# Original Article Multiplex amplification refractory mutation system (MARMS) for the detection of β-globin gene mutations among the transfusion-dependent β-thalassemia Malay patients in Kelantan, Northeast of Peninsular Malaysia

Sarifah Hanafi<sup>1</sup>, Rosline Hassan<sup>2</sup>, Rosnah Bahar<sup>2</sup>, Wan Zaidah Abdullah<sup>2</sup>, Muhammad Farid Johan<sup>2</sup>, Noor Diana Rashid<sup>1</sup>, Nurul Fatihah Azman<sup>1</sup>, Ariffin Nasir<sup>1</sup>, Syahzuwan Hassan<sup>3</sup>, Rahimah Ahmad<sup>3</sup>, Azizah Othman<sup>1</sup>, Mohd Ismail Ibrahim<sup>4</sup>, Surianti Sukeri<sup>4</sup>, Sarina Sulong<sup>5</sup>, Surini Yusoff<sup>1</sup>, Nor Sarwany Mohamad<sup>1</sup>, Adil Hussein<sup>6</sup>, Rozita Hassan<sup>7</sup>, Narazah Yusoff<sup>3</sup>, Badrul Hisyam Yahaya<sup>8</sup>, Endom Ismail<sup>9</sup>, Nik Khairuddin Nik Yussof<sup>10</sup>, Sinari Salleh<sup>11</sup>, Bin Alwi Zilfalil<sup>1</sup>

<sup>1</sup>Department of Pediatrics, School of Medical Sciences, Universiti Sains Malaysia, Malaysia; <sup>2</sup>Department of Hematology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia; <sup>3</sup>Hematology Unit, Cancer Research Centre, Institute for Medical Research, Kuala Lumpur; <sup>4</sup>Department of Community Medicine, School of Medical Sciences, Universiti Sains Malaysia, Malaysia; <sup>5</sup>Human Genome Center, School of Medical Sciences, Universiti Sains Malaysia, Malaysia; <sup>6</sup>Department of Ophthalmology, Universiti Sains Malaysia, Malaysia; <sup>7</sup>School of Dental, Universiti Sains Malaysia, Malaysia; <sup>8</sup>Regenerative Medicine Cluster, Advanced Medical & Dental Institute, Universiti Sains Malaysia, Malaysia; <sup>9</sup>School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Malaysia; <sup>10</sup>Department of Pediatrics, Hospital Raja Perempuan Zainab II, Kota Bharu Kelantan, Malaysia; <sup>11</sup>Department of Medical, Hospital Kuala Krai, Kelantan, Malaysia

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Abstract: The aim of this study was to adapt MARMS with some modifications to detect beta mutation in our cohort of thalassemia patients. We focused only on transfusion-dependent thalassemia Malay patients, the predominant ethnic group (95%) in the Kelantanese population. Eight mutations were identified in 46 out of 48 (95.83%) beta thalassemia alleles. Most of the patients (54.2%) were compound heterozygous with co-inheritance Cd 26 (G>A). The frequencies of spectrum beta chain mutation among these patients are presented in Table 2. Among the transfusion dependent beta thalassemia Malay patients studied, 26 patients were found to be compound heterozygous and the main alleles were Cd 26 (G>A). Compound heterozygous mutation of Cd 26 (G>A) and IVS 1-5 (G>C) were 12 (46.2%), Cd 26 (G>A) and Cd 41/42 (TTCT) were 9 (34.6%), Cd 26 (G>A) and IVS 1-1 (G>C) were 2 (7.7%) respectively. Meanwhile the minority were made of a single compound heterozygous of Cd 26 (G>A) and Cd 71/72, Cd 26 (>A) and Cd 17 (A>T), Cd 26 (G>A) and -28 (G>A) respectively. Twenty out of forty six patients were shown to have homozygous of IVS 1-5 (G>C) were 2 (10.0%), Cd 26 (G>A) were 15 (75.0%), Cd 19 (A>G) were 1 (5.0%), and IVS 1-1 (G>T) were 2 (10.0%). The beta chain mutations among the Kelantanese Malays followed closely the distribution of beta chain mutations among the Thais and the Malays of the Southern Thailand. The G-C transition at position 5 of the IVS 1-5 mutation was predominant among the Malay patients. In conclusion, this method has successfully identified the mutation spectrum in our cohort of transfusion-dependent beta thalassemia patients, and this method is equally effective in screening for mutation among thalassemia patients.

Keywords: MARMS-PCR, β-globin gene, thalassemia, Malay, mutation

#### Introduction

In Malaysia, seven  $\beta$ -thalassemia mutations are responsible for the majority of  $\beta$ -thalassemia in the Malays and the Chinese ethnic groups. These include the  $\beta$ -mutations at -28 TATA Box, codon 17, codon 41-42 and IVS 2-654 among the Malaysian Chinese [1, 2]. In the Malaysian Malays, the common mutations responsible for the majority of the beta thalassemia are codon 26 (HbE), IVS 1-1 and IVS 1-5 [3].

Various molecular techniques have been developed to characterize these mutations. The choice of the detection protocol used in the molecular characterization and prenatal diagnosis in each region depends on the diversity of the ethnic groups and types of  $\beta$ -thalassemia mutations present in the region [4, 5].

The ARMS technique for detecting known point mutations was first described by Newton et al. [6]. It has been developed for the diagnosis of all the common  $\beta$ -thalassemia mutations found in all the main ethnic groups [7]. Many researchers conducted their studies by using this technique and it is proven that this method is suitable for its rapid, simple, cost-effective, and safe (non-radioactive method) for detection of most common  $\beta$ -mutation worldwide [8]. The other technique includes reverse dot-blot, which requires the construction of biotinylated probe spotted onto a membrane, and followed by PCR and hybridization of amplicon onto the labelled membrane. The probe construction and hybridization are time consuming, whereas the MARMS-PCR only requires targeted PCR amplification and gel electrophoresis of the amplicon. Although reverse dot-blot is available commercially, the method is generally expensive and not suitable for routine test, as well as limited access to laboratories with limited funds.

The technique involves multiplex different PCR of five MARMS-A, B, C, D, E and one ARMS-F. Different specific primers are used for each type of MARMS (Details as described in the methodology part). Six regions of interest within the β-globin gene in a single reaction containing a common oligoprimer and either normal or mutant oligonucleotides corresponding to a few common mutations such as IVS-1 nucleotide 1 or IVS-1 nucleotide 5, codon 26, and IVS-2 nucleotide 654 regions will be detected. Multiple primers are chosen so that the sizes of the six PCR products are different, thereby facilitating the detection on agarose gels following the amplification process. This approach simplifies mutation detection and is promising for automation by employing fluorescent-tagged primers. In addition to the design of these systems, type of mutation, frequency of the mutation in our population, and whether the mutation's amplicon size is separable by gel electrophoresis should be considered in order to adopt MARMS. By using this method, we could detect 20 different mutations of the β-globin gene, followed by direct sequencing for mutation confirmation for those with negative results.

The aim of this study was to adapt MARMS with some modifications to detect beta mutation in our cohort of thalassemia patients. We only focused on transfusion-dependent thalassemia Malay patients, which is the predominant ethnic group (95%) in the Kelantanese population [9].

# Materials and methods

# Subjects

A total of 48 transfusion-dependent patients, comprising HBE/B thalassemia and ß thalassemia major, were randomly selected. There were 27 females and 21 males with the age ranged from 9 to 26 years old. Six milliliter of whole blood samples were collected in EDTA bottle after the informed consent was obtained from each individual or parents if they were younger than 18 years old. The protocols of this study were approved by the Human Research Ethics Committee, Universiti Sains Malavsia (FWA Reg. No: 00007718; IRB Reg. No: 00004494) and ethics committee of the Ministry of Health, Malaysia. The molecular test was performed at Hospital Universiti Sains Malaysia (HUSM) and Instituted Medical Researcher (IMR), Kuala Lumpur.

# DNA extraction

DNA was extracted using QiAmp® DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). The kit was used according to the manufacturer's instructions. The extracted genomic DNA was used as a template and was kept at 4°C until further use.

# Multiplex amplification refractory mutation system

The mutations within  $\beta$ -globin gene were detected using 5 different multiplex ARMS-PCR and one single ARMS-PCR reaction. For MARMS-A, B, C and D, 4 types of mutations in each multiplex system were examined, whereas in MARMS-E, 3 types of mutations were examined followed by 1 mutation in ARMS-F (**Table 1**). The primers for DNA amplification were synthesized by First BASE Laboratories Sdn Bhd. The oligonucleotide primers to detect all these mutations and two internal controls with 861 bp and 493 bp used in this study have been described previously by Syahzuwan et al., [10]. All samples were analyzed simultaneously with positive controls for a particular mutation.

Multiplex	List of mutation	Amplicon size (bp)
MARMS-A	Internal control	861
	CD 41/42 (-TTCT)	476
	IVS 1-5 (G>C)	319
	CD 26 (G>A)	301
	CD 17 (A>T)	275
MARMS-B	Internal control	861
	CD 71/72 (+A)	569
	IVS 1-1 (G>T)	315
	CD 8/9 (+G)	250
	-28 (A>G)	145
MARMS-C	Internal control	861
	CD43 (G>T)	482
	Poly A (A>G)	393
	IVS1-1 (G>A)	315
	CD 16 (-C)	273
MARMS-D	Internal Control	861
	-88 (C>T)	369
	Initiation codon	248
	Cd 15 (G>A)	203
MARMS-E	Internal Control	861
	-86 (C>G)	367
	CAP +1 (A>C)	173
	Codon 19 (A>G)	173
ARMS F	IVS 2-654	826
	Internal codon	493

**Table 1.** List of mutations for each MultiplexARMS-PCR

The positive control used in this study was from the IMR, which have been confirmed and validated by sequencing method.

#### Polymerase chain reaction

DNA amplification was carried out using HotStarTaq Plus Master Mix kit (1X PCR Buffer, 1.5 MM MgCl<sub>2</sub>, HotStarTaq, DNA polymerase and 200 UM of each DNTP) (Qiagen GmbH, Hilden, Germany) and 0.1 ug of genomic DNA in a total volume of 20 ul. The PCR consisted of 30 cycles at 94°C for 45 s, 64°C for 45 s, and 72°C for 90 s, followed by a final extension step at 72°C for 7 min. All primers were annealed at 64°C except for IVSII-654, which was annealed at a lower temperature (60°C). Amplified DNA was visualized after electrophoresis in 3% agarose, followed by FloroSafe DNA staining.

# Genomic sequencing

2.2 kb fragment of the whole  $\beta$ -globin gene was amplified by a set of forward and reverse prim-

ers namely 833/1338 and PAA respectively, followed by purification. The amplicon integrity was in the range of 1.8-2.0 of 260/280 wavelength. Based on the results, those which were not detected by MARMS were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems Division, USA). The sequencing was performed by First BASE Laboratory Sdn Bhd (Selangor, Malaysia).

#### Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 20.0 (IBM SPSS Statistics 20, U.S government) was used for data entry and data analysis. All the data was double checked and prepared properly whilst being documented. The Pearson Chi-square test (crosstabs) and Fisher exact test were applied to the different types of mutation between homozygous and compound heterozygous respectively. The results were presented as frequency (n) and percentage (%). Fisher exact test was used for categorical variables with 2×2 contingency tables, and Pearson Chi-square ( $\phi$ 2) was selected for statistical comparison of each categorical data, if not more than 20% of cell with expected frequencies less than 5: Pearson Chisquare was valid. Whereas fisher exact test was used if more than 20% of cell with expected frequencies should more than 5, Fisher exact test was valid.

# Results

A total of eight mutation types were identified in forty six out of forty eight (95.83%) transfusiondependent beta thalassemia alleles. All samples were analyzed simultaneously with positive controls for a particular mutation (**Figure 1**). All the positive controls used in this study were obtained from the IMR, which have been confirmed and validated by sequencing method.

In this study, 26 patients with heterozygous mutation and 20 patients with homozygous were determined. Cd 26 (G>A) HbE was the most common beta chain mutation defect which accounted for 84.78% of the transfusion-dependent beta thalassemia among Malay patients in Kelantan.

Most of the patients (54.2%) were compound heterozygous Cd 26 Hb E/beta-thalassemia. The frequencies of spectrum beta chain muta-



MARMS-PCR β-globin gene Malay thalassemia patient

**Figure 1.** Agarose gel for MARMS A-PCR. Lane 1 is 100 bp marker; lane 2 is positive control for Cd 41/42 (-TTCT); lane 3 is positive control for IVS 1-5 (G>C); lane 4 is positive control for Cd 26 (G>A); lane 5 is positive control for Cd 17 (A>T); lane 6 is negative control; lane 7 sample patient 1 positive for compound heterozygous Cd 41/42 (-TTCT) and Cd 26 (G>A); lane 8 and 12 are sample patient 2 and 6 both are negative for any listed mutation in MARMS A; lane 9 is sample patient 3 and lane10 is sample patient 4, both are positive IVS 1-5 (G>C); lane 11, 14, 15 are sample patient 5, 8, 9 are positive for compound heterozygous Cd 26 (G>A) and IVS 1-5 (G>C); lane 13 is sample patient 7 positive Cd 26 (G>A).

tion among these patients are presented in Table 2. Among the transfusion dependent beta thalassemia Malay patients studied, 26 patients were found to be compound heterozygous and the main alleles were Cd 26 (G>A). Compound heterozygous mutation of Cd 26 (G>A) and IVS 1-5 (G>C) were 24 (92.3) and 12 (46.2%) respectively. Compound heterozygous mutation of Cd 26 (G>A) and IVS 1-5 (G>C) were 12 (46.2%), Cd 26 (G>A) and Cd 41/42 (TTCT) were 9 (34.6%), Cd 26 (G>A) and IVS 1-1 (G>C) were 2 (7.7%) respectively. Meanwhile the minority were made of a single compound heterozygous of Cd 26 (G>A) and Cd 71/72, Cd 26 (>A) and Cd 17 (A>T), Cd 26 (G>A) and -28 (G>A) respectively. Twenty out of forty six patients were homozygotes. Fifteen (75.0%) of the homozygous patients were Cd 26 (G>A), 2 (10.0%) IVS 1-5 (G>C), 2 (10.0%) IVS 1-1 (G>T), and 1 (5.0%) Cd 19 (A>G) respectively.

Two patients that were not detected by MAR-MS were further investigated by direct sequencing. Interestingly, from the sequencing result of one patient, three single nucleotides polymorphism were observed. One of these polymorphisms was reported in several Mediterranean populations. They were IVSII-74 T>G (**Figure 2**), rs1609812 (NG\_000007.3.g.7170-5C>T.C.316-285C>T and rs10768683 (NG\_-000007.3.g.71055G>C C.315+16G>C). On the other hand, there were no mutation and single nucleotide polymorphism detected in another patient.

Based on the genotype, patients can be categorized into 4 different groups. The groups were TM ( $b^{0}/b^{0}$ ), TI ( $b^{0}/b^{+}$  or  $b^{+}/b^{+}$ ), HbE/b thal (HbE/b<sup>0</sup> or HbE/b<sup>+</sup>), and HbE homozygosis HbE/HbE). Within this classification, 15 patients were determined as HbE homozygosis (HbE/HbE), 13 patients as HbE/b thal (HbE/b<sup>0</sup>), 11 patients as HbE/b thal (HbE/b<sup>+</sup>), 5 patients as TI( $b^{+}/b^{+}$ ), and the remaining 2 patients as TM ( $b^{0}/b^{0}$ ).

Turne of Mutation	Homozygous	Compound Heterozygous	Total n (%)	P-value
Type of Mutation	(n=46)	(n=46)		
	n (%)	n (%)		
IVS 1-5 (G>C)				
No	18 (90.0)	14 (53.8)	32 (69.6)	0.008ª
Yes	2 (10.0)	12 (46.2)	14 (30.4)	
CD 26 (G>A)				
No	5 (25.0)	2 (7.7)	7 (15.2)	0.114 <sup>b</sup>
Yes	15 (75.0)	24 (92.3)	39 (84.8)	
CD19 (A>G)				
No	19 (95.0)	24 (92.3)	43 (93.5)	0.599 <sup>b</sup>
Yes	1 (5.0)	2 (7.7)	3 (6.5)	
IVS 1-1 (G>T)				
No	18 (90.0)	24 (92.3)	42 (91.3)	0.590 <sup>b</sup>
Yes	2 (10.0)	2 (7.7)	4 (8.7)	
CD 41/42 (-TTCT)				
No	20 (100.0)	17 (65.4)	37 (80.4)	0.003ª
Yes	0 (0.0)	9 (34.6)	9 (19.6)	
CD 71/72 (+ A)				
No	20 (100.0)	25 (96.2)	45 (97.8)	0.565 <sup>b</sup>
Yes	0 (0.0)	1 (3.8)	1 (2.2)	
CD 17 (A>T)				
No	20 (100.0)	25 (96.2)	45 (97.8)	0.565 <sup>b</sup>
Yes	0 (0.0)	1 (3.8)	1 (2.2)	
-28 (A>G)				
No	20 (100.0)	25 (96.2)	45 (97.8)	0.565⁵
Yes	0 (0.0)	1 (3.8)	1 (2.2)	

Table 2. Types of homozygous and compound heterozygous mutations for transfusion-dependent  $\beta$ -thalassemia among Malay patients

<sup>a</sup>Pearson Chi-Square was applied. <sup>b</sup>Fisher Exact test was applied.

#### Discussion

The frequently detected mutations found in this study were closely related with the previous study [2, 10-12]. The main alleles mutation found in our cohort of transfusion-dependent beta thalassemia were Cd26 (G>A), Cd 41/42 (-TTCT) and IVS 1-5 (G>C). IVS II-74 (T>G) commonly found in Mediterranean populations affected two alleles in this study. This defect could be due to the intermarriage. Therefore unexpected mutations and nucleotide polymorphisms can now be included in the existing Malaysian spectrum to devise a better strategy in detecting  $\beta$  defects in a cost-efficient manner in the area [2]. This variant was detected using DNA sequencing performed by First BASE Laboratory Sdn Bhd (Selangor, Malaysia).

The molecular of this heterogeneity represents strong evidence for the recurrent and metacentric origins of the mutation. In this study, the majority of the patients were compound heterozygous mutation, where both alleles defected instead of one allele. This result is significantly different from the previous study, and it could be due to the subject of the study. The current study only focuses on the transfusion-dependent beta thalassemia among Malay patients.

β-globin genotypes were systematically analyzed and characterized according to the previous report. CD19, CD26 (HbE), -29, -28, Cap +1, -30, Poly A and Initiation CD defects were categorized as mild mutation (mild b<sup>+</sup>), IVS 1-5 and IVS 2-654 as severe intermediate type (severe b<sup>+</sup>), whereas CD 41/42, IVS 1-1, Cd 17, CD 8/9, IVS 1-1 (G>A), CD 37, IVS 1-2, CD 35, and Cd 71/72 were categorized as severe (b°) [13-15]. Normally mild disease and nontransfusion in our study consisted of 15 patients, which means 1/3 out of 46 patients (32%) who initially had homozygous HBE disease but then became transfusion dependent. Therefore, there must be some other factors that contributed to the severity of the disease.

Carrier screening in Southeast Asia revealed a high frequency of β-thalassemia ranging from 3-10% in Indonesia, 0.5-1.5% in Myanmar, 0.93% in Singapore, 1.5% in Vietnam, and 3-9% in Thailand [13]. Previously, only Cd 26 (A>G) and IVS 1-5 (G>C) were reported as common among the Malays. The high frequencies of Cd 26 (A>G) HbE and IVS 1-5 (G>C) were closely similar to an earlier finding in Indonesia [16]. The higher frequency of Cd 26 (G>A) in this study was also relatively similar to that neighboring countries [18-21]. Cd 41/42 (-TTCT), the mutation that was commonly found in the Chinese population, was also found with higher frequency in this study. All this relativeness genetic mutation could be due to the history of human migration and admixture that cause large scale of intermarriage and integration



**Figure 2.** Sequencing analysis of Intron 2 transfusion dependent beta thalassemia patient showing IVSII-74 T>G (Rs7480526) single nucleotides polymorphism at beta nt 569 T>G, HGVS nomenclature HBB: c.315+74T>G.

between them and the Malays. This phenomenon has been explained in detail as described in a previous report [22].

The justification for the focus on the Malay patients is because of their unique genetic structure. The existence of the Chinese and the Indian in the Malay Peninsula with different timelines throughout the centuries brought varying degree of genetic mixture to the Malay population [22]. Prior to British colonization, the Chinese and the Indian traders had established strong trading links with the Malay Peninsula, which caused the large scale of intermarriage and integration between them and the Malays [23]. In addition, the presentday Malays of the Malay Peninsula are described as Deutro-Malays, which are the descendants of the proto-Malays who had admixed with Siamese, Javanese, Sumatran, Indian, Thai, Arab and Chinese traders [24]. Interestingly, it is believed that the genetic structure of the Malay Peninsula population is related together with aboriginal people [22]. Hence, it is interesting to know the spectrum of beta globin mutation among this population, as it is noted that different populations consist of different mutations with their own unique [25].

ARMS is a rapid and direct molecular technique in which  $\beta$ -thalassemia mutations are visual-

ized immediately after DNA amplification by gel electrophoresis [7]. It has been developed for the diagnosis of common β-thalassemia mutations found in all main ethnic groups. The method is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is present within the sample. The application of the MARMS method to 48 cases of transfusion-dependent B-thalassemia among Malay patients in this study revealed a specific mutation in 46 cases (95.83%), which means the method is highly sensitive. This proved that the method is only slightly less sensitive than that of the laborious DNA sequencing technique. DNA sequencing is an accurate technique, but it is more time-consuming and expensive compared to the MARMS.

In this study, a robust, accurate, simple, speedy and cost-effective multiplex amplification refectory mutation system technique has been successfully set up to access 20 known mutations in  $\beta$ -globin chain region, namely Cd 26 (G>A), IVS 1-5 (G>C), IVS 1-1 (G>T), Cd 19 (A>G), Cd 41/42 (-TTCT), -28 (A>G), cap +1 (A>C), initiation Cd (AT>AGG), Cd 8/9 (+G), Cd 15 (TGG>TAG), Cd 17 (A>T), Cd 43 (G>T), Cd 71/72 (+A), IVS 1-1 (G>A), Cd 16 (-C), Poly-A (AATAA-AATAGA), -86 (C>G), -88 (C-T), -29 (A>G) and IVS 2-654. The methods were minimally modified from those of the previous MARMS assays described in Syahzuwan et al. 2013. The optimization of specific amplification for  $\beta$ -globin chain mutations were carried out by the manipulation of annealing temperature and the duration of initial denaturation and extension. The amplified products were electrophoresed in 3% agarose gel, followed by FloroSafe DNA stained at 70 V for 1 h.

In conclusion, this method has successfully identified the mutation spectrum in our cohort of transfusion-dependent beta thalassemia patients, and this method is equally effective in screening for mutation among thalassemia patients.

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# Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bin Alwi Zilfalil, Department of Pediatrics, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia. Tel: 09-7676531; Fax: 09-7676543; E-mail: zilfalil@ usm.my

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