β-globin Gene Mutations Among β-thalassemia and β-variant patients in a teaching Hospital in Universiti Sains Malaysia, Kubang Kerian, Kelantan

Universiti Sains Malaysia, Kubang Kerian, Kelantan'daki Bir Eğitim Hastanesinde β-talasemi ve β-varyant hastaları arasında β-globin Gen Mutasyonları

Syahzuwan Hassan[1], Zefarina Zulkafli[2,3], Rozieyati Mohamed Saleh[4], Mohd Nazri Hassan[2,3], Rahimah Ahmad[1]
Wan Zaidah Abdullah[2,3]

[1]Hematology Unit, Cancer Research Centre, Institute for Medical Research (IMR), National Institutes of Health (NIH), Block C, No.1, Jalan Setia Murni, U13/52 Seksyen U13, Setia Alam, 40170, Shah Alam, Selangor
[2]Department of Hematology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[3]Hospital Universiti Sains Malaysia, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[4]School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

ABSTRACT

Background: Thalassemia is one of the most common single gene disorders worldwide with hundreds of mutations involving beta (β)-globin gene alone. Nevertheless, each ethnic population has its own unique type of mutations. The aim of this study is to characterize types of β-globin gene mutations among β-thalassemia patients in a teaching hospital; Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia.
Methods: Hematological profile for each patient was studied, and this was followed by screening and characterizations of β-globin mutations using multiplex amplification-refractory mutation system (ARMS)-PCR. In addition, direct DNA sequencing was also performed for the selected number of samples. Multiplex ARMS-PCR was carried out on 102 patient samples taken from three different ethnic groups: Malays, Chinese and Thais.
Results: This study finding indicated that majority of the patients were found to be heterozygous for Hb E (47.5%), heterozygous for β-thalassemia (31.3%) and homozygous for Hb E (7.5%). Heterozygous Hb Malay and compound heterozygous β-thalassemia/Hb E were observed at the equivalent frequency of 5.0%. Other genotypes include compound heterozygous β-thalassemia/ Hb Malay and compound heterozygous Hb E/ Hb Malay. However, 22 cases remain uncharacterized. Overall, 12 types of mutations were successfully identified which were depicted as 16 different genotypes. Cd 26 (G-A) mutation was found to be the most observed anomaly in this study.
Conclusion: In conclusion, together with complete red cell indices as well as information on ethnic background, the multiplex ARMS-PCR can be an effective tool in dissecting specific regional mutations of the β-globin gene particularly in multi-ethnic populations of Malaysia.

Keywords: Multiplex ARMS-PCR, β-globin gene mutations, thalassemia.

ÖZET

Arka plan: Talasemi, yalnızca beta (β)-globin genini içeren yüzlerce mutasyonla dünya çapında en yaygın tek gen hastalıklarından biridir. Bununla birlikte, her etnik popülasyonun kendine özgü mutasyon tipi vardır. Bu çalışmanın amacı, Hastane Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malezya'daki bir eğitim hastanesindeki β-talasemi hastalarında β-globin gen mutasyonlarının tiplerini karakterize etmektir.
Yöntemler: Her hasta için hematolojik profil çalışıldı ve bunu, multipleks amplifikasyon-refrakter mutasyon sistemi (ARMS)-PCR kullanılarak β-globin mutasyonlarının taranması ve karakterizasyonları takip etti. Ek olarak, seçilen örnek sayısı için doğrudan DNA dizilimi de yapıldı. Malaylar, Çinliler ve Taylandlılar olmak üzere üç farklı etnik gruptan alınan 102 hasta numunesi üzerinde multipleks ARMS-PCR uygulandı.
Bulgular: Bu çalışma bulgusu hastaların çoğunluğunun Hb E için heterozigot (%47,5), β-talasemi için heterozigot (%31,3) ve Hb E için homozigot (%7,5) olduğunu göstermiştir. Heterozigot Hb Malay ve bileşik heterozigot β-talasemi/Hb E, %5,0'lik eşdeğer frekansta gözlendi. Diğer genotipler, bileşik heterozigot β-talasemi/ Hb Malay ve bileşik heterozigot Hb E/ Hb Malay'ı içerir. Bununla birlikte, 22 vaka tanımlanmamıştır. Genel olarak, 16 farklı genotip olarak gösterilen 12 tip mutasyon başarıyla tanımlandı. Bu çalışmada en çok gözlenen anomalinin Cd 26 (G-A) mutasyonu olduğu tespit edildi**.**
Sonuç: Tam kırmızı hücre indeksleri ve etnik geçmişe ilişkin bilgilerle birlikte multipleks ARMS-PCR, özellikle Malezya'nın çok etnikli popülasyonlarında β-globin geninin spesifik bölgesel mutasyonlarını incelemede etkili bir araç olabilir.

Anahtar Sözcükler: Multipleks ARMS-PCR, β-globin gen mutasyonları, talasemi

Received: 07.19.2020 **Accepted:** 01.26.2021 **Geliş Tarihi:** 19.07.2020 **Kabul Tarihi:** 26.01.2021

ORCID IDs: S.H.0000-0002-9886-504X,Z.Z.0000-0003-2029-2234,R.M.S.0000-0002-3817-0932,M.N.H.0000-0003-4975-2038,R.A.0000-0002-1809-1479,W.Z.A.0000-0002-4008-6772

Address for Correspondence / Yazışma Adresi: Zefarina Zulkafli,MD Department of Hematology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. E-mail: zefarinazulkafli@gmail.my

doi:http://dx.doi.org/10.12996/gmj.2022.51

INTRODUCTION

Thalassemia hemoglobinopathies represent a major public health problem in many areas around the world, which classically described to be concentrated in tropical and subtropical countries (1). However, due to mobility and migration flows, the prevalence of thalassemia is also found to be high in countries with diverse ethnicity, high immigrant rate and in a society where consanguineous marriage are still widely practise (2). The disease is believed to affect 300,000 children annually and predicted to reach 900,000 by the year 2025 (3,4). It is well established that the frequency and prevalence of thalassemia vary from one country to another, even within different regions in a country. This scenario is known as ethnic-specific, in which each ethnic has its own unique spectrum of β-thalassemia mutations (5). Despite hundreds of β-globin gene mutations that have been described globally, fortunately only a few common mutations and several rare variants occur in each population (6,7).

Malaysia is a multi-ethnic country with a population of over 30 million, comprising of Malays (62%), 21% Chinese, 6% Indians and the remaining 1% constitutes of other minority ethnics (8). It is estimated that about 4.5% of the Malaysians are heterozygous for β-thalassemia, and carrier couples are at risk of producing a thalassemia major offspring (9). As of 2017, there were 7,509 registered thalassaemia patients, with 35% of these patients were transfusion dependent (10). In Malaysia, identifications and characterisations of β-thalassemia mutations have been systematically delineated since the mid-80s and molecular diagnostic approach of the local populations is progressively being studied and published (11- 14).

Geographically, Peninsular Malaysia shares its north border with Thailand, south with Singapore and west maritime border with the Indonesia Island of Sumatera. Kelantan state which is in the north-eastern corner of Peninsular Malaysia is bordered by Narathiwat Province of Thailand. Unlike any other states, it has most Malays populations (> 93%) (8). As such, inter-racial marriages with the southern Thais are common and the criss-crosses border has existed as long as history's been recorded. The recent phylogenetic study has also revealed that Malay Kelantan formed a distinct clade with genetical differences from the other Malay sub-ethnics and with a limited link with populations from the Indonesian archipelago (15).

For these reasons, it is fundamental to have effective strategies to understand the spectrum of β-thalassaemia based on the current regional situation. It is also imperative to know and understand different types of β-thalassaemia anomalies occurring within the Kelantanese population so that a cost-effective approach can be designed to characterize these mutations. This study reported screening and compilation of β-globin mutations data in Kelantan which can be useful for future health management to reduce the number of thalassemia major patients in this region.

MATERIALS and METHODS

Patient Selection

This is a cross-sectional study, where a total of 102 patients attended Hospital Universiti Sains Malaysia (HUSM); teaching hospital in Kubang Kerian, Kelantan, Malaysia was enrolled in the period of January 2009 to December 2010. Subjects were walk-in patients with various medical conditions that were found to have abnormal blood cells, either by RBC indices or Hb electrophoresis, mostly with MCV < 76 fL, MCH < 27 pg and Hb A2 > 2.9%. Two milliliters (2 mL) of peripheral venous blood was collected with Ethylenediaminetetraacetic acid (EDTA) tube after the informed consent was obtained from each individual. This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg. No.: 00007718; IRB Reg. No.: IRB00004494). The patient's ethnicity was recorded to determine the sequence of the Multiplex amplification-refractory mutation system (ARMS)-PCR test. The molecular tests were carried out at the Institute for Medical Research (IMR), Malaysia.

DNA Extraction

The DNA was extracted using QIAamp DNA Mini Kits® (QIAGEN, Hilden, Germany), according to the manufacturer's instruction. The extracted genomic DNA was used as a template and was kept at 4°C until further use. The amount and purity of DNA were quantified using NanoDrop ND-1000 UV-VIS Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Mutation Identification & Analysis - DNA Amplification Using Multiplex ARMS-PCR and ARMS-PCR Twenty (20) different mutations within the β-globin gene were detected using five sets of multiplex ARMS-PCR as previously described (12). Two internal controls (861 bp and 493 bp) were used. All samples were analysed simultaneously together with positive controls for a particular mutation.

DNA amplification was carried out in Eppendorf Mastercycler pro-S (Eppendorf AG, Hamburg, Germany), using HotStarTaq® Master Mix; (1X PCR Buffer, 1.5 mM $MgCl_2$, HotStarTaq® DNA Polymerase and 200 µM of each dNTP) (Qiagen GmbH, Hilden, Germany). For each PCR reaction, 100 ng of genomic DNA was used with a final volume of 20 µL. The thermal cycling conditions were set-up as described earlier (12). The resultant PCR products were electrophoresed with 3.0 % Bioline™ Multipurpose Agarose gel in 1X TBE buffer, followed by 15 minutes staining with Ethidium bromide (EtBr). The gel was visualized using transilluminator (Vilber Lourmat, Marne-la-Vallée, France). Confirmation methods: Reverse Dot-Blot Hybridization & Direct DNA Sequencing

Reverse Dot-Blot Hybridization

Commercially available reverse dot-blot hybridization of β-Globin StripAssay SEA™ (ViennaLab Diagnostics GmbH, Vienna, Austria) which covers 22 different types of mutations was used for mutation confirmations following the manufacturer's recommendations. Biotinylated primer products were detected using streptavidin-alkaline phosphatase and colour substrates.

Direct DNA Sequencing

Direct DNA sequencing was carried out on 4 samples out of 76 subjects with mutation detected by Multiplex ARMS-PCR: 1 sample with cd 26 mutation but having moderate anemia and another 3 samples with mutations such as Poly A (A-G) and IVS 1-1 (G-A) but not detectable using reverse dot-blot hybridization technique. Direct DNA sequencing was also performed on 12 out of 26 subjects without mutation detected by multiplex ARM-PCR. The 12 subjects were selected because having HbA2 of 3.4% and above. The sequencing was performed by First Base Laboratory Sdn. Bhd. (Selangor, Malaysia). The whole region of β-globin gene was amplified by a set of primers namely 833/1338 and PAA Reverse that generated a 2020 bp amplicon. The amplicon was subjected to sequencing by primer walking using 5 sequence segments: 833/1338, PAA Reverse, CA, PC0 R, and PC07 F. The oligonucleotide primers were synthesized by First Base Laboratories (Selangor, Malaysia). Purification and sequencing were performed by First Base Laboratories (Selangor, Malaysia). The sequencing data were analysed using CLC Bio Main Workbench (Aarhus, Denmark). The sequence data were compared to reference β-globin sequence (National Center for Biotechnology Information, NCBI Reference Sequence: NM_000518) exported from NCBI.

RESULTS

Multiplex and a single ARMS-PCR were used in this study to characterize β-globin gene mutations in a small cohort of the Kelantanese population. A total of 102 suspected β-thalassemia and β-variant patients were selected based on their hematological profiles (Table 1). Most of the patients enrolled were Malays (91.2%), followed by seven Chinese (6.9%) and two Thais (2.0%) (Table 2).

Table 1: Summary of Full Blood Counts and Hemoglobin Analysis

Parameter	Value
No. of Subjects Enrolled (n)	102
No. of Ethnics (n)	3 Malays: 93 (91.2%) Chinese: 7 (6.9%) Thais: 2 (2.0%)
[+]**Mean Hb (**g/dL)	10.8 ± 4.3
[+]**Mean Red Blood Count** (10^{12}/L)	5.1 ± 2.1
[+]**Mean MCH** (pg)	21.6 ± 5.3
[+]**Mean MCV** (fL)	67.1 ± 15.2
RDW (range - %)	11.8 – 35.5
Hb A2 (range - %)	2.1 –8.1
Hb F (range - %)	0.3-79.2

[+]Mean ± 2SD. Abbreviation: Hb: hemoglobin; MCH: mean cell hemoglobin; MCV: mean cell volume; RDW: red cell distribution width.

In general, out of 102 patients enrolled, mutation was detected in 80 cases; 75 cases by multiplex ARMS-PCR, 4 cases (heterozygous IVS 1-2 (T-C)) were detected by direct sequencing and 1 case noted to have mutation Cd 26 and Cd 38 (ACC>-CC) which has been detected by multiplex ARMS-PCR and direct sequencing, respectively. In this study 12 types of β-globin gene mutation were detected; 67 were identified as carriers, six were found to be homozygous for Hb E and four patients were identified as compound heterozygous Hb E/β-thalassemia (Table 2). Also, anomalies such as compound heterozygous β-thal / Hb Malay and Hb E/ Hb Malay recorded a single case and two cases respectively. However, genotyping for 22 patients were not able to be resolved using this test algorithm.

Further analysis revealed that the most common β-gene mutation in our study cohort was Cd 26 (G-A)/Hb E that reached almost half from the total mutations characterized (Table 2). This was followed by mutations in IVS-I-5 (G-C) and homozygous of Cd 26 (G-A) with the prevalence of 11.3% and 7.5% respectively. The remaining top six of β-globin defects include Cd 19 (A-G), Cd 8/9 (+G) and IVS 1-2 (T-C) that recorded the equivalent occurrence of 5%. Other heterozygous compounds such as Cd 26 (G-A), Cd 41/42 (-TCTT), CD 26 (GAG>AAG), CD 38 (ACC>-CC), as well as IVS 1-1 (G-T) and Cd 19 (A-G) were rarely found in our cohort with cases of <2%.

Table 2: Distribution of β-globin Gene Mutations Detected among β-thalassaemia and β-variant Patients in HUSM

	Types of β mutation	**HGVS nomenclature**	**Clinical classification**	**Hb level g/dl (range)**	**Ethnicity**			[*]**Total number** (n)	[#]**Prevalence** (%)
					Malay	**Thais**	**Chinese**		
	Cd 26 (G-A)	HBB:c.79G>A	thalassemia minor	9.8-11.4	38			38	47.5
	-28 (A-G)	HBB:c.-78A>G	thalassemia minor				1	1	1.3
	Cd 17 (A-T)	HBB:c.52A>T	thalassemia minor				2	2	2.5
	Cd 41/42 (-TTCT)	HBB:c.126_129delCTTT	thalassemia minor		2		1	3	3.8
	Cd 8/9 (+G)	HBB:c.27dupG	thalassemia minor		4			4	5.0
	IVS 1-1 (G-T)	HBB:c.92+1G>T	thalassemia minor	9.8-11.8	1			1	1.3
	IVS 1-2 (T-C)	HBB:c.92+2T>C	thalassemia minor		4			4	5.0
	IVS 1-5 (G-C)	HBB:c.92+5G>C	thalassemia minor		9			9	11.3
	IVS 2-654 (C-T)	HBB:c.316-197C>T	thalassemia minor		1			1	1.3
	Cd 19 (A-G)	HBB:c.59A>G	thalassemia minor		4			4	5.0
	Cd 41/42 (-TCTT) / Cd 26 (G-A)	HBB:c.126_129delCTTT / HBB:c.79G>A	thalassemia intermedia to major	6.5-10.0	1			1	1.3
Compound heterozygous β thal/ Hb Malay	Poly A (A-G) / Cd 26 (G-A)	HBB:c.*111A>G/ HBB:c.79G>A			2			2	2.5
	CD 38 (ACC>-CC) / Cd 26 (G-A)	HBG2:c.115A>C/ HBB:c.79G>A			1			1	1.3
	IVS 1-1 (G-T) / Cd 19 (A-G)	HBB:c.92+1G>T/ HBB:c.59A>G			1			1	1.3
	Cd 26 (G-A) / Cd 19 (A-G)	HBB:c.79G>A/ HBB:c.59A>G			2			2	2.5
	Cd 26 (G-A)/ Cd 26 (G-A)	HBB:c.79G>A/ HBB:c.79G>A	thalassemia intermedia	8.8-10.1	4	2		6	7.5
Total					74	2	4	80	100

[*] Only samples that were successfully characterised were taken into considerations, i.e. (102 screening cases – 22 uncharacterised = 80 cases characterised)

Based on overall 80 cases that were successfully characterized.

The details analysis of the carrier's status revealed that majority of the carriers were traced to be heterozygous for Hb E (47.5%), with nearly one-third (30.5%) of patients were carrying different variants of β-thalassemia traits and four patients were identified to be heterozygous of Cd 19 (A-G) Hb Malay.

If the data were stratified according to the ethnicity and based on the cases that have been successfully characterized, half of the Chinese patients (4 out 2) in this study were typed to be heterozygous Cd 17 (A-T), while a total of two patients were found to have -28 (A-G) and Cd 41/42 (-TTCT). The latter mutation was also observed in 2.7% (2 out of 74) in Malay ethnic. Meanwhile, both Thais patients that were recruited in this study were identified as homozygous Cd 26 (G-A), which was also observed in Malays (5.4%, 4 out of 74). Furthermore, in Malay ethnic, the two most common mutations of the β-globin gene were Cd 26 (G-A) and IVS 1-5 (G-C) with the prevalence of 47.5% and 11.3% respectively. The remaining β-thalassemia trait and variants were observed in the range of 1.3-5.0%.

In addition, the β-Globin StripAssay SEA™ was also performed to confirm the above Multiplex ARMS-PCR findings. The results showed 100% concordance with the Multiplex ARMS-PCR (data not shown). Regarding 22 cases with no mutation detected: the Hb level range between 10-11.3 g/dl and HbA2 level range between 2.9-3.1%.

DISCUSSION

The reported prevalence of β-globin gene mutations varies considerably from region to another and was found to be ethnic specific (5). As such, this study was undertaken to characterize β-globin gene mutations in a selected cohort of the Kelantan population, where most of its residence is Malay ethnic (8). Furthermore, giving the geographical location of Kelantan itself, which is bordered by Thailand, it is interesting to observe if there is any impact of gene flow on the local spectrum of β-thalassemia mutations. This will provide useful information regarding the frequency distribution of such mutations and subsequently enabling better strategies for the management of the disease.

In this study, 102 walk-in HUSM patients suspected of having β-thalassemia or its variants based on their hematological and hemoglobin profiles were enrolled. Patients' blood samples were molecularly characterised using multiplex ARMS-PCR as previously described (12). Additionally, all these findings were being validated using a commercially available test; β-globin strip. The results were found to be 100% in agreement with each other. Direct DNA sequencing was also performed on subjects with HbA2 of 3.4% and above. Overall, 78% of the cases have been molecularly characterized. Twelve different mutations were successfully identified which were distributed as 16 different genotypes, either as single or as combinations of mutations. Multiplex ARMS-PCR identified 10 of these mutations, while the other two anomalies were discovered by direct sequencing. Genotyping for 22 patients were not able to be resolved using this test algorithm. This can be explained by the specificity of the primers used which only enable to detect a specific set of given mutations. After this, any uncharacterized mutations should ideally be followed by the Multiplex ligation-dependent probe amplification (MLPA) study on HBB and HBA genes for the big deletion/duplication and gene dosage changes to exclude complex thalassemia as well as next generation sequencing (NGS) technique. However, given the cost of these techniques, mass screening of samples is usually not favourable.

This study indicated that the most common β-globin mutation was heterozygous Cd 26 (G-A) or also known as Hb E, occurring at the percentage of 47.5, with all carriers were from the Malay ethnic. This finding is consistent with previous studies carried out nationally (12, 16). In addition, 7.5% of our cohort was also identified to be homozygous for this genotype. In fact, homozygous Hb E was the third most frequently observed genotype in our list after the IVS 1-5 (G-C). The Hb E disease in this study was traced to four Malays as well as to both of our Thai patients. Given the geographical location of our cohort, it is anticipated to observe this mutation sharing between Malay Kelantanese and its neighbouring Thais. The previous study has shown that the mutation spectrum of the Kelantan population was almost equivalent to those with the Southern Thailand (17).

Globally, Hb E is found to cause one-half of all severe cases of β-thalassemia (4). High frequency is reported mainly in Northeast India18 and in the Southeast Asia region, particularly in the 'Hb E triangle' borders of Thailand, Cambodia, and Vietnam where its prevalence can reach up to 50–60% (19). Hb E allele (G > A), a base substitution of lysine for glutamic acid in cd 26, can induce cryptic splice site, hence producing an abnormal messenger RNA splicing process, resultant in the reduced rate of β-chain of Hb E (βE) synthesis (20). This usually gives rise to a mild phenotype form of β-thalassemia. However, co-inheritance of Hb E with other variants (α and β) have been shown to cause a wide range of clinical syndromes with varying severity (1). This study has also revealed of such interactions wherein a total of four cases of compound heterozygous HbE / β-thal were recorded (prevalence of 5%). This includes mutations with Cd 26 (G-A) & poly A (2 cases) and a single case for Cd 26 & Cd 41/42 (-TCTT) and Cd 26 & Cd 38 (ACC>-CC).

The second most frequently observed mutation in this study was heterozygous IVS 1-5 (G-C) which accounted for just over 10% and was only found in the Malay ethnic. This mutation together with Hb E (G-A) and Hb Malay (A-G) have been shown to constitute > 73% of β-thalassemia in Malaysian Malays (21). The change from G-C at nucleotide 5 of the IVS-1 greatly reduces the efficiency of splicing of the normal 5' splicing site and combination with other β-thalassemia polymorphisms will give rise to variable pathogenic conditions (22). The IVS 1-5 mutation has also been reported to be common elsewhere (23, 24). Interestingly, in Thailand, this polymorphism was found to be four times higher in the southern Thais Muslim population compared to the central region which was believed to be spilled over from Malaysia (25).

Other predominant mutations in this study include heterozygous Cd 19 (A-G) Hb Malay, heterozygous Cd 8/9 (+G) and heterozygous IVS 1-2 (T-C) that were observed an equivalent frequency of 5%. All these mutations were traced to Malay patients. As stated earlier, Hb Malay is already established as one of the most common anomalies observed in this ethnic (21). Furthermore, our data also revealed different variants of Hb Malay namely compound heterozygous β-thal / Hb Malay and compound heterozygous Hb E / Hb Malay recorded at 1.3% and 2.5% respectively. Other anomalies identified were the heterozygous Cd 8/9 (+G). This frameshift mutation is phenotypically expressed as a mild form in heterozygous condition and chiefly associated with the Asian-Indians origin (26). Locally, this mutation has also been described in a Kedayan individual (12).

Three mutations namely Cd 17 (A-T), -28 (A-G) and Cd 41/42 (-TTCT) were observed in Chinese patients, with the mutation rates of 2.5%, 1.3%, and 3.8%, respectively. This finding concurs well with the previously published data related to the mutation spectrum in Chinese Malaysian (21, 27). Beyond this study, the mentioned anomalies were also reported to be prevalent in Thailand population and in certain parts of China (28, 29). This is expected as the Chinese Malaysians were historically originated from the Southern regions of China (30). Interestingly, although the genotypic of Cd 41/42 (-TTCT) is normally prominent among the Chinese, yet we also noted that this mutation was also shared between Malays and Chinese patients with double and a single case, respectively. Likewise, Yatim and colleagues (2014) have also observed the same in some of their Malay Penang patients (31). This demonstrates the heterogeneity of β-thalassemia and its variants which may be attributed to the assimilation of living in multi-ethnic society and miscegenation among the races.

CONCLUSION

We have successfully identified 12 different β-thalassemia mutations in the selected Kelantanese cohort, and the findings were compared with the allelic distribution in the general Malaysian population and outside. Our data have explicitly shown how multiple genes can contribute to the complex genotypic and phenotypic, rendering the importance of molecular diagnosis. This regional mutation rate is beneficial for prognostic information for the establishment of more specific and effective molecular characterisation, confirmation of diagnosis, treatment, prenatal and counselling program for a specific community.

Conflict of interest
No conflict of interest was declared by the authors.

Acknowledgements
This work was supported by the Short-Term grant schemes [Grant no: 304/PPSK/61312038] from Universiti Sains Malaysia. We would like to thank Puan Selamah Ghazali for her help in carrying out this study and the management of the Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan for granting the permission to the investigators to use patients' medical record; space and assets belong to the hospital during the process of conducting the research.

REFERENCES

1.Higgs DR, Engel JD, and Stamatoyannopoulos G. Thalassaemia. *Lancet*. 2012; 379 (9813): 373-83.
2.De Sanctis V. β-thalassemia: Distribution in the old world: A historical standpoint of an ancient disease. Mediterr J Hematol Infect Dis. 2017; 9 (1).
3.Modell B. and Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull. World Health Organ. 2008; 86: 480-7.
4.Vichinsky EP. Changing patterns of thalassemia worldwide. Ann N Y Acad Sci. 2005; 1054: 18-24.
5.Thein SL. Beta-thalassaemia. Baillieres Clin Haematol. 1998; 11 (1): 91-126.
6.Patrinos GP, Giardine B, Riemer C, Miller W, Chui DHK, Anagnou N, et al. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations
for population and sequence variation studies. Nucl Acids Res. 2004; 32 (Database issue): D537-41.
7.Steinberg MH, Forget BG, Higgs DR, and Weatherall DJ. Disorders of hemoglobin: Genetics, pathophysiology, and clinical management. 2nd. ed. New York. USA: Cambridge University Press; 2009. 323 p.
8.Department of Statistics Malaysia. Population Quick Info 2016 [cited 2017 August 1]. Available from: https://www.dosm.gov.my/.
9.George E. Beta-thalassemia major in Malaysia, an ongoing public health problem. Med J Malaysia. 2001; 56 (4): 397-400.
10. Pharmacy Practice & Development Division Ministry of Health. Protocol thalassaemia medication therapy adherence clinic (TMTAC). 2018. 1st ed.
11. George E. Beta-thalassaemia mutations in West Malaysia: a new thalassaemia clinical score system. Ann Acad Med Singapore. 1994; 23 (1): 89-93.
12. Hassan S, Ahmad R, Zakaria Z, Zulkafli Z, and Abdullah WZ. Detection of beta-globin gene mutations among beta-thalassaemia carriers and patients in Malaysia:
Application of multiplex amplification refractory mutation system-polymerase chain reaction. Malays J Med Sci. 2013; 20 (1): 13-20.
13. Abdullah WA, Jamaluddin NB, Kham SK and Tan JA. The spectrum of beta-thalassemia mutations in Malays in Singapore and Kelantan. Southeast Asian J Trop Med Public Health. 1996; 27 (1): 164-8.
14. Tan KL, Tan JA, Wong YC, Wee YC, Thong MK and Yap SF. Combine-ARMS: a rapid and cost-effective protocol for molecular characterization of beta-thalassemia in Malaysia. Genet Test. 2001; 5 (1): 17-22.
15. Hatin WI, Nur-Shafawati AR, Zahri MK, Xu S, Jin L, et al. Population genetic structure of Peninsular Malaysia Malay sub-ethnic groups. PloS one. 2011; 6 (4): e18312.
16. George E and Khuziah R. Malays with thalassaemia in West Malaysia. Trop Geogr Med. 1984; 36 (2): 123-5.
17. Rozitah R, Nizam MZ, Nur Shafawati AR, Nor Atifah MA, Dewi M, Kannan TP, Ariffin N, Norsarwany M. et al. Detection of beta-globin gene mutations among Kelantan Malay thalassaemia patients by polymerase chain reaction restriction fragment length polymorphism. Singapore Med J. 2008; 49 (12): 1046-9.
18. Sikdar M. Hemoglobin E in Northeast India: A review on its origin, distribution, migration and health implication. Anthropological Review. 2016; 79 (3): 241-63.
19. Fucharoen S and Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin. 1987; 11 (1): 65-88.
20. Orkin SH, Kazazian HH, Jr., Antonarakis SE, Ostrer H, Goff SC and Sexton JP. Abnormal RNA processing due to the exon mutation of beta E-globin gene. Nature. 1982; 300 (5894): 768-9.
21. George E and Ann TJ. Genotype-phenotype diversity of beta-thalassemia in Malaysia: treatment options and emerging therapies. Med J Malaysia. 2010; 65 (4): 256-60.
22. Treisman R, Orkin SH and Maniatis T. Specific transcription and RNA splicing defects in five cloned beta-thalassaemia genes. Nature. 1983; 302 (5909): 591-6.
23. Bang-Ce Y, Hongqiong L, Zhuanfong Z, Zhengsong L and Jianling G. Simultaneous detection of alpha-thalassemia and beta-thalassemia by oligonucleotide microarray. Haematologica. 2004; 89 (8): 1010-2.
24. Baysal E. Molecular basis of beta-thalassemia in the United Arab Emirates. Hemoglobin. 2011; 35 (5-6): 581-8.
25. Laosombat V, Fucharoen SP, Panich V, Fucharoen G, Wongchanchailert M, Sriroongrueng W. et al. Molecular basis of beta thalassemia in the south of Thailand. Am J Hematol. 1992; 41 (3): 194-8.
26. Old J. Thalassemias and Abnormal Hemoglobins of the Indian Subcontinent. In: Kumar D, editor. Genomic in Medicine & Health: Indian Subcontinent: Oxford Universit Press Inc.; 2012.
27. Tan JA, Chin PS, Wong YC, Tan KL, Chan LL and George E. Characterisation and confirmation of rare beta-thalassaemia mutations in the Malay, Chinese and Indian ethnic groups in Malaysia. Pathology. 2006; 38 (5): 437-41.
28. Mirasena S, Shimbhu D, Sanguansermsri M and Sanguansermsri T. Detection of beta-thalassemia mutations using a multiplex amplification refractory mutation system assay. Hemoglobin. 2008; 32 (4): 403-9.
29. Yin A, Li B, Luo M, Xu L, Wu L, Zhang L, et al. The prevalence and molecular spectrum of alpha- and beta-globin gene mutations in 14,332 families of Guangdong Province,China. PloS One. 2014; 9 (2): e89855.
30. Lockard CA. Chinese migration and settlement in Southeast Asia before 1850: Making fields from the sea. History Compass. 2013; 11 (9): 765-81.
31. Yatim NF, Rahim MA, Menon K. Al-Hassan FM, Ahmad R, Manocha AB, et al. Molecular characterization of α- and β-thalassaemia among Malay patients. Int J Mol Sci.2014; 15 (5): 8835-45.