and clinical presentations of the patients are summarized in Table 2. Patient 4 and 5 were siblings.

Haematological Values and Haemoglobin Analysis

Haemoglobin levels of homozygous

Table 2: Demographic data and clinical manifestations of patients with HbS haemoglobinopathy.

Patient	Age* / Gender	Race	Diagnosis	Clinical Presentations
1	23 / Female	Nigerian	Homozygous HbS	· History of recurrent painful crises requiring hospitalization. · Demised at the age of 23 due to massive subarachnoid haemorrhage secondary to ruptured anterior communicating artery aneurysm.
2	27 / Male	Nigerian	Homozygous HbS	· Recurrent pain crises requiring hospitalization and intravenous opioids
3	31 / Male	Malay	Heterozygous HbS	· Detected upon family screening (daughter known case of HbS trait). · No documented HbS related sign and symptom.
4	11 / Female	Indian	Homozygous HbS	· Detected during investigations for neonatal jaundice (mother known case of homozygous HbS) · Presented with single episode of haemolytic crisis and acute chest syndrome (? triggered by infection).
5	2 / Male	Indian	Homozygous HbS	· Detected during anaemia workout and family screening at infancy (siblings of patient no. 4) · History of neonatal jaundice; otherwise presented with asymptomatic anemia.
6	14 / Female	Malay	Homozygous HbS	· Jaundice since 11 years old, developed deep vein thrombosis (at 14 years old) and bilateral lower limb bone infarct (no avascular necrosis). · Multiple pain crises requiring hospitalization. · Cholecystectomy and splenectomy were performed in the later years.
7	2 / Male	Malay	HbS/β thalassemia	· Diagnosed at 1 year old in a private hospital. · Recurrent haemolytic crises, first presented with jaundice at 4 months old.

*age upon diagnosis at UKMMC

HbS patients ranged from 7.4-10.2 g/dL, 5.8 g/dL in the compound heterozygous HbS/β-thalassaemia patient and was normal in heterozygous HbS patient (Table 2). Four out of five homozygous HbS patients showed normochromic normocytic RBCs. In the homozygous patient with hypochromic microcytic feature, concomitant iron deficiency and α-thalassemia could not be fully excluded. HbS, HbF and HbA2 levels ranged from 58.3-94.7%, 1.5-35.5%, 1.1-3.8%, respectively among homozygous HbS; 67%, 27.2%, 4.2% in compound heterozygous HbS/β-thalassemia and 38.6%, 0.1%, 2.8%, respectively in the heterozygous HbS. HbA was absent in the homozygotes of HbS and compound heterozygotes of HbS/β-thalassemia patients.

Molecular Analysis

MLPA successfully detected HbS mutation (HBB:c.20A>T, rs334) in all seven cases (Figure 1). However, this method was unable to distinguish between homozygous and heterozygous states of the mutation. Four cases (Patient 2, 3, 6, 7) were

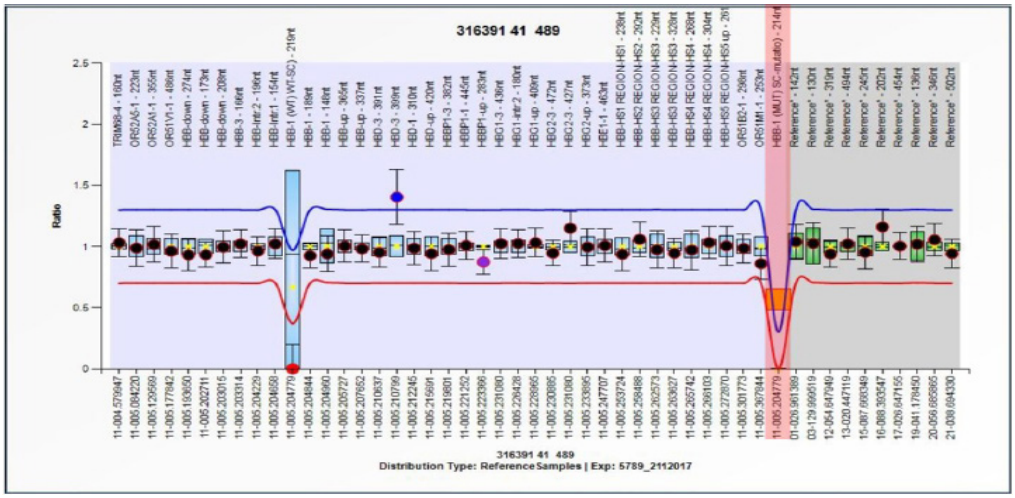

Figure 1: MLPA demonstrating presence of HbS mutation (HBB:c.20A>T, rs334) (highlighted red).

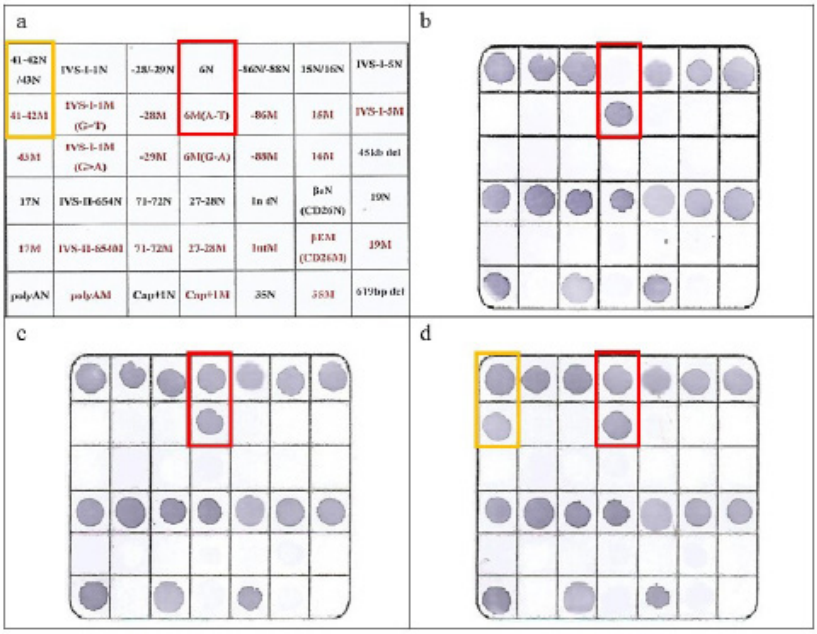

Figure 2: Flow-through Hybridization using HybriMem (Hybribrio, Hong Kong, China). (a) Location of probes designed in the membrane template. Red box indicates codon 6 and yellow box for Codon 41/42 of β-gene. Top rows show wild type alleles and bottom rows show alleles with mutation / deletion. (b) Homozygous HbS mutation showing presence of CD6 (A>T) (HbS) with absence of the wild type allele (Patient 6): a single dot within red box. (c) Heterozygous HbS mutation showing presence of both CD 6 (A>T) (HbS) and wild type allele (Patient 3): double dots within red box. (d) Compound heterozygous CD 6 (A>T) HbS and Codon 41/42 (-TCTT) β-gene mutations (Patient 7): double dots in both red and yellow boxes.

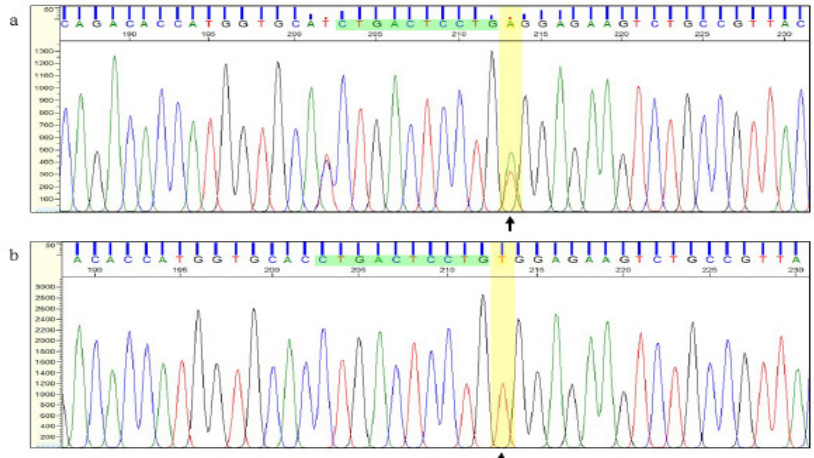

Figure 3: DNA sequencing showing HbS mutation (CD 6 A>T). (highlighted yellow) (a) Heterozygous HbS mutation. (b) Homozygous HbS mutation.

subsequently analysed with FTH. Patient 3 showed heterozygous HbS mutation, Patient 2 and 6 showed homozygous HbS mutation and Patient 7 showed compound heterozygous for HbS and Codon 41/42 (-TCTT) mutations (Figure 2). DNA sequencing confirmed the HbS genotypes of all seven cases (Figure 3) and concomitant β-thalassemia in patient 7. In addition, patients 4 and 5 were found to carry additional mutations of heterozygous c.9T>C and homozygous IVS-II-16 G>C (Table 3). DNA analysis for α-thalassemia was also carried out on patient 2 in view of hypochromic microcytic picture but failed to detect presence of any HBA gene mutation or deletion based on panels available in our laboratory (Azma et al. 2014).

DISCUSSION

Sickle haemoglobinopathy is an inheritable condition secondary to A>T mutation in codon 6 of the β-globin gene leading to HbS formation. The resultant replacement of hydrophilic glutamic acid by the hydrophobic valine causes the intracellular HbS to become insoluble and polymerize upon deoxygenation. The long polymers of HbS in turns distort the RBCs into stiff, sickle shape with reduced deformability. Repeated or prolonged sickling progressively damages the RBC membrane, contributing to shortened RBC lifespan and haemolytic anemia. The reduced deformability and increased stiffness, along with abnormal adhesion of the sickle RBCs to vessel endothelium, lead to vaso-occlusion and splenic sequestration. These events eventually gives rise to a broad range of acute and chronic ischaemic clinical complications (Kato et al. 2017). Several genetic modifiers have been described to influence HbS haemoglobinopathy phenotypes. This includes coinheritance with α-thalassemia trait and one's ability to produce HbF. (Steinberg & Sebastiani 2012; Thein 2017). Compound heterozygous HbS/

Table 3: Summary of molecular analysis of patients with HbS haemoglobinopathy

Patient	MLPA	Flow-through Hybridization	DNA Sequencing
1	HbS mutation detected	Not performed	Homozygous HbS mutation
2	HbS mutation detected	HbS mutation detected	Homozygous HbS mutation
3	HbS mutation detected	HbS mutation and wild type alleles detected	Heterozygous HbS mutation
4	HbS mutation detected	Not performed	Homozygous HbS mutation Heterozygous HBB c.9T>C Homozygous IVS-II-16 G>C
5	HbS mutation detected	Not performed	Homozygous HbS mutation Heterozygous HBB c.9T>C Homozygous IVS-II-16 G>C
6	HbS mutation detected	HbS mutation detected	Homozygous HbS mutation
7	HbS mutation detected	HbS mutation and wild type alleles detected Codon 41/42 (-TCTT) and wild type alleles detected	Heterozygous HbS mutation Heterozygous Codon 41/42 (-TCTT)

β^0-thalassemia generally has similar clinical picture to homozygous HbS but with less severe haemolysis (Sokal et al. 2016). Heterozygous HbS are generally asymptomatic due to the lower level of intracellular HbS in the RBCs. However, complications related to vaso-occlusion such as renal papillary necrosis, splenic infarction and haematuria have been observed (El Ariss et al. 2016)

HbS haemoglobinopathy is uncommon in Malaysia, with isolated case reports describing the disease mainly in Malaysia Indians and occasionally Malays. The cases were either homozygous HbS or compound heterozygous with beta thalassaemia, another inherited beta globin disorder common in Malaysia. Both forms showed similar clinical spectrum and disease presentation (Lie-Injo et al. 1986; Hafiza et al. 2010). Rahimah et al. reported a rare case of sickle/HbD-Punjab disease in a young Indian boy presented with severe anaemia during infancy (Rahimah et al. 2014).

Under basal conditions, heterozygous HbS individuals show no obvious haematologic abnormality with normal haemoglobin concentration and RBC indices. Concomitant α-thalassaemia or iron deficiency anaemia may produce a hypochromic microcytic picture. The usual partition of HbA and HbS is approximately 60:40 ratio, owing to the greater post-translational affinity of α-globin chains for β^A than for β^S (Sauntharajah & Vichinsky 2013).

Homozygous HbS usually has haemoglobin levels around 6-9 g/dL (Yawn et al. 2014) and anaemia is usually normochromic normocytic without α-thalassaemia or iron deficieny. HbS usually constitutes 80-95% of total haemoglobins, with HbF levels of 5-15%, HbA2 of <3.5% and absence of HbA (Ware et al. 2017). Compound heterozygous HbS/β^0-thalassemia shows hypochromic microcytic anemia with similar ranges

of HbS, HbF and absence of HbA as in homozygous HbS, but typically has raised HbA2 level (>3.5%)(Ware et al. 2017). However, HbA2 higher than 3.5% has been reported in homozygous HbS, some being linked to concomitant α-thalassaemia (Da Fonseca et al. 2015).

High level of HbF is observed in homozygous HbS. HbF level varies across different HbS haplotypes, in the descending order of Arab-Indian, Senegal, Benin and Bantu haplotypes. (Steinberg & Sebastiani 2012). However, HbF level shows considerable variation within any haplotype group as well (Steinberg & Sebastiani 2012). A study by Akinsheye et al. reported HbF levels of 18-41% in a group of Saudi Arabian homozygous HbS patients. Other than that, coinheritance with non-deletional HPFH inevitably raises the HbF level. Several quantitative trait locus (QTL) has also been linked to high HbF level in homozygous HbS, namely *Xmn1-HBG2*, *HBS1L-MYB* intergenic region (HMIP) and *BCL11A* (Akinsheye et al. 2011; Thein 2017). Because of this overlap in values, HbF level is sometimes less useful in discriminating HbS/β^0-thalassaemia from homozygous HbS.

HbF has been recognized as an important modulator of homozygous HbS phenotypes due to its inhibitory effect on deoxy HbS polymerization. This is because HbF cannot enter the deoxy HbS polymer phase and it also reduces intracellular HbS concentration (Steinberg et al. 2014). Therefore, HbS patients with higher HbF level are generally associated with milder haematologic and clinical features. Patient 5 from our study showed the highest level of HbF (35.5%) with the least eventful clinical course among the homozygous HbS patients (Table 2**)**. His sister, Patient 4, with a similarly high HbF level (29.9%), presented with less frequent complications. However, recent studies mentioned that it was the amount of HbF per F-cell (HbF/F cell) and the proportion of F-cells with sufficient HbF to inhibit HbS polymerization, rather than F-cell number or overall HbF concentration, were better predictors of clinical severity in homozygous HbS (Steinberg et al. 2014). It was proposed that a HbF concentrations nearing 30% is required for amount of protected cells to reach 70% (Steinberg et al. 2014). This may explain why multiple vaso-occlusive events still occurred in Patient 6 who had HbF level of 17.1%.

Molecular studies is vital to confirm the presence of HbS mutation. Some molecular methods can simultaneously detect the zygosity status of the mutation. This is crutial in cases where clinical presentations or test results are atypical or in patients previously exposed to blood transfusions or hydroxyurea that may affect haemoglobin analysis interpretation. Various methods had been established for the purpose, including RDB, RFLP and ASA. Other available molecular methods include MLPA, RDB-FTH and High Resolution Melting analysis (HRM).

MLPA is one of the molecular methods useful for multiple detection of genetic deletions and duplications in HBA and HBB genes (Greene et al. 2015). The MLPA kit for the

β-thalassaemia also includes HbS mutation (HBB:c.20A>T, rs334). However, unlike in gene deletions or duplications, MLPA is not useful in identifying the homozygosity or heterozygosity status of the mutation. Limited literature is available on the study of HbS mutation using MLPA up to the current date.

FTH, initially developed for HPV detection, was later introduced for the diagnosis of α-, β-thalassaemia and haemoglobinopathy. FTH has the advantage of genotyping samples as heterozygotes, homozygotes or compound heterozygotes in a single run (Lin et al. 2012). The method is also less laborious and yields more rapid results in comparison to some of the conventional molecular techniques. However, the limitation lies in its ability to detect unknown mutations, as it is only based on the selected primer panel used (Lin et al. 2012). As with MLPA, HbS mutation analysis using this technique has not been widely studied.

DNA sequencing provides confirmation for the presence of HbS mutation and the zygosity status. It also identifies other concomitant gene deletion and/or mutation in the β-globin genes clusters, depending on the segments of HBB tested. In this case series, Codon 41/42 (-TCTT) β-gene mutation, a type of β^0 thalassaemia, was detected along with HbS mutation in the HbS/β-thalassaemia patient (Patient 7).

Other than that, DNA sequencing had also detected two additional mutations, *HBB* c.9T>C and IVS-II-16 G>C, in Patient 4 and 5. A silent mutation, *HBB* c.9T>C, was found to be the most common mutation on *HBB* gene among an urban eastern Indian population (19.57%) in Odisha, India (Sahoo et al. 2014). The same study also detected an increased frequency of IVS-II-16 G>C in the population. Another study from Bangladesh also reported that these two mutations were common among their local population (Sultana et al. 2016). The second study observed that *HBB* c.9T>C produced no pathogenic effect when present alone or in the presence of homozygous IVS-II-16 G>C,but may generate thalassaemia when present in homozygous state or coinherits with particular HBB mutations. Nevertheless, both mutations were found in healthy controls in either heterozygous or homozygous forms. Therefore, the author proposed that both *HBB* c.9T>C and IVS-II-16 G>C might be only a polymorphism with no pathogenic effect.

Molecular analysis for α-thalassaemia was performed on Patient 2 and showed no deletion or mutation detected. However, as the genotypes for α-thalassemia vary geographically, deletion or mutation uncommon in our setting and hence were not tested for, could have been missed.

CONCLUSION

HPLC and CE have been widely employed for the detection and quantification of HbS. HbS level varies widely among homozygous HbS patients, being affected by the level of HbF. Overlapping HbF and

HbA2 levels can be seen between homozygous HbS and HbS/β^0-thalassemia. Both MLPA and FTH are effective in detecting the presence of HbS mutation but MLPA is unable to determine the zygosity status. On the contrary, FTH can concurrently identify HbS mutation, wild type allele and other concomitant deletional or non-deletional β-globin gene mutations. This advantage enables the determination of HbS mutation and to simultaneously identify homozygosity, heterozygosity and compound heterozygosity of the mutation. Nevertheless, more studies should be carried out to establish the roles of MLPA and FTH in the diagnosis of HbS haemoglobinopathy.

ACKNOWLEDGEMENT

Authors acknowledge the Malaysian Ministry of Higher Education Exploratory Research Grant Scheme (Code: FRGS/1/2014/SKK06/UKM/02/1) for financial support recieved

REFERENCES

Akinsheye, I., Alsultan, A., Solovieff, N., Ngo, D., Baldwin, C.T., Sebastiani, P., Chui, D.H., Steinberg, M.H. 2011. Fetal hemoglobin in sickle cell anemia. *Blood* **118**(1): 19-27.

Azma, R.Z., Ainoon, O., Hafiza, A., Azlin, I., Noor Farisah, A.R., Nor Hidayati, S., Noor Hamidah, H. 2014. Molecular characteristic of alpha thalassaemia among patients diagnosed in UKM Medical Centre. *Malays J Pathol* **36**(1): 27-32.

Da Fonseca, S.F., Amorim, T., Purificacao, A., Goncalves, M., Boa-Sorte, N. 2015. Hemoglobin A2 values in sickle cell disease patients quantified by high performance liquid chromatography and the influence of alpha thalassemia. *Rev Bras Hematol Hemoter* **37**(5): 296-301.

El Ariss, A.B., Younes, M., Matar, J., Berjaoui, Z. 2016. Prevalence of sickle cell trait in the southern suburb of Beirut, Lebanon. *Mediterr J Hematol Infect Dis* **8**(1): e2016015.

Greene, D.N., Vaughn, C.P., Crews, B.O., Agarwal, A.M. 2015. Advances in detection of hemoglobinopathies. *Clin Chim Acta* **439**: 50-7.

Hafiza, A., Noor, H.H., Noor, F.A., Azlin, I., Ainoon, O. 2010. A family study of HbS in a Malay family by molecular analysis. *Malays J Pathol* **32**(2): 137-41.

Harteveld, C.L. 2014. State of the art and new developments in molecular diagnostics for hemoglobinopathies in multiethnic societies. *Int J Lab Hematol* **36**(1): 1-12.

Kato, G.J., Steinberg, M.H., Gladwin, M.T. 2017. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J. Clin. Invest* **127**: 750-60.

Lie-Injo, L.E., Hassan, K., Joishy, S.K., Lim, M.L. 1986. Sickle cell anemia associated with alpha-thalassemia in Malaysian Indians. *Am J Hematol* **22**(3): 265-74.

Lin, M., Zhu, J.J., Wang, Q., Xie, L.X., Lu, M., Wang, J.L., Wang, C.F., Zhong, T.Y., Zheng, L., Pan, M. C., Wu, J.R., Wen, Y.F., Liu, G.R., Zhan, X.F., Lin, F., Yang, L.Y. 2012. Development and evaluation of a reverse dot blot assay for the simultaneous detection of common alpha and beta thalassemia in Chinese. *Blood Cells Mol Dis* **48**(2): 86-90.

Magdoff-Fairchild, B., Poillon, W.N., Li, T., Bertles, J.F. 1976. Thermodynamic studies of polymerization of deoxygenated sickle cell hemoglobin. *Proc Natl Acad Sci USA.* **73**(4): 990-4.

Marotta, C.A., Wilson, J.T., Forget, B.J., Weissman, S.M. 1977. Human B-globin messenger RNA III. Nucleotide sequences derived from complementary DNA. *J Biol Chem* **252**: 5040-51.

Norunaluwar, J., Aushikin, K., Rusilawaty, A., Azma, R.Z., Emida, M., Hafiza, A., Azlin, I., Zarina, A.L., Hamidah, A., Rafeah, T., Noor Farisah, N.R., Malisa, M.Y., Ainoon, O. 2015. Molecular analysis of β-globin gene mutations in UKMMC using multiplex amplification refractory mutation system and flow-through hybridization kit: A comparison of two methods. *Malaysian J Pathol* **37**(2): 199.

Rahimah, A., Syahira Lazira, O., Siti Hida, H. M., Faidatul Syazlin, A.H., Nur Aisyah, A., Nik Hafidzah, N.M., Zubaidah, Z. 2014. Haemoglobin sickle d punjab: - a case report. *Med J Malaysia* **69**(1): 42-3.

Sahoo, S.S., Biswal, S., Dixit, M. 2014. Distinctive mutation spectrum of the HBB gene in an urban eastern Indian population. *Hemoglobin* **38**(1): 33-8.

Sauntharajah, Y., Vichinsky, E.P. 2013. Sickle cell disease: Clinical features and management. In

Hematology: Basic Principles and Practice., 6th edition; Elsevier Health Sciences 548-572.

Sokal, A., Dubert, M., Diallo, D., Diop, S., Tolo, A., Belinga, S., Sanogo, I., Diagne, I., Wamba, G., Boidy, K., Deme Ly, I., Seck, M., Faye, B. F., Kamara, I., Traore, Y., Offredo, L., Arlet, J.B., Jouven, X., Ranque, B. 2016. African sickle beta zero-thalassemia patients vs sickle cell anemia patients: similar clinical features but less severe hemolysis. *J Hematol Transfus* **4**(3): 1053.

Steinberg, M.H., Chui, D.H., Dover, G.J., Sebastiani, P., Alsultan, A. 2014. Fetal hemoglobin in sickle cell anemia: a glass half full? *Blood* **123**(4): 481-5.

Steinberg, M.H., Sebastiani, P. 2012. Genetic modifiers of sickle cell disease. *Am J Hematol* **87**(8): 795-803.

Sultana, G.N.N., Begum, R., Akhter, H., Shamim, Z., Rahim, M.A., Chaubey, G. 2016. The complete Spectrum of beta (β) thalassemia mutations in Bangladeshi population. *Austin Biomark Diagn* **3**(1): 1024.

Thein, S.L. 2017. Genetic Basis and Genetic Modifiers of beta-Thalassemia and Sickle Cell Disease. *Adv Exp Med Biol* **1013**: 27-57.

Wajcman, H., Moradkhani, K. 2011. Abnormal haemoglobins: detection & characterization. *The Indian journal of medical research* **134**(4): 538-46.

Ware, R.E., De Montalembert, M., Tshilolo, L., Abboud, M.R. 2017. Sickle cell disease. *Lancet* **390**(10091): 311-23.

Yawn, B.P., Buchanan, G.R., Afenyi-Annan, A.N., Ballas, S.K., Hassell, K.L., James, A.H., Jordan, L., Lanzkron, S.M., Lottenberg, R., Savage, W. J., Tanabe, P.J., Ware, R.E., Murad, M.H., Goldsmith, J.C., Ortiz, E., Fulwood, R., Horton, A., John-Sowah, J. 2014. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *Jama* **312**(10): 1033-48.

Received: 23 Aug 2019
Accepted: 5 Feb 2020