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β-GLOBIN GENE CLUSTER MUTATION AND DELETION AMONG ANEMIC PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA USING MULTIPLEX AMPLIFICATION REFRACTORY MUTATION SYSTEM POLYMERASE CHAIN REACTION (MARMS-PCR) AND GAP-PCR

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Abstract

Anemia associated with high fetal hemoglobin (HbF) levels of more than 1.0%, can be impacted by several possible factors, including acquired and inherited causes. The aim of this study is to screen for β-globin gene cluster mutations among anemic patients with high HbF (HbF >1%). The study involved 150 blood samples of anemic patients from Hospital Universiti Sains Malaysia. High performance liquid chromatography (HPLC) was performed on 150 anemic samples to observe the levels of HbF and hemoglobin A2 (HbA2). One hundred six patients reported with high HbF levels (HbF >1%). The samples with HbA2 levels >3.2% were subjected to multiplex amplification refractory mutations system-polymerase chain reaction (MARMS-PCR), while those with HbA2 level ≤3.2% utilised gap-PCR. For MARMS-PCR, 61 out of 106 patients had the most frequent mutations detected in Cd 26 (35), followed by IVS1-5 (6), Cd 41/42 (3), Cd 8/9 (2) and IVS 1-1 (1). Furthermore, only one sample showed compound heterozygosity for Cd26 and Cd 8/9. However, for gap-PCR, there was no deletion detected in the 45 samples. This study shows the importance and significance of screening for high HbF levels and molecular characterisation in detecting various types of β-globin gene cluster mutations/deletions among anemic patients for establishing a proper diagnosis and management.

INTRODUCTION

β-globin gene cluster is a functional gene that located on chromosome 11, responsible for the expression of hemoglobin [1]. The mutation and/or deletion of the β-globin gene cluster lead to an imbalance in β-globin chain production, manifesting as β-thalassemia, hereditary persistence of fetal hemoglobin (HPFH), δβ-thalassemia or γδβ-thalassemia [1]. β-thalassemia is the most common hemoglobin disorder while the deletion of β-globin gene cluster is still rare among the Malaysian population [2–4]. β-thalassemia is an autosomal recessive disorder that involves more than 200 mutations and, least commonly, deletions of the β-globin gene [5]. According to the data from 2007 to 2018, there was a total of 91.97% thalassemia patients in the Malaysian population (7984 out of 8681 patients) registered in the Malaysian Thalassemia Registry were highly distributed in Malay ethnicity [6]. Approximately 4.5% of β-

thalassemia carriers are among Malays and Chinese-Malaysians [7]. Thalassemia is also associated with anemia due to an imbalance in the globin chain, presenting with symptoms that vary in severity from asymptomatic, mild to severe microcytic anemia [8,9].

Anemia is a common condition that is related to lack of red blood cells (RBCs) or hemoglobin concentration, hindering the transport of an adequate oxygen amount [9]. This condition remains a major public health concern due to the inherited causes such as thalassemia and sickle cell anemia, or acquired causes such as pregnancy, drug induced anemia, chronic disease and nutritional deficiencies [10]. In Malaysia, 13.8% of the population is affected by anemia, which is more prevalence among women, with hypochromic anemia accounts for 59.7% of the overall anemia prevalence [11].

Furthermore, the reactivation or persistence of HbF levels was delineated as a biomarker in numerous inherited or acquired conditions, including mutations of the β-globin gene cluster [12]. An unusually high HbF level can also be observed in acquired conditions such as pernicious anemia, tumours, acute blood loss and pregnancy [13]. The high HbF level is significantly associated with the deletion/mutation of the β-globin gene cluster. The HbF level can increase till 100% in β-thalassemia major, hereditary persistence of fetal hemoglobin (HPFH), and sickle cell disease, and it has been shown that high HbF levels are beneficial in reducing the anemia severity [12,14].

Previously, MARMS-PCR and gap-PCR were used as molecular diagnostic tools to detect common mutations and deletions in thalassemia [15]. The MARMS-PCR and gap-PCR were preceded based on the HbA2 level, which is an important part in the thalassemia screening. If the value of HbA2 is more than 3.2%, the patients are assumed to have a β-thalassemia mutation while a normal HbA2 level, below 3.2%, is considered as β-thalassemia deletion [16]. Thus, the aim of this study was to detect the β-globin gene cluster mutations among anemic patients with high HbF levels in Hospital Universiti Sains Malaysia, Kelantan.

MATERIALS AND METHODS

Materials

The genomic DNA of the samples was extracted using the DNA extraction kit from Macharey-Nagel (Duren, Germany). The primers for MARMS and gap-PCR were purchased from Integrated DNA technologies (IDT) Malaysia [15,17]. The amplification contained HotstartTaq® Master Mix (1.5mm $MgCl_2$, PCR buffer, HotstarTaq® DNA polymerase and 200µm of each DNTP) (Qiagen GmbH, Hilden, Germany). The DNA ladder 100bp (Promega, Madison, United State), agarose powder (Thermo Fisher Scientific, Waltham, United States), Florosafe DNA stain (1st Base Laboratories, Selangor, Malaysia) were used for gel electrophoresis.

Blood Samples Collection

Approximately 2 mL of peripheral blood was collected from 150 Malay anemic patients in the Medical Ward at Hospital Universiti Sains Malaysia (Hospital USM), Kelantan. This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/19090552).

Full blood Count (FBC) Analysis

A full blood count was carried out using a hematological analyzer (Sysmex XN-1000™). Red cell indices such as RBC, Hb, mean cell volume (MCV), and mean cell hemoglobin (MCH) were measured.

Hemoglobin (Hb) Analysis

The quantification of Hb was carried out to determine HbA, HbA2/E and HbF levels using cation exchange high performance liquid chromatography (CE-HPLC) (Bio-rad Variant II System, USA). One hundred six out of 150 samples had high HbF level (>1%) and were chosen for consequent molecular studies.

DNA Extraction

DNA was extracted using the commercial kit Nucleospin blood L (Macherey-Nagel, Germany), following to the manufacturer's instructions. The concentration and the purity of the DNA were quantified using Nanodrop ND-2000 Spectrophotometer (ThermoFisher). The extracted genomic DNA was kept at 4℃ until further analysis.

DNA Amplification using MARMS and Gap-PCR

The β-globin gene cluster was screened using MARMS-PCR for several mutation, such as are Cd 26 (G-A), IVS 1-5 (G-C), Cd 41/42 (-TTCT), Cd 8/9 (+G), Cd 17 (A-T), IVS 1-1 (G-A), and Cd 71/72 (+A), each with specific molecular weight product [15]. Out of 106 samples, 61 with HbA2 levels >3.2% proceeded with MARMS. While, the large deletion in β-globin gene cluster were determined by DNA amplification using gap-PCR, including deletions observed in conditions such as Thai (δβ)°-thalassaemia, Hb Lepore, HPFH-6 and Siriraj J Gγ(Aγδβ)°-thalassaemia [18]. Primer sequences were obtained from previous studies [4,15,18]. Therefore, 45 samples with HbA2 level ≤3.2% utilised the gap-PCR. DNA amplification was carried out using Veriti™ 96-well thermal cycler (Applied Biosystem, USA). The PCR products were analysed using gel electrophoresis with 1.5% (w/v) agarose gel for MARMS-PCR and 1.2% (w/v) agarose gel for multiplex gap-PCR.

Statistical Analysis

The data were expressed as frequency, percentage, mean, ± SD and median and analysed using the Statistical Package for Social Sciences (SPSS) version 26.

RESULTS AND DISCUSSION

The mean of Hb levels was 9.49 ± 0.22 g/dL which showed that most of the studied anemia patients had mild anemia. As shown in Table 1, the hematological profile showed calculated means for MCV was 22.42 ± 0.41 fL and MCH was 68.27 ± 1.19 pg, which were low compared to the normal range. The mean of HbF in this study was high which was 5.37 ± 1.47% and the range level of HbF was 1.10 – 96.70%. The hematological findings in the study subjects were consistent with previous studies conducted among individuals with β-thalassemia, HPFH, and sickle cell disease with elevated HbF patients [19–21].

Table 1. Hematological profile among anemic patients with high HbF levels (n=106)

Red Blood Indices (Unit)	Mean ± SEM	Median	Range value
RBC (x10^{12}/L)	4.45 ± 0.11	4.58	1.12 – 7.89
Hb (g/dL)	9.49 ± 0.22	10.10	3.40 – 12.70
MCV (fL)	68.27 ± 1.19	67.80	71.1 – 107.60
MCH (pg)	22.42 ± 0.41	21.90	14.90 – 39.30
HbF (%)	5.37 ± 1.47	1.95	1.10 – 96.70
HbA2 (%)	3.60 ± 0.19	3.00	1.20 – 9.30
Presumed HbE (%)	46.05 ± 3.78	30.60	15.00 – 95.80

Abbreviation; MCV=mean corpuscular volume, fL= femtolitre, MCH=mean corpuscular hemoglobin, pg = picogram, dL= decilitre

There was a significant correlation between HbF level and hematological parameter observed in anemic patient with high HbF levels (p-value <0.05), as shown in Table 2. Similar finding showed a statistically significant correlation between HbF with RBCs indices among HbE/β-thalassemia [23]. According to Gupta et al. 2016, a negative correlation between HbF and hematological parameters, except MCH level, among thalassemia patients [24]. Similarly, this study also showed a direct correlation between HbF and MCH but an inverse correlation with all others parameters among anemic patient (Table 2). The different types of correlations between HbF levels and hematological parameters may due to the selective sample and clinical presentation of the patient in this study [25].

Table 2. Correlation between HbF and hematological paramaters

	HbF	
Red Blood Indices (Unit)	***r***	***P value***
RBC (x10^{12}/L)	-0.334	0.0005
Hb (g/dL)	-0.271	0.005
MCV (fL)	-0.286	0.002
MCH (pg)	0.273	0.005

P-value >0.05, r= Pearson correlation

Figure 1 and Figure 2 show the gel electrophoresis of the PCR products on a 1.5% (w/v) agarose gel of MARMS and gap-PCR on a 1.2% (w/v) agarose gel. Each mutation/deletion in the β-globin gene was amplified with specific molecular weight by appearing specific fragment. Using MARMS, β-globin gene mutations were detected in 48 patients. The observed mutations are presented in decreasing frequency: Cd 26 (G-A) (73%), IVS 1-5 (G-C) (13%), Cd 41/42 (-TTCT) (6%), Cd 8/9 (+G) (4%), and IVS 1-1 (G-A) (2%) as well as 2% compound heterozygous Cd 26 (G-A) and Cd 8/9 (+G). According to previous studies, the Cd26 (G-A), IVS 1-5 (G-C) and Cd 41/42 (-TTCT) were the most frequent mutations detected among Malay patients [4,15]. Furthermore, Cd 26 (G-A) was also highly prevalent in neighbouring countries such as Singapore, Indonesia and Thailand. [15,26,27].

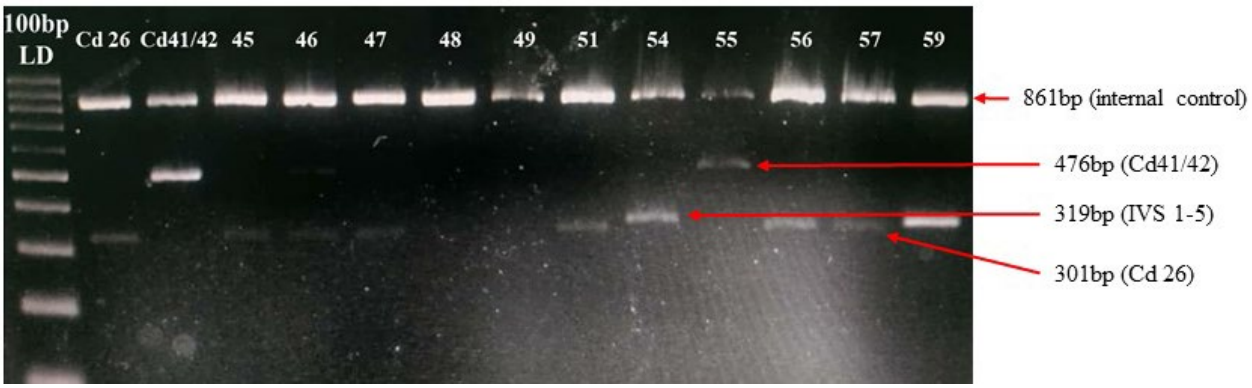

Figure 1. MARMS-PCR product was conducted using high HbF (>1%) and HbA2 (>3.2%) DNA samples with 1.5% agarose gel electrophoresis at 100V. Lane 100bp indicates as a DNA ladder. Lane Cd26 and Cd41/42 indicate positive control. Lane 45, 46, 47, 48, 49, 51, 54, 55, 56, 57, and 59, were PCR products. Key cd = codon IVS= intervening sequence bp=base pair.

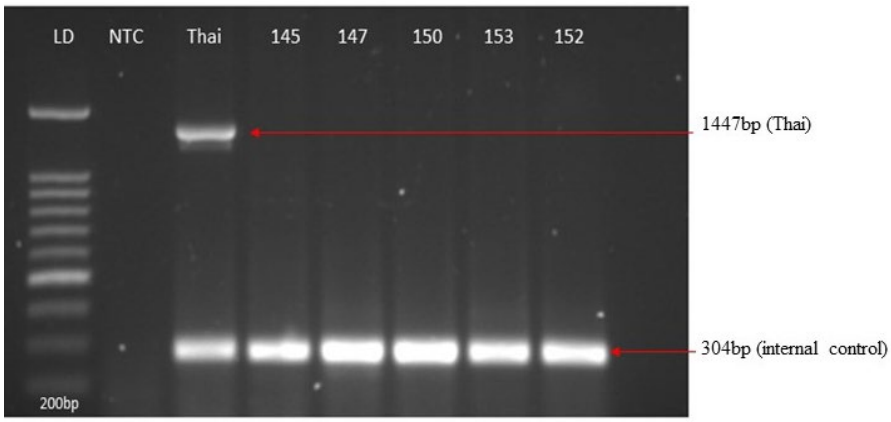

Figure 2. Gap-PCR product was conducted using high HbF (>1%) and HbA2 (≤3.2%) DNA samples with 1.2% agarose gel electrophoresis at 100V. Lane 200bp indicates as a DNA ladder. Lane NTC indicates the negative control. Lane Thai indicates the positive control. The lanes with number tagging were patient's DNA sample. Key cd=codon IVS= intervening sequence bp=base pair

However, there was no deletion detected by multiplex gap-PCR in this study. This may be due to the scarcity of gene cluster deletions among Malaysian population [1]. Previous study showed that (δβ)⁰-thalassaemia was found only in one sample among Malay patients [28]. The (δβ)⁰-thalassaemia is commonly found in Thailand while Hb Lepore is more distributed in Southern Europe and Siriraj J in central Asia (India) [18,29,30]. This study highlights the importance of molecular characterisation by screening different types of β-globin gene cluster mutations or deletions among the anemic patients with high levels of HbF in establishing a definitive diagnosis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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