β -globin gene cluster mutations and large deletions among anaemic patients with elevated HbF level in a tertiary teaching hospital in Kelantan, Malaysia

Siti Nor Assyuhada Mat Ghani^a, Rozieyati Mohamed Saleh^a, Wan Suriana Wan Ab Rahman^{c,d},
Mohd Nazri Hassan^{b,d}, Wan Zaidah Abdullah^{b,d}, Maryam Azlan^a, Zefarina Zulkafli^{b,d*}

^aSchool of Health Sciences, Universiti Sains Malaysia Health Campus, 16150 Kota Bharu, Kelantan

^bSchool of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kota Bharu, Kelantan

^cSchool of Dental Sciences, Universiti Sains Malaysia Health Campus, 16150 Kota Bharu, Kelantan

^dHospital USM, Universiti Sains Malaysia Health Campus, 16150 Kota Bharu, Kelantan

Received 22nd October 2020 / Accepted 14th December 2020

Abstract. Mutations in the β -globin gene cluster can lead to β -thalassaemia, $\delta\beta$ -thalassaemia, hereditary persistence of foetal haemoglobin (HPFH) and some of the haemoglobin variants. The clinical and haematological spectrum of thalassaemia range from benign to pathogenic conditions including severe transfusion dependent thalassaemia. Therefore, it is important to accurately diagnose β -globin gene cluster mutations to prevent thalassaemia major or intermedia offspring. The objective of this study is to detect β -globin gene cluster mutations and large deletions among anaemic patients with elevated HbF level in a tertiary teaching hospital in Kelantan, Malaysia. This study involved 144 anaemic patients with HbF level $>1.0\%$. High-Performance Liquid Chromatography (HPLC) was used to determine the HbF and HbA₂ levels. Multiplex-ARMS (ARMS)-PCR and gap-PCR were performed for those patients with high HbA₂ level ($>3.2\%$) and normal HbA₂ level ($\leq 3.2\%$) to detect β -globin gene cluster mutations and large deletions respectively. The majority of patients were Malays (99.3%) with mean age of 19.99 ± 1.64 years and female 61.1% predominance. Out of 87 samples tested using multiplex ARMS-PCR against eight targeted single mutation; Cd 41/42, IVS 1–5, Cd 26, Cd 17, Cd 71/72, IVS 1–1, Cd 8/9 and -28 mutations, 65 (74.7%) patients were detected which comprises of Cd 26 (56.3%), Cd 41/42 (11.5%), compound Cd 26 and Cd 41/42 (3.4%) and IVS 1–1 (3.4%). Meanwhile, for multiplex gap-PCR which detect four types of large deletions; Thai ($\delta\beta$)^o-thalassaemia, HPFH-6, Siriraj J and Hb Lepore, one out of 57 patients (1.8%) was found positive with Thai ($\delta\beta$)^o-thalassaemia. There was a significant difference between the mean of HbF level, MCV level as well as MCH level of patients with and without β -globin gene mutations and large deletions ($P < 0.05$). This study highlighted the presence of various types of β -globin gene cluster mutations detection in establishing a definitive diagnosis among this selected group of patients for the large-scale screening of the thalassaemia gene.

Keywords: Anaemia, ARMS-PCR, β -globin gene, HbF level, gap-PCR

INTRODUCTION

To date, numerous mutations affect the β -globin gene cluster with most cases being single nucleotide substitutions also known as single point mutations, insertions, as well as short

deletions (Thein, 2011). Deletions and/or mutations of various sizes and positions within the β -globin gene cluster are responsible for certain clinical disorders, namely hereditary

*Author for correspondence: Dr. Zefarina Zulkafli, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kota Bharu, Kelantan, Malaysia.
Email – zefarinazulkafli@gmail.com

persistence of foetal haemoglobin (HPFH), β -thalassaemia, sickle cell anaemia, HbE disorder, and delta beta ($\delta\beta$)-thalassaemia (Carrocini *et al.*, 2011). Sickle cell anaemia and β -thalassaemia mutations are characterised by significant elevations of both HbF and HbA₂ levels while HPFH and $\delta\beta$ -thalassaemia mutations are characterised by elevations of HbF level and a normal or decreased HbA₂ level (Galanello, 2013).

In a routine clinical setting, HbA₂ level of more than 4.0% indicates β -thalassaemia or severe β -thalassaemia while in mild β -thalassaemia, HbA₂ level is usually more than 3.6%. Fucharoen *et al.* (2002) observed that HPFH-6 and Thai ($\delta\beta$)^o-thalassaemia patients have high HbF (>5%) and normal HbA₂ (2.2%) levels. However, patients are considered to have β -globin gene cluster mutations when the HbF level is more than 1.0%, HbA₂ level is above 3.2%, and/or show the presence of other abnormal haemoglobin (Koh *et al.*, 2017). Therefore, in this study, samples with high HbF level (>1.0%) and HbA₂ level (>3.2%) were chosen to detect β -globin gene cluster mutation while samples with high HbF level (>1.0%) and normal HbA₂ level ($\leq$ 3.2%) were used to detect β -globin gene cluster large deletion.

β -thalassaemia is a heterogenous group of autosomal recessive disorders that cause a hereditary insufficient synthesis of β -globin chain in haemoglobin (Thein, 2011). World Health Organization (WHO) reported that approximately 1.5% of global population are carriers of β -thalassaemia and 17% of 330 000 affected infants are born each year with various thalassaemia syndromes (Roth *et al.*, 2018). Database of Hb variants and thalassaemia mutations have been recorded that there are more than 800 mutations involving β -globin gene cluster (Patrinos *et al.*, 2004). It was reported that the carriers for β -thalassaemia among the Malaysian population are 4.5% which are commonly found in Malays and Chinese (George *et al.*, 2001). Malaysia is a multi-ethnic country comprising three major ethnic groups consist of Malays, Chinese, and Indians along with other minor ethnic groups, which include *Orang Asli*.

Each ethnic group in Malaysia dominates its own specific set of mutations (Tan *et al.*, 2006). According to Tan *et al.* (2004), Cd 26 or HbE is usually found among the Malays by 76.0%. Another study showed that HbE is the most

observed β -globin mutation by 84.8% in transfusion-dependent β -thalassaemia Malay patients in Kelantan (Hanafi *et al.*, 2014). Furthermore, HbE is common among Malays with 5% of carrier rate. *Orang Asli* in Peninsular Malaysia were identified to have high prevalence of HbE (George, 2013). This is supported by a study conducted on the Senoi group of *Orang Asli*, where HbE is the most common mutation with a rate of 18.6% (Koh *et al.*, 2017). Globally, individuals with HbE/ β -thalassaemia comprised almost 50% of those affected with thalassaemia (Olivieri *et al.*, 2011).

β -globin genes cluster large deletion is the elimination of the δ and/or β genes leaving one or both γ genes. This leads to the occurrence of β -thalassaemia, HPFH, $\delta\beta$ -thalassaemia, Hb Lepore, and $\gamma\delta\beta$ -thalassaemia (Galanello, 2013). Deletions that eliminate parts or entire of the β -globin gene are classified as β -thalassaemia deletions. Meanwhile, deletions that eliminate the δ - and β -globin genes are termed $\delta\beta$ -thalassaemia, and this is often associated with moderate increases in HbF due to the expression of both γ -globin genes at high level to balance the absence of β -globin production. Individuals with HPFH/ β -thalassaemia may have mild anaemia but are clinically asymptomatic (Thein, 2011).

In Asia, approximately 12 types of large deletions in the β -globin gene cluster have been identified within the Southeast Asian populations. These include Siriraj J (~118 kb deletion), Southeast Asian (SEA) (~27 kb deletion), Filipino (~45 kb deletion), Hb Lepore (~7.4 kb deletion), Thai ($\delta\beta$)^o-thalassaemia (~12.5 kb deletion), Chinese G γ (A $\gamma\delta\beta$)^o-thalassaemia (~100 kb deletion), and the Asian Indian deletion-inversion G γ (A $\gamma\delta\beta$)^o-thalassaemia, HPFH-6 deletion, HPFH-7 deletion, as well as β^o -thalassaemia 105 bp, 619 bp and 3.5 kb deletion (Tritipsombut *et al.*, 2012).

MATERIALS AND METHODS

Patient recruitment and sample collection

Patients with β -thalassaemia and $\delta\beta$ -thalassaemia are associated with anaemia and high level of HbF. The sample size was calculated based on previous studies. It was estimated that

approximately 4% of Malaysian populations are β -thalassaemia carriers and the prevalence of $\delta\beta$ -thalassaemia was found to be around 0.01% (Karimi *et al.*, 2012). By using the stated formula (sample size required to achieve a 95% confidence interval with $\pm 5\%$), the sample size was calculated as in Appendix 1.

The calculation of adequate sample size is crucial in any clinical study to determine the optimum number of patients required. Based on the calculation, a minimum sample size required for this study was 82 patients. Despite that, 144 patients agreed to participate in this study, exceeding the calculated sample size. Larger sample size is preferable as it may reflect the distribution of the studied objectives in a population.

This cross-sectional study was carried out among 144 patients (HbF > 1%), who attended Hospital Universiti Sains Malaysia (HUSM) in Kubang Kerian, Kelantan, Malaysia from March 2018 to October 2019. This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/16090283). Peripheral blood samples were withdrawn after written consents were obtained. The full blood counts (FBCs) were carried out using the automated haematology analyser (Sysmex XN-1000TM, USA). The Hb analysis was performed using cation exchange high performance liquid chromatography (CE-HPLC) (Bio-rad Variant II System, USA).

Multiplex Amplification Refractory Mutation System (ARMS)-PCR and gap-PCR

Genomic DNA was extracted from blood samples using a commercially available kit (Macherey Nagel, Germany) according to the manufacturer's instructions. The extracted genomic DNA concentration was measured by using Nanodrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, USA). The DNA samples were stored in -20°C before molecular analysis.

Multiplex amplification refractory mutation system polymerase chain reactions (ARMS-PCR) was performed for detection of common β -globin gene cluster mutations in those samples with high HbA₂ level (>3.2%) (Koh *et al.*, 2017). These eight common mutations include Cd 41/42, IVS 1–5, Cd 26, Cd 17, Cd 71/72, IVS 1–1, Cd 8/9 and -

28 mutations (Hassan *et al.*, 2013). The ARMS-A consisted of 0.30 μM forward (F) primer IVS 1-5, 0.04 μM F primer Cd 41/42 (-TTCCT), 0.05 μM F primer Cd 17 (A-T), 0.04 μM F primer Cd 26 (G-A), 0.18 μM F primer Internal Control, 0.135 μM reverse (R) primer Internal Control, 0.10 μM Common E (reverse primer for eight β -globin gene cluster mutations), 2.50 U/ μL HotStarTaq master mix (Qiagen, Netherlands) and 40 ng/ μL DNA template. Meanwhile, PCR reactions for ARMS-B were performed in 20 μL volume containing 0.30 μM F primer IVS 1-1 (G-T), 0.16 μM F primer Cd 8/9 (+G), 0.12 μM F primer -28 (A-G), 0.10 μM F primer Cd 71/72 (+A), 0.18 μM F primer Internal Control, 0.135 μM R primer Internal Control, 0.10 μM Common E, 2.50 U/ μL HotStarTaq master mix (Qiagen, Netherlands) and 40 ng/ μL DNA template. The amplification process began with initial denaturation at 95°C for 5 minutes followed by 30 cycles of 94°C for 25 seconds, 64°C for 45 seconds, 72°C for 1.5 minutes, with a final extension of 7 minutes at 72°C . The PCR product was separated by electrophoresis on 1% agarose gel.

Meanwhile multiplex gap-PCR were carried out in those samples with normal or low HbA₂ level ($\leq 3.2\%$) to detect large deletions at β -globin gene cluster including Siriraj J $\text{G}\gamma(\text{A}\gamma\delta\beta)^{\circ}$ -thalassaemia (~118 kb deletion), Thai ($\delta\beta)^{\circ}$ -thalassaemia (~12.5 kb deletion), HPFH-6 and Hb Lepore (Tritipsombut *et al.*, 2012). The detailed preparation for master mix components for multiplex gap-PCR were consisted of 0.10 μM F and R primer Siriraj J $\text{G}\gamma(\text{A}\gamma\delta\beta)^{\circ}$ thalassaemia, 0.2 μM F and 0.8 μM R primer Thai ($\delta\beta)^{\circ}$ -thalassaemia, 0.50 μM F and R primer HPFH-6, 0.4 μM F and 0.8 μM R primer Hb Lepore, 0.8 μM F and 0.4 μM R primer Internal Control, 1X Colourless GoTaq® Flexi Buffer (Promega, USA), 1.50 mM MgCl Solution (Promega, USA), 0.20 mM PCR Nucleotide Mix (dNTPs)(Promega, USA), 1.00 U/ μL GoTaq® DNA Polymerase (Promega, USA) and 50 ng/ μL DNA template. The amplification conditions include initial denaturation 94°C for 1 minute followed by 30 cycles of 94°C for 30 seconds, 62°C for 2 minutes, 59°C for 1 minute, 72°C for 1 minute, with a final extension of 10 minutes at 72°C . The PCR product was separated by electrophoresis on 1.2% agarose gel. All these primers were commercially purchased from

Integrated DNA Technologies (Malaysia). All amplifications were carried out using PCR machine Veriti 96 Well Thermal Cycler (Applied Biosystem, USA). Electrophoresis of PCR products was carried out on agarose gel pre-stained with Florosafe DNA Stain (Apical Scientific, Malaysia), and the results were observed using Gel Documentation System Molecular Imager® Gel Doc™ XR+ System (Bio Rad Laboratories, USA). However, our limitation here was we did not have positive control for Hb Lepore.

Statistical analysis

All statistical analyses were performed using independent t-test by GraphPad Prism 7.0 software. Qualitative data were expressed as frequency and percentage while quantitative data were expressed as mean, $\pm$ SEM and median. The independent t-test was used to compare the data between patients with and without β -globin gene cluster of eight types mutations and four types large deletions screened in this study.

RESULTS

Demographic distribution of patients

A total of 144 anaemic patients with HbF > 1.0% have participated in this study and met our study criteria. Their age range was between 2 to 71 years and the mean $\pm$ SEM was 19.99 ± 1.64 years. Female patients contributed largely to this study with approximately 61.1% of the total patients. Majority of the patients were Malays (99.3%).

β -globin gene cluster mutation by multiplex ARMS-PCR

From 144 anaemic patients in which HPLC analysis was carried out to identify HbF > 1.0%, 87 samples were identified HbA₂ > 3.2% and subsequently the samples were analysed using

multiplex ARMS-PCR. Figure 1 showed the representative gel electrophoresis of PCR products using multiplex ARMS-PCR that was run for 40 minutes at 100 volts. Following multiplex ARMS-PCR, 65 (74.7%) mutations were detected which comprises of 49 patients with Cd 26 (56.3%), 10 patients with Cd 41/42 (11.5%), 3 patients with compound Cd 26 and Cd 41/42 (3.4%) and 3 patients with IVS 1–1 (3.4%), while 22 patients were not detected for any of the mutations screened (Table 1).

Table 1. Frequency of β -globin gene cluster mutations (n=87).

Mutations	No. of patients	Percentage (%)
Cd 26	49	56.3
Cd 41/42	10	11.5
Compound Cd 26 and Cd 41/42	3	3.4
IVS 1–5 (G–C)	0	0
Cd 17 (A–T)	0	0
IVS 1–1	3	3.4
Cd 8/9 (+G)	0	0
–28 (A–G)	0	0
Cd 71/72 (+A)	0	0

β -globin gene cluster large deletion by multiplex gap-PCR

From HPLC analysis, 57 patients with HbA₂ $\leq$ 3.2% were analysed using multiplex gap-PCR. The results indicated an internal control band of 304 base pair (bp) size has been successfully amplified. However, out of these only one patient (1.8%) was found positive with Thai ($\delta\beta$)^o-thalassaemia type large deletions when subjected to the multiplex gap-PCR consisted of four target large deletions; Siriraj J G γ (A $\gamma\delta\beta$)^o-thalassaemia, Thai ($\delta\beta$)^o-thalassaemia, HPFH-6, and Hb Lepore (Table 2). Figure 2 showed the representative gel electrophoresis of PCR products using multiplex gap-PCR that was run for 60 minutes at 120 volts.

Table 2. Frequency of β -globin gene cluster large deletions (n=57)

Mutations	No. of patients	Percentage (%)
Siriraj J G γ (A $\gamma\delta\beta$) ^o – thalassaemia (~118 kb deletion)	0	0
Thai ($\delta\beta$) ^o - thalassaemia (~12.5 kb deletion)	1	1.8
HPFH-6	0	0
Hb Lepore	0	0

Abbreviations; kb: kilobase pair, HPFH-6: hereditary persistence of foetal haemoglobin type-6.

Figure 1. A) Multiplex ARMS-A. B) Multiplex ARMS-B. Representative of multiplex ARMS-PCR gel electrophoresis. Multiplex ARMS-PCR was conducted using 40 ng of DNA samples and was run on 1.0% (0.5 g/50 mL) agarose gel.

Figure 2. Representative of multiplex gap-PCR gel electrophoresis. Multiplex gap-PCR consisted of four target large deletions; Thai ($\delta\beta$)^o-thalassaemia, HPFH-6, Siriraj J $G\gamma(A\gamma\delta\beta)$ ^o - thalassaemia and Hb Lepore was conducted using 50 ng of DNA samples and was run on 1.2% (0.6 g/50 mL) agarose gels.

Figure 3. Mean difference between (A) HbF level, (B) Hb level, (C) MCV level, (D) MCH level and (E) RBC count of patients with and without 12 types of β -globin gene cluster mutations and large deletions screened.

Mean difference between haematological profiles of patients with and without β -globin gene cluster mutations and large deletions

Mean difference between HbF, Hb, MCV, MCH and RBC levels of patients with and without 12 types of β -globin gene cluster of mutations and

large deletions screened in this study are shown in Figure 3. Analysis of mean difference using independent t-test had shown significant difference between the mean of HbF, MCV as well as MCH levels of patients with and without 12 types of β -globin gene cluster mutations and

large deletions ($P < 0.05$). In contrast, there was no significant difference between the mean of Hb level and RBC count of patients with and without 12 types of β -globin gene cluster mutations and large deletions ($P > 0.05$).

DISCUSSION

Thalassaemia is an inherited disorder which normally occurs in the tropical and sub-tropical regions. In areas with a high frequency of thalassaemia, the carrier rate of thalassaemia mutation among the population is more than 20%. Thalassaemia brings great harm to human health. Several thalassaemia disorders cause the most severe degree of harm including severe β -thalassaemia (Long *et al.*, 2013). Patients with high HbF $> 1.0\%$, microcytosis (MCV < 80.0 fL) and/or hypochromia (MCH < 27.0 pg) can be considered as having thalassaemia, haemoglobinopathy or acquired anaemia (Langlois *et al.*, 2008). Some genetic conditions are known to influence γ -globin gene expression in adults, including β -thalassaemia, β -variants, HPFH and $\delta\beta$ -thalassaemia (Carrocini *et al.*, 2011). HPFH and $\delta\beta$ -thalassaemia comprise a heterogeneous group of disorders characterised by reduced HbA level and elevated HbF level (Pant *et al.*, 2014). This study showed that there was a significant difference between the mean HbF level of patients with and without 12 types of β -globin gene cluster mutations and large deletions screened in this study. β -thalassaemia is a genetic disorder and many studies have shown that the frequencies of β -thalassaemia mutations significantly differ between ethnic groups and geographical areas. In Malaysia, β -thalassaemia has been detected among major ethnic groups such as Malays, Chinese and Indians along with in other indigenous communities including *Orang Asli* of Peninsular Malaysia, Kadazandusun, Bajau, Murut and Bisayah of Malaysia Borneo (Koh *et al.*, 2017; Hassan *et al.*, 2013). This study employed multiplex ARMS-PCR which has been established previously (Hassan *et al.*, 2013). Multiplex ARMS-PCR is one of the rapid and inexpensive methods that can be performed to detect *HBB* gene mutations among multi-ethnic

population of Malaysia (Hanafi *et al.*, 2014; Hassan *et al.*, 2013). In this study, Cd 26 (G-A) was the most common mutation followed by Cd 41/42 (–TTCT) and IVS 1–1 (G–T), which is similar to the findings by Hassan *et al.* (2013), as the most common β -globin gene cluster mutation in Malaysia are Cd 26, Cd 41/42, IVS 1–1, and IVS 1–5. The highest frequency of Cd 26 allele among the population in this study is also similar to the previous report in Penang, Malaysia (Yatim *et al.*, 2014). In Kelantan, Cd 26 was the most common β -globin gene mutation faulty, which accounted for 84.8% of the transfusion-dependent β -thalassaemia among Malay patients (Hanafi *et al.*, 2014). Cd 26 mutation was the highest β -globin anomaly detected among the *Orang Asli* in Gombak and has been shown to have higher prevalence among the *Orang Asli* in Peninsular Malaysia (George, 2013). This has also been confirmed by a previous study conducted on the Senoi group of *Orang Asli*, where the most common mutation reported was Cd 26 with 18.6% occurrence (Koh *et al.*, 2017). The mutations IVS 1–5 (G–C), Cd 17 (A–T), Cd 8/9 (+G), –28 (A–G), and Cd 71/72 (+A) were not detected in this study.

$\delta\beta$ -thalassaemia caused by the elimination of both δ and β genes but with preservation of the γ genes. Homozygotes $\delta\beta$ -thalassaemia cannot synthesise HbA₂ or HbA as they have no δ and β genes but comprises 100% of HbF concentration (Khunger *et al.*, 2014). $\delta\beta$ -thalassaemia such as Siriraj J $G\gamma(A\gamma\delta\beta)^{\circ}$ -thalassaemia (~118 kb deletion), Thai ($\delta\beta)^{\circ}$ -thalassaemia (~12.5 kb deletion), HPFH-6 and Hb Lepore have been commonly associated with individuals with high HbF ($> 1.0\%$), but having a low to normal value of HbA₂ ($\leq 3.2\%$) (Tritipsombut *et al.*, 2012). The screening of samples for this study using multiplex gap-PCR showed that only one patient was positive with Thai ($\delta\beta)^{\circ}$ -thalassaemia. In Malaysia, most of the large deletional mutation cases reported are from the α -thalassaemia. Published studies involving the large deletion in the β -globin gene cluster are limited. We managed to obtain only one positive Thai ($\delta\beta)^{\circ}$ -thalassaemia in Malaysia. The low positive rate result in this study may be due to limited samples and types of large deletions that were screened. Large deletions that affect the β -globin gene cluster are rare (Cao & Galanello, 2010). Nevertheless, the prevalence

of carrier for Thai ($\delta\beta$)^o-thalassaemia is as high as 4% in Thailand. Therefore, these findings can be clarified by the neighbouring proximity and frequent travelling of Malaysians and Thais. Data obtained in this study also demonstrates that the ($\delta\beta$)^o-thalassaemia is associated with hypochromic microcytic red blood cell indices. Finding from this study is in agreement with previously published data by Fucharoen *et al.* (2002), which reported low mean values of MCV and MCH in HPFH-6 and Thai ($\delta\beta$)^o-thalassaemia patients, together with high HbF (>5.0%), but normal HbA₂ (2.2%). Furthermore, the large deletion at the β -globin gene cluster that is associated with $\delta\beta$ -thalassaemia trait leads to the elevated level of HbF (5% to 20%), while a moderate increment of HbF (< 10%) is associated with β -thalassaemia trait (Hoyer *et al.*, 2002). Hb Lepore also occurs globally and in various ethnic groups. Southern Europe is the region with the most common Hb Lepore variant occurrences. However, it is uncommon in African Americans with few cases have been reported (Nussenzveig *et al.*, 2012). HPFH-6 is sporadically found in Thailand (Fucharoen *et al.*, 2002). According to Tritipsombut *et al.* (2012), Siriraj J G γ (A $\gamma\delta\beta$)^o-thalassaemia is a novel mutation. It has been found in two unrelated families from Hat Yai and Phuket. Similar to this study, we found no patients with Hb Lepore, Siriraj J G γ (A $\gamma\delta\beta$)^o-thal, and HPFH-6.

CONCLUSION

This study highlighted the presence of different types of β -globin gene cluster mutations and the importance of haematological characterisation as well as these large deletions detection in establishing a definitive diagnosis among this selected group of patients.

ACKNOWLEDGEMENTS

This work was supported by the Research University Individual grant (1001/PPSK/8012213), and Bridging Incentive grant (304.PPSK.6316463) of Universiti Sains Malaysia.

We would like to thank the Institute for Research in Molecular Medicine (INFORMM) for technical assistance, and management of the Hospital USM, Kubang Kerian, Kelantan for permitting the investigators to use patients' medical record; space and assets belong to the hospital during the process of conducting this research.

REFERENCES

- Cao, A., & Galanello, R. 2010. Beta-thalassemia. *Genetics in Medicine* 12(2): 61-76.
- Carrocini, G. C. D. S., Zamaro, P. J. A., & Bonini-Domingos, C. R. 2011. What influences Hb fetal production in adulthood?. *Brazilian Journal of Hematology and Hemotherapy* 33(3): 231-236.
- Fucharoen, S., Pengjam, Y., Surapot, S., Fucharoen, G., & Sanchaisuriya, K. 2002. Molecular and hematological characterization of HPFH-6/Indian deletion-inversion Ggamma(Agammadeltabeta)0-thalassemia and Ggamma(Agammadeltabeta)0-thalassemia/HbE in Thai patients. *American Journal of Hematology* 71(2): 109-113.
- Galanello, R. 2013. CHAPTER 4 - Screening and diagnosis for haemoglobin disorders. In: Prevention of Thalassaemias and Other Haemoglobin Disorders: Volume 1: Principles [Internet] (Second Edition). Ed. Old, J. Thalassaemia International Federation.
- George, E. 2013. HbE^{#2}-Thalassaemia in Malaysia: revisited. *Journal of Hematology and Thromboembolic Diseases* 1: 1-3.
- George, E., Jamal, A. R., Khalid, F., & Osman, K. A. 2001. High performance liquid chromatography (HPLC) as a screening tool for classical Beta-thalassaemia trait in Malaysia. *Malaysian Journal of Medical Sciences* 8(2): 40-46.
- Hanafi, S., Hassan, R., Bahar, R., Abdullah, W. Z., Johan, M. F., Rashid, N. D., Azman, N. F., Nasir, A., Hassan, S., Ahmad, R., Othman, A., Ibrahim, M. I., Sukeri, S., Sulong, S., Yusoff, S., Mohamad, N. S., Hussein, A., Hassan, R., Yusoff, N., Yahaya, B. H., Ismail, E., Nik-Yusoff, N. K., Salleh, S., & Zilfalil, B. A. 2014. Multiplex amplification refractory mutation system (MARMS) for the detection of β -globin gene mutations among the transfusion-dependent β -thalassaemia Malay patients in Kelantan, Northeast of Peninsular Malaysia. *American Journal of Blood Research* 4(1): 33-40.
- Hassan, S., Ahmad, R., Zakaria, Z., Zulkafli, Z., & Abdullah, W. Z. 2013. Detection of β -globin gene mutations among β -thalassaemia carriers and patients in Malaysia: Application of multiplex amplification refractory mutation system-polymerase chain reaction. *Malaysian Journal of Medical Sciences* 20(1): 13-20.
- Hoyer, J. D., Penz, C. S., Fairbanks, V. F., Hanson, C. A., & Katzmann, J. A. 2002. Flow cytometric measurement of hemoglobin F in RBCs: diagnostic usefulness in the distinction of hereditary persistence of fetal hemoglobin (HPFH) and hemoglobin S-HPFH from other conditions with elevated levels of hemoglobin F. *American Journal of Clinical Pathology* 117(6): 857-863.
- Karimi, M., Marvasti, V. E., Mehrabanejad, S., Mohaghegh, P., Afrasiabi, A., Dehbozorgian, J., Silavizadeh, S., Bazrafshan, A. 2012. Prevalence of delta beta thalassemia minor in Southern Iran. *Iranian Journal of Blood and Cancer* 4(4): 153-155.

- Khunger, J. M., Gupta, M., Singh, R., Kapoor, R., & Pandey, H. R. 2014. Haematological characterisation and molecular basis of asian Indian inversion deletions delta Beta thalassemia: a case report. *Journal of Clinical and Diagnostic Research* 8(9): FD01-FD02.
- Koh, D. X. R., Raja Sabudin, R. Z. A., Mohd Yusoff, M., Hussin, N. H., Ahmad, R., Othman, A., & Ismail, E. 2017. Molecular characterisation of α - and β -thalassaemia among indigenous Senoi Orang Asli communities in Peninsular Malaysia. *Annals of Human Genetics* 81(5): 205-212.
- Langlois, S., Ford, J. C., & Chitayat, D. 2008. Carrier screening for thalassemia and hemoglobinopathies in Canada. *Journal of Obstetrics and Gynaecology Canada* 30(10): 950-959.
- Long, J., Ye, X., Lao, K., Pang, W., Weng, X., Fu, K., Yan, S., & Sun, L. 2013. Detection of three common α -thalassemia in non-deletion types and six common thalassemia in deletion types by QF-PCR. *Clinical Biochemistry* 46(18): 1860-1864.
- Modell, B., & Darlison, M. 2008. Global epidemiology of haemoglobin disorders and derived service indicators. *Bulletin of the World Health Organization* 86: 480-487.
- Nussenzveig, R. H., Vanhille, D. L., Hussey, D., Reading, N. S., & Agarwal, A. M. 2012. Development of a rapid multiplex PCR assay for identification of the three common Hemoglobin-Lepore variants (Boston-Washington, Baltimore, and Hollandia) and identification of a new Lepore variant. *American Journal of Hematology* 87(10): E74-75.
- Olivieri, N. F., Pakbaz, Z., & Vichinsky, E. 2011. Hb E/beta-thalassaemia: a common & clinically diverse disorder. *The Indian Journal of Medical Research* 134(4): 522-531.
- Pant, L., Kalita, D., Singh, S., Kudesia, M., Mendiratta, S., Mittal, M., & Mathur, A. 2014. Detection of Abnormal Hemoglobin Variants by HPLC Method: Common Problems with Suggested Solutions. *International Scholarly Research Notices*: 257805.
- Patrinos, G. P., Giardine, B., Riemer, C., Miller, W., Chui, D. H., Anagnou, N. P., Wajcman, H., & Hardison, R. C. 2004. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies. *Nucleic Acids Research* 32(Database issue): D537-541.
- Tan, J. A., Chin, P. S., Wong, Y. C., Tan, K. L., Chan, L. L., & George, E. 2006. Characterisation and confirmation of rare beta-thalassaemia mutations in the Malay, Chinese and Indian ethnic groups in Malaysia. *Pathology* 38(5): 437-441.
- Tan, J. A., George, E., Tan, K. L., Chow, T., Tan, P. C., Hassan, J., Chia, P., Subramaniam, R., Chandran, R., & Yap, S. F. 2004. Molecular defects in the beta-globin gene identified in different ethnic groups/populations during prenatal diagnosis for beta-thalassemia: a Malaysian experience. *Clinical and Experimental Medicine* 4(3): 142-147.
- Thein, S. L. 2011. CHAPTER 9 - Abnormalities of the structure and synthesis of hemoglobin. In: *Blood and Bone Marrow Pathology (Second Edition)*. Ed. Porwit, A., McCullough, J. and Erber, W. N. pp. 131-155. Edinburgh: Churchill Livingstone.
- Tritipsombut, J., Phylipsen, M., Viprakasit, V., Chalaow, N., Sanchaisuriya, K., Giordano, P. C., Fucharoen, S., & Harteveld, C. L. 2012. A single-tube multiplex gap-polymerase chain reaction for the detection of eight β -globin gene cluster deletions common in Southeast Asia. *Hemoglobin* 36(6): 571-580.
- Yatim, N. F. M., Rahim, M. A., Menon, K., Al-Hassan, F. M., Ahmad, R., Manocha, A. B., Saleem, M., & Yahaya, B. H. 2014. Molecular characterization of α - and β -thalassaemia among Malay patients. *International Journal of Molecular Sciences* 15(5): 8835-8845.