

# Detection of beta-globin gene mutations among Kelantan Malay thalassaemia patients by polymerase chain reaction restriction fragment length polymorphism

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## ABSTRACT

**Introduction:** Beta-thalassaemia major is an autosomal recessive disorder that results in severe microcytic, hypochromic, haemolytic anaemia among affected patients. Beta-thalassaemia has emerged as one of the most common public health problems in Malaysia, particularly among Malaysian Chinese and Malays. This study aimed to observe the spectrum of mutations found in Kelantan Malay beta-thalassaemia major patients who attended the Paediatrics Daycare Unit, Hospital Universiti Sains Malaysia, Kelantan, Malaysia, the data of which was being used in establishing the prenatal diagnosis in this Human Genome Centre.

**Methods:** This was a cross-sectional study conducted with 35 Kelantan Malay beta-thalassaemia major patients. DNA was extracted from the blood collected from the patients and subjected to polymerase chain reaction (PCR) amplification. Six restriction enzymes were used to digest the PCR products for the detection of mutations.

**Results:** Five out of the six beta-globin gene defects were detected, namely, IVS-I nt5 (G > C), IVS-I nt1 (G > T), codon 26 (G > A), codon 41–42 (4 bp del) and codon 19 (A > G). The mutation which was not observed in this study was in codon 15 (G > A). The two most common mutations observed were codon 26 (G > A) and IVS-I nt5 (G > C), which was detected in 26 and 17 patients, respectively. Two patients did not show any of the six mutations.

**Conclusion:** Our results added to the existing data on the common beta-globin gene defects in Kelantan Malay beta-thalassaemia patients.

**Keywords:** beta thalassaemia, genetic disease,  $\beta$ -globin gene, haemolytic anaemia, mutations, polymerase chain reaction, restriction fragment length polymorphism, thalassaemia

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## INTRODUCTION

The thalassaemias are a group of anaemia that results from a genetic defect which reduces the rate of synthesis of normal globin chains. The thalassaemias are among the most common genetic disorders worldwide, occurring frequently in the Indian subcontinent, Southeast Asia and West Africa.<sup>(1)</sup> Recently,  $\beta$ -thalassaemia (BT) has emerged as one of the most common public health problems in Malaysia, particularly among Malaysian Chinese and Malays.<sup>(2)</sup> Thalassaemia is classified according to the chain of the globin molecule that is affected. BT major is an autosomal recessive disorder that results in severe microcytic, hypochromic, haemolytic anaemia among affected patients.<sup>(3)</sup> BT is known to occur due to the mutations in the  $\beta$ -globin gene (HBB) on chromosome 11.<sup>(4)</sup> To date, there are over 200 known mutations in HBB found to be associated with thalassaemia, but it is believed that each population has its own spectrum of mutations.<sup>(5)</sup>

Molecular characterisation of HBB mutation among the Chinese and Malays in Malaysia has been previously reported,<sup>(6,7)</sup> where the presence of nine mutations in HBB were confirmed; viz. IVS-1 nt5 (G > C), IVS-1 nt1 (G > T), codon 15 (G > A), codon 26 (G > A), the mutation which leads to HbE, codon 19 (A > G), codon 41–42 (4 bp del), codon 17 (A > T), -28 (A > G) and IVS2-654 (C > T). However, this study focused on six BT mutations which are believed to occur commonly among the Malay ethnic group.<sup>(8)</sup> This study aimed to observe the spectrum of mutations found in BT major patients who attended the Paediatrics Daycare Unit, Hospital Universiti Sains Malaysia, Kelantan, the data of which is being used in the establishment of prenatal diagnosis in this Human Genome Centre. Our results added to the existing data on

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**Table I. The number and percentage of mutations found in 35 Kelantan Malay beta-thalassaemia patients.**

Types	Beta-thalassaemia mutations																			
	IVS-1 nt5 (G > C)				Codon 15 (G > A)				IVS-1 nt1 (G > T)				Codon 26/HbE (G > A)				Codon 19/HbMalay (A > G)			
	N	H	M	%	N	H	M	%	N	H	M	%	N	H	M	%	N	H	M	%
HbE	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
HbE/B-Thal (n = 32)	14	11	3	26.6	—	—	—	—	8	6	2	15.6	26	8	18	68.8	2	2	—	3.1
BTM (n = 3)	3	3	—	50.0	—	—	—	—	1	—	1	33.3	—	—	—	—	—	—	—	—

HbE: patients with haemoglobin E; HbE/B-Thal: patients who are compound heterozygous HbE and B-Thal; BTM: beta-thalassemia major (homozygous B-Thal); n: total number of patients; N: number of patients positive for that particular type of mutation; H: heterozygous; M: homozygous mutant; %: allele frequencies of that particular mutation.

the most common mutations of HBB existing among the Malay ethnic group in Kelantan, Malaysia.

## METHODS

This was a cross-sectional study and was approved by Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia. 35 Kelantan Malay patients diagnosed to have BT major and requiring a regular blood transfusion were included in this study. Informed consent was obtained from their parents prior to blood collection. All patients were interviewed to complete a questionnaire. Blood samples were later sent to the Human Genome Centre, Universiti Sains Malaysia, for molecular analysis.

DNA was extracted from the blood using a commercially-available DNA extraction kit, QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) based on restriction endonucleases that recognise naturally-occurring or PCR-generated restriction sites was applied to detect the BT mutations. Three sets of primers were used to amplify the exon 1, exon 2 and a part of exon 1 of HBB that created a recognition site for *AluI* in the presence of the codon 19 (A > G) mutation. The reaction mixture for PCR amplification consisted of ~100 ng DNA template, 1× PCR buffer (PE Applied Biosystems), 1.875 mM MgCl<sub>2</sub> (PE Applied Biosystems), 0.375 mM dNTPs (PE Applied Biosystems), 0.5 μM of each primer and 1 unit of AmpliTaq Gold DNA Polymerase (PE Applied Biosystems).

PCR grade water was added to a final volume of 20 μL. The PCR conditions were as follows: 96°C of pre-denaturation for 5 mins, 95°C of denaturation for 60 s, annealing at 66°C (exon 1), 65°C (exon 2) and 62°C (exon 1 for *AluI* enzyme) for 1 min and 72°C of extension (1 min 30 s) for 40 cycles, followed by 72°C of final extension for 7 mins in the Eppendorf Mastercycler Gradient (Eppendorf, Germany). Amplicons were then detected by gel electrophoresis in a 2% agarose gel. Subsequently, the PCR product that was obtained from the PCR amplification

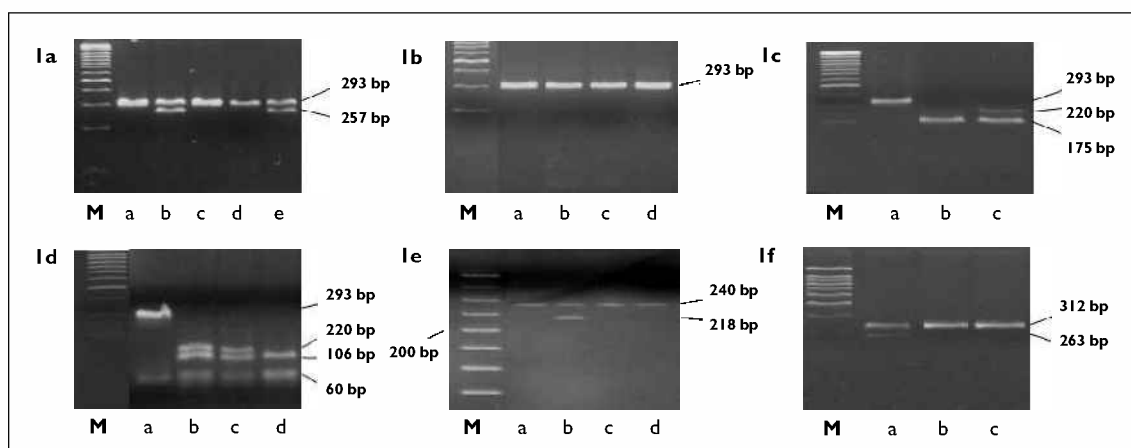
was subjected to restriction enzyme digestion. PCR primers set and restriction enzymes as suggested by Pramoonjago et al were used in this PCR-based restriction fragment length polymorphism (PCR-RFLP) method.<sup>(9)</sup>

## RESULTS

PCR amplification successfully generated 293 bp, 240 bp and 312 bp of DNA fragments from the exon 1 region, exon 1 region with special site for enzyme *AluI* and exon 2 region, respectively. Amplicon (293 bp) was used for the detection of IVS-1 nt5 (G > C), IVS-1 nt1 (G > T), codon 26/HbE (G > A) and codon 15 (G > A), and amplicon (312 bp) was used for codon 41–42 (4 bp del) mutation detection. The results revealed five mutations in the HBB in Kelantan Malay BT patients; viz. IVS-1 nt5 (G > C), IVS-1 nt1 (G > T), codon 26/HbE (G > A), codon 41–42 (4 bp del) and codon 19/Hb Malay (A > G) (Fig. 1). The two most common mutations observed were codon 26 (G > A) and IVS-1 nt5 (G > C). The mutation at codon 26/HbE (G > A) was observed in 26 patients, and other mutation IVS-1 nt5 (G > C) was observed in 17 patients. The remaining mutations, IVS-1 nt1 (G > T), codon 41–42 (4 bp del) and codon 19/Hb Malay (A > G) were detected in nine, four and two patients, respectively. The allele frequencies of mutant allele for the five mutations detected are shown in Table I. Two Malay patients in the present study, however, did not show any mutation in the six loci studied.

## DISCUSSION

BT major is one of the most common single gene disorders in the multiracial population in Malaysia.<sup>(8)</sup> The heterozygous carriers of BT in Malaysia are about 4.5% among the Malay and Chinese populations,<sup>(10)</sup> and have clearly emerged as one of the most common public health problems in Malaysia. The World Health Organisation has highlighted the importance of characterisation of the spectrum of BT mutations as one of the ways for community control of BT;<sup>(10)</sup> thus, characterisation of the patients in this study is essential for the patient



**Fig. 1** Gel electrophoresis profile of the detection of beta thalassaemia mutations by PCR-RFLP analysis. (a) Lane a – undigested PCR product (293 bp); Lanes b and e – heterozygous mutations (IVS-1 nt5 G>C); Lanes c and d – normal subjects (restriction enzyme-Cac81); (b) Lane a – undigested PCR product (293 bp); Lanes b, c and d – normal subjects (Codon 15 G>A) (restriction enzyme-sfcl); (c) Lane a – undigested PCR product (293 bp); Lane b – normal subjects; Lane c – heterozygous mutations (IVS-1 nt1 G>T) (restriction enzyme-Bs/I); (d) Lane a – undigested PCR product (293 bp); Lanes b and c – heterozygous mutations (Codon 26/HbE G>A); Lane d – normal subjects (restriction enzyme - Mn/I); (e) Lane a – undigested PCR product (240 bp); Lane b – heterozygous mutations (Codon 19/HbMalay A>G); Lanes c and d – normal subjects (restriction enzyme - *AluI*); (f) Lane a – heterozygous mutations (Codon 41–42 4bp del); Lane b – undigested PCR product (312 bp); Lane c – normal subjects (restriction enzyme - *TaqI*).

management in this country. This study has enriched the current database.<sup>(6,7)</sup> The distribution of BT mutations among the Kelantan Malays was close to the distribution of BT mutations in Thais and Malays of southern Thailand and Malays of West Malaysia. In addition, single base substitution (A > G) in codon 19 could only be detected within these populations.<sup>(6)</sup> Therefore, we planned to compare our findings with the spectrum of BT mutations that has previously been reported among Kelantan Malays and as well as in other populations.

Seven common mutations have been identified among individuals originating from several provinces in southern Thailand, viz. codon 41–42 (4 bp del), IVS1-nt5 (G > C), codon 19 (A > G), codon 17 (A > T), IVS1-nt1 (G > T), -28 (A > G) and 3.5 kb deletion, which accounted for about 91.5% of all mutations.<sup>(11)</sup> A study conducted by Abdullah et al revealed that HbE was the most common mutation among Kelantan Malays, followed by IVS1-nt5 (G > C).<sup>(6)</sup> The occurrence of both mutations was in agreement with our findings in this study. In addition, the mutation that was found only among Kelantan Malays, codon 19 (A > G), as reported by Abdullah et al, was also detected among the present study subjects. Although two patients did not show any of the six mutations, it may be likely that they carry other types of mutations of this gene.

The earlier study conducted by Abdullah et al to characterise the spectrum of BT mutations among Malays in Kelantan used allele specific priming and direct sequencing method.<sup>(6)</sup> In the current study, a simpler method was applied by using PCR-RFLP to

detect the BT mutations in the same population. PCR-RFLP proved to be a reliable and easy assay in identifying the BT mutations and could detect mutations in 90% of the Malaysian population,<sup>(9)</sup> making this method superior to the previous methods of dot blot or reverse dot blot hybridisation and amplification refractory mutation system, which require more experienced hands, specialised laboratories and certain conditions suitable to the method itself, thus making them less reproducible in less advanced laboratories.<sup>(12)</sup> In addition, this assay does not involve any allele-specific PCR amplification or hybridisation which is prone to non-specific reactions that could lead to false-positive results. Even though this method has been shown to be effective in detecting mutations, it cannot yet be used to detect the presence of unknown mutations. Also, the complexity of the human genome may make it difficult to develop this method as it may produce several restriction sites within a certain sequence that we wish to amplify.

The molecular characterisation of BT mutations can be considered as a stepping stone in identifying the spectrum of mutations in a population. The results from this study add to the existing data on the spectrum of BT mutations among Kelantan Malay patients, although a larger number of patients would provide a more accurate representation of the spectrum of mutations. The characterisation of BT mutations in Kelantan Malays will help to bolster the establishment of a rapid and effective prenatal diagnosis programme, or genetic counselling in this ethnic group in future as one of the effective ways to control the prevalence of BT cases.

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