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CASE REPORT

A family study of HbS in a Malay family by molecular analysis

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Abstract

Sickle cell disease (SCD) is an inherited red cell disorder, characterized by the tendency of haemoglobin S or sickle haemoglobin to polymerize and assume a characteristic sickle shape. Molecular analysis has been the mainstay of detection method when confirmation is required. Previously a polymerase chain reaction (PCR)-based restriction enzyme analysis was used for this purpose. A simple bidirectional allele-specific amplification, recently described by Waterfall in 2001 was used to detect the GAG -> GTG mutation on codon 6 of the β globin gene. Two sets of primers for the mutant and the wild type alleles were used in a single PCR reaction to amplify the regions of interest. The resultant PCR products will produce two fragments at 517 and 267 base pair (bp) respectively. This report highlights the investigations for SCD in the family of a 16-year old girl with recurrent painful crisis affecting the lower limbs whereby the family members are asymptomatic for the disease. Her haemoglobin electrophoresis at an alkaline pH showed dense bands at the HbS and HbF regions, while her father and two sisters had bands at HbS, HbF and HbA. The PCR analysis showed that she was homozygous for the mutation by the presence of only one band at 267 bp fragment, while the father and her sisters were heterozygotes, with the presence of two bands at 267 as well as 517 bp fragments. DNA sequencing of the sample confirmed the mutation. In conclusion, this case report highlighted the simple and cheap yet practical method for molecular confirmation of the presence of HbS gene in subjects with homozygous or heterozygous state of the condition.

Key words: Sickle cell disease, HbS, allele-specific amplification PCR, sickle cell mutation

INTRODUCTION

Sickle cell disease is a severe form of inherited haemolytic anaemia due to an A > T point mutation in codon 6 of the β globin gene. The resulting haemoglobin S (HbS)(α2β26Glu→Val) has reduced solubility and is prone to polymerize under low oxygen tension. The detection of HbS are usually made by using electrophoresis and liquid chromatography techniques, supported by either the sickling test or the sickle solubility test. Although these methods are sufficient in diagnosing a large majority of the clinical cases, they are by no means confirmatory. Molecular analysis provides confirmatory evidence for the presence of HbS. This is particularly useful in the regions where the initial clinical presentation of sickle cell disease or the usual test results are atypical. Molecular diagnosis is also increasingly used for prenatal and even preimplantation diagnosis of sickle cell disease.

Since the sickle cell mutation involves a known point mutation in the sixth codon of the β globin gene, methods that are conventionally employed for the purpose of molecular diagnosis are polymerase chain reaction (PCR) based restriction fragment length polymorphism (RLFP) and allele-specific amplification (ASA). RLFP makes use of restriction enzyme that is able to cleave Bsu36 site on the normal β globin gene. This site is lost in the typical GAG > GTG mutation in codon 6 of sickle cell disease.[1] The use of restriction enzyme for this method can be quite cumbersome and expensive for some laboratories. The ASA technique is relatively simpler and

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cheaper where two separate reactions involving a mutant primer and a wild-type oligonucleotide primer sequences are utilized. The results of the two reactions are interpreted simultaneously to determine the presence or absence of sickle cell mutation.

A modification of the conventional ASA technique was described by Waterfall in 2001, where a single-tubed, bi-directional ASA was used to detect the sickle cell mutation.[2] In this system, both the wild type (β-gene) and the mutant gene (βS-gene) can be detected in a single reaction. This method has the potential to reduce laboratory work as well as the cost of the test performed. We applied this method to a family with an index case affected by sickle cell disease (SCD) to test whether this modified method was able to detect the presence or absence of both the wild-type and mutant alleles in a single reaction.

CASE REPORT

The patient was a 16-year-old Malay female whose initial presentation was deep vein thrombosis in the left lower limb at the age of 14 years and who was treated with warfarin for 6 months. Four months after completing the warfarin treatment, she presented with intramuscular haematoma and was then diagnosed with sickle cell disease. From then on, she had multiple admissions for veno-occlusive disease mainly involving the lower limbs. Hydroxyurea 500 mg was given every other day but she developed side effects of neutropaenia and aplastic crisis requiring hospital admissions, rendering the treatment to be stopped. Her father and three sisters were asymptomatic. Her mother passed away due to an unrelated condition.

Clinical examination revealed no thalassaemic facies but there was splenomegaly of 2.0 cm. Other clinical examinations were unremarkable. The patient's peripheral blood film showed moderate normochromic normocytic anemia with occasional sickle cells seen. Her sickling test was negative. The family's blood investigation results are listed in Table 1. We proceeded to confirm the diagnosis by DNA analysis and family study.

Molecular analysis: methodology

Blood samples from the patient, father and three sisters were taken with informed consent. DNA was extracted from peripheral blood using GeneAll Exgene Blood SV Minikit (GeneAll Biotechnology Korea) according to the manufacturer's protocol. DNA analysis was not carried out for Sister 3 since she was normal as implicated by the full blood count and haemoglobin analysis.

The patient, her father, her two sisters, a positive control and a normal control were genotyped initially by bi-directional allele-specific PCR assay. The positive control used here was a confirmed case with HbSS. The primer sets used were the same as those described by Waterfall[2] and PCR conditions were adapted in our laboratory as described here. The standard reaction mixture contained 1 µg DNA, 10 pmol of each primer and 1.5 U of HotStart DNA polymerase (i-Taq, Intron Biotechnology) and was subjected to PCR amplification at 94°C for 5 minutes followed by 35 cycles of 94°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec and final extension of 2 minutes at 72°C. All PCR amplifications were carried out using the DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystem). An aliquot of 1µl of each PCR product was subjected to Lab-on-chip analysis, using the Agilent 1000 DNA Lab Chip Kit and the Agilent 2100 Bioanalyzer. The mutations identified in this study by using the ASA-PCR were confirmed by DNA sequencing.

TABLE 1: Haematology results of the family

	Haemoglobin (g/dl)	MCV (fl)	MCH (pg)	%HbS (HPLC)	%HbF (HPLC)	%HbA2 (HPLC)
Patient	9.8	84.5	27.6	77.3	17.1	3.0
Father	14.6	83.0	28.0	35.2	<1.0	3.5
Sister1(SS)	12.9	81.1	26.3	35.0	<1.0	3.3
Sister2(SN)	13.5	80.5	26.7	35.3	<1.0	3.2
Sister3(SH)	13.3	91.9	30.0	0	<1.0	3.3

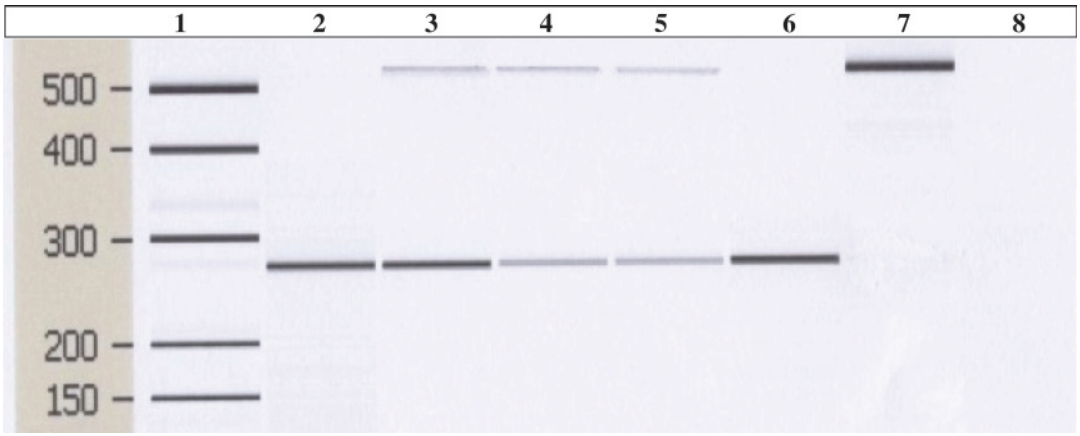

FIG. 1: Agilent gel electrophoresis of the allele-specific amplification (ASA) PCR products. Lane 1 is 100 bp marker. Lane 2 patient showing 267 bp DNA fragment, lane 3 (SS), 4 (SN) and 5 (father) showing both the 267 bp and 517 bp DNA fragments. Lane 6 is the positive control showing 267 bp DNA fragment, lane 7 is the normal control showing 517 bp DNA fragment and lane 8 is negative control.

Molecular analysis: findings

The ASA PCR products of the DNA samples from the patient, her father and her two sisters are shown in Figure 1. The patient's sample clearly showed only one band at position 267 indicating that she was homozygous for the GAG > GTG mutation in codon 6 similar to the positive control sample taken from a confirmed patient with HbSS. The sample from a normal individual showed a band at the 517 bp position. Her father and the other two siblings showed two bands at the position 267 bp and 517 bp designating their heterozygosity (HbAS). DNA sequencing results performed on the patient's sample confirmed the presence of the A > T mutation at codon 6 of the β-globin gene (Figure 2) as compared to the normal sample (Figure 3).

DISCUSSION

Sickle cell anaemia is not common in Malaysia. The few reported cases of sickle cell disease and sickle cell trait involved mainly Malaysian Indians, although there were some Malays affected.[3,4] The clinical picture of SCD in the reported population was relatively mild and many even reached old age. This was explained by the fact that all of them also had α-thalassaemia[3] which could ameliorate the haemolysis effects of Hb S in red cells. In contrast, β-thalassaemia genes are more commonly found in Malaysia, with a carrier rate of 4.5%[5]. In a study performed by Ehsan Valavi *et al* in 2010 that compared homozygous Hb S and compound heterozygous Hb S/β^0, the mean age of presentation for both Hb SS and Hb S/β^0 was 4 years old where newborn

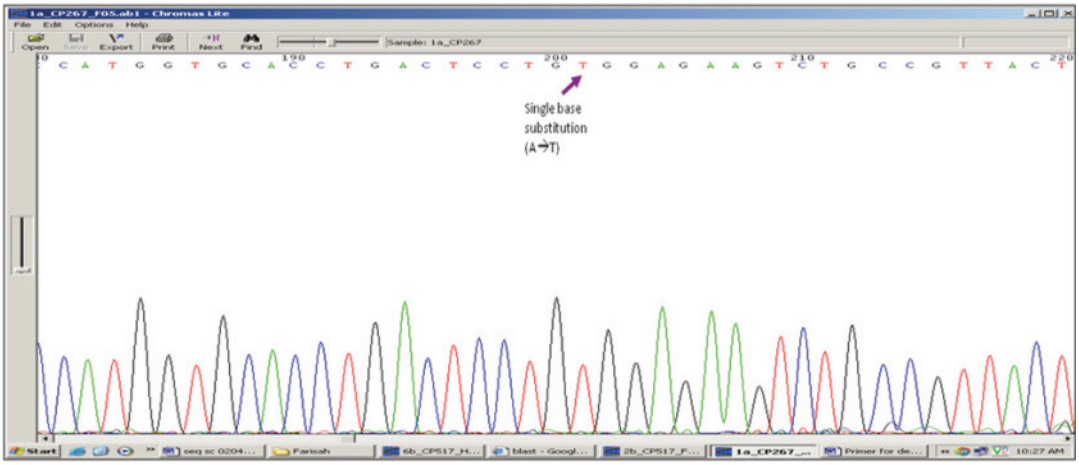

FIG. 2: DNA analysis sequence electropherogram of the mutant sample. The arrow points towards the single base substitution A >T in codon 6 of the β-globin gene.

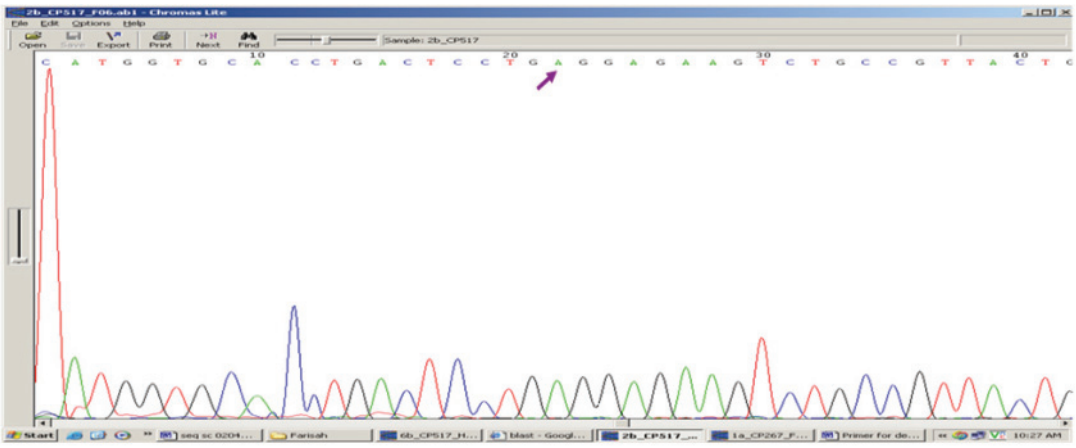

FIG. 3: DNA analysis sequence electropherogram of a normal sample. The arrow indicates the same position in codon 6 of β-globin gene as compared to figure 2.

screening for sickle cell was not routinely practiced. The clinical presentations were also similar where veno-occlusive disease and tissue infarction predominate. Laboratory features of Hb SS may differ slightly from Hb S/β^0. Anaemia in Hb SS is more severe, the MCV and MCH are higher than Hb S/β^0 and Hb A2 is normal as compared to Hb S/β^0.[6] However there is considerable overlap in the homozygous HbS and compound heterozygous Hb S/β^0 diagnoses that render the distinction more difficult and thus the need for molecular diagnosis. In this patient, her initial full blood count showed normochromic, normocytic anaemia and her haemoglobin electrophoresis revealed Hb S, Hb F and Hb A2 bands with Hb A2 quantification of 3%, consistent with homozygous Hb SS. The DNA analysis however, was still performed because her sickling tests were repeatedly negative and a complete family study was not possible since her mother's blood was not available. Furthermore, compound heterozygosity between sickle and β^0-thalassaemia (Hb S/β^0) should also be excluded by molecular methods for a confirmatory diagnosis.

Polymerase chain reaction (PCR) based techniques such as allele specific amplification (ASA) have been reported to be accurate and reliable.[7,8] Conventionally, ASA involves the use of two separate tubes, each containing a common primer and either the primer for wild type (WT) or mutant (MUT) respectively. Interpretation of the results involves comparison of the ASA PCR products of the two reactions simultaneously to detect for the presence or absence of the target mutation. More recently, a single tube adaptation was developed by Waterfall and Cobb, where two sets of primers (WT-AS and WT-CP517; MUT-AS and MUT-CP267) were used to amplify the normal β-globin and the mutant β^S globin genes respectively.[3] The WT outer primer (WT-CP517) was positioned at 517 bp downstream on the opposite strand of WT-AS, while the mutant outer primer (MUT-CP267) was at 267 bp upstream of MUT-AS[2]. The WT-AS and MUT-AS primers were designed in opposing directions, such that both primers would terminate at the mutation site. WT-AS primer had a single A nucleotide at the 3'-end, matching the T nucleotide on the non-coding strand of the normal β-globin gene. MUT-AS primer had a single A nucleotide at the 3'-end, matching the T base on the coding strand of the sickle cell gene. The A:A or T:T mismatches of the WT-AS:sickle cell gene sequence and MUT-AS:normal β-globin gene sequence respectively were completely refractory to amplification by PCR under optimized conditions. This allowed rapid detection of heterozygous and homozygous sickle cell disease status in a simple single reaction for each sample.[2]

We employed the primer design of the described method[2] on the samples of our patient and her family to confirm the diagnosis This analysis has successfully demonstrated the presence of the sickle cell mutations in the samples tested. The patient's PCR product showed presence of only one band at position 267 bp indicating homozygosity for HbSS, while her heterozygous father and two sisters had two bands at positions 267 and 517 bp.

Although a compound heterozygosity for Hb S/β^0 would also give a similar result to that of HbS carrier because the DNA amplification

target for this method is specific only for the HbS mutation, this method could still enable the distinction of HbSS and Hb S/β^0 .

Determination of zygosity of sickle cell allele was also made possible by this method. In the analysis result, the heterozygotes (HbAS) showed the presence of both the wild-type and the mutant alleles, on the other hand the homozygote (HbSS) and the normal control (HbAA) only showed the mutant and the normal alleles respectively.

The primer designed by Waterfall was expected to amplify the whole target region to act as an internal control but his PCR reaction was unable to amplify this large amplicon because the HotStart enzyme used in the PCR reaction was less efficient at amplifying larger fragments.[2] Similarly, we were also unable to amplify this large region of the target sequence. However, in our opinion this was immaterial since each successful reaction would definitely result in a PCR product, either wild-type or mutant, to act as an internal control for that particular reaction. In contrast to the conventional ASA, an internal control PCR product, normally at a site away from the target sequence is compulsory in each reaction to signify a negative reaction.

In conclusion, the bi-directional ASA for a single tube genotyping of the SNP responsible for sickle cell anaemia is a rapid, reproducible technique and easy to perform.

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