

Original article:

Characterization of β -Globin Gene Cluster Deletions Using Multiplex-Gap Polymerase Chain Reaction (PCR) and Multiplex Ligation-Dependent Probe Amplification (MLPA)

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Abstract

Background : Deletions in the β -globin gene cluster are usually rare, problematic to detect, and subsequently possess a challenge in many diagnostic laboratories. They are normally related to the heterozygous of the delta beta ($\delta\beta$) thalassemia, hereditary persistence of fetalhemoglobin (HPFH) and some of the hemoglobin variants. These disorders are typically presented by elevated levels of hemoglobin F (Hb F), but with low to normal hemoglobin A2 (Hb A2). However, despite their existence, there is still a limited number of studies focusing on this area, hence no definitive diagnosis could be conclusively established. Therefore, this pilot study was carried out to fill this knowledge gap. **Methods:** In this study, screening of the selected deletional mutations in the β -globin gene cluster among patients with Hb F (>1%) and Hb A2 (<4%) were performed using multiplex Gap-PCR and multiplex ligation-dependent probe amplification (MLPA). **Results:** The results showed that out of 54 samples tested using multiplex Gap-PCR against four target deletions; Thai ($\delta\beta$)^o-thalassemia, HPFH-6, Siriraj J and HbLepore, one sample was found positive with Thai ($\delta\beta$)^o-thalassemia. Further findings from the MLPA screening on 12 randomly selected samples revealed that another patient was positive with double deletions within the region of the β -globin gene cluster. These deletions occur at the gamma-globin gene 1 (HBG1) and gamma-globin gene 2 (HBG2) in exon 3. **Conclusions:** In conclusion, this study highlighted the importance of these deletions' characterization using multiplex Gap-PCR and MLPA which helps in establishing a definitive diagnosis among this selected group of patients.

Keywords: Deletion mutation; β -globin gene cluster; Hb F, Hb A2; multiplex Gap-PCR; MLPA

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Introduction

Beta (β) globin gene cluster are arranged in a single cluster (5'- ε- Gγ- A γ-δ- β-3') on chromosome 11p15.¹ Several mutations have been shown to occur within this cluster which results in reduction or absence of synthesis of one or more of globin chains.² Most of these mutations are primarily point mutations, single base substitutions, small insertions or minor deletions³, yet major deletions are rare.⁴ Deletions in the β-globin gene cluster cause inactivation of the structural genes, which leads to disorders such as β-thalassemia, delta-beta (δβ)-thalassemia, hemoglobin (Hb) Lepore, gamma delta beta (γδβ)-thalassemia and hereditary hemoglobin persistence of fetalhemoglobin (HPFH).⁵⁻⁸

In a routine clinical setting, a high Hb A2 (>4%) indicates a presumed β-thalassemia traits.^{2,4} However, this is not always a clear-cut diagnosis, particularly for those individuals with a borderline Hb A2 value, but concurrently harbouring a β-globin abnormality at the molecular level, which may be missed during thalassemia screening programs.⁹⁻¹⁰ Cases involving deletions in the β-globin gene cluster are usually problematic to detect, and subsequently possess a challenge for many diagnostic laboratories.

In addition, the Hb F levels too may also be used as one of the hallmarks to many syndromes. It was observed that large deletions within the β-globin locus are associated with high Hb F levels and vice versa.¹¹ In a heterozygous δβ-thalassemia, γδβ-thalassemia and HbLepore, moderate increased of Hb F were noted.¹² Similarly, a HPFH heterozygous essentially would have normal red blood cell indices but with higher Hb F in comparison to δβ-thalassemia condition.¹³ Significant increase of Hb F can also be expected in the case of homozygous HPFH.¹² Furthermore, the coinheritance of this deletion with other β-thalassemia mutations or hemoglobin variants can lead to mild, intermediate and severe clinical conditions.¹⁴ As such, it is imperative to include an unknown and uncharacterized deletions in any thalassemia screening program, so that carriers can be effectively identified even at the molecular level.

In Malaysia, the distribution and spectrum of the β-globin gene cluster mutations from different parts of the country have been systematically delineated since mid-19th century¹⁵, and are progressively being studied and published. However, cases on the deletional modifications related to the HPFH and

δβ-thalassemia are sparse. To date, only a limited number of reports among the local populations have been documented.¹⁶⁻¹⁸

Various techniques have been used for the characterization of rare and unknown β-globin gene cluster deletions, which are commonly PCR-based methods.⁴ These include multiplex Gap-PCR¹⁹ and multiplex ligation-dependent probe amplification (MLPA).²⁰ In this study, both techniques were chosen to screen a selected cohort of hematology patients suspected of having β-globin gene cluster deletions based on their hematological profile. The multiplex Gap-PCR is reported to be fast, simple and cost-effective in comparison to other molecular methods.¹⁹⁻²¹ The latter technique is commonly applied to detect a large deletion in β-thalassemia gene clusters which is based on the principle of detecting copy number variants in genomic sequences.²² MLPA is often described as a robust, quick and effective way of screening for the spectrum and frequency of β-globin gene cluster deletion mutations^{20,23} with a potential of replacing the laborious method of Southern blotting.²⁴

Although cases involving the deletions in β-globin gene cluster can be diagnostically challenging, this however should not underscore the importance of giving accurate diagnosis to the affected individuals. As such, this pilot study is aimed to detect and molecularly characterized deletion and mutations in the β-globin gene cluster in a selected cohort of hematology patients using both multiplex gap-PCR and MLPA techniques.

Materials And Methods

Patient selection

Approximately 2 mL of peripheral blood was collected from a cohort of 54 patients (Hb F > 1%, Hb A2 <4%) that attended Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. In addition, normal healthy individuals (n = 6) were also enrolled as a control group or reference samples for this study. This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/16090283).

Full Blood Count (FBC)

Full blood count was carried out using hematological analyzer Sysmex XN-1000™ in Hematology Laboratory, Hospital USM. Red cell indices such as Hb, mean cell volume (MCV), mean cell hemoglobin (MCH), Hb F and Hb A2 were measured.

Hemoglobin (Hb) analysis

Quantifications of hemoglobin subtypes such as Hb A, Hb A2 and Hb F were carried out using cation exchange high performance liquid chromatography (CE-HPLC) (Bio-rad Variant II System, USA) in Hematology Laboratory, HUSM. Analysis was based on both peak resolution and peak integration quantifications. Samples with a high level of Hb F (> 1%) and normal Hb A2 (<4.0%) as confirmed by HPLC were chosen for molecular studies.

DNA extraction

DNA was extracted from peripheral venous blood using commercial kit Nucleospin Blood L (Macherey-Nagel, Germany) according to the manufacturer's instructions. 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 Spectrophotometer (Marshall Scientific, USA).

Multiplex Gap-PCR

Four types of mutations namely Thai ($\delta\beta$)-thalassemia, HPFH-6, Siriraj J and Hb Lepore which commonly occur within the β -globin gene cluster were detected using multiplex Gap-PCR as previously described¹⁹, but with some modifications. DNA amplification was carried out in Veriti™ 96-well thermal cycler (Applied Biosystem™, USA), using 5X Colorless GoTaq® Flexi Buffer1 (Promega, USA), 25 mM MgCl₂ solution (Promega, USA), 10 mM PCR Nucleotide Mix 5 U/ μ L GoTaq® DNA Polymerase (Promega, USA) and 5 U/ μ L GoTaq® DNA Polymerase (Promega, USA). For each PCR reaction, DNA concentrations of 50–200 ng/ μ L were tested in a reaction containing 1.5 mM MgCl₂, together with the optimized primer concentrations. PCR product was mixed with 6X DNA loading dye (Thermo Fisher Scientific, USA) and loaded into the wells. Alongside the samples, 200 bp DNA ladder (Promega, USA) was also loaded into the gel.

Multiplex ligation-dependent probe amplification (MLPA)

Screening of deletions in the β -globin gene cluster were also determined using the MLPA procedure; SALSA MLPA P102 HBB kit (MRC-Holland, Netherland). The screening was carried out according to the manufacturer's instructions.

Fragment separation by capillary electrophoresis & data analysis

All MLPA samples were sent to the First Base Laboratories Sdn. Bhd. for fragment separation using ABI sequencer 3730XL DNA analyzer. The data were analyzed using the Coffalyser. Net software (MRC-Holland, Netherland).

Ethical clearance: This study was ethically approved by School of Medical Sciences, Health Campus Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan,

Results

A total of 54 patients with high Hb F (Hb F > 1%), but with normal Hb A2 (Hb A2 < 4%) from Hospital USM were recruited (Table 1). Summarized data on the hematological profiles which include MCV, MCH and quantifications of Hb A2 and Hb F showed that the calculated mean for MCV = 72.44 ± 11.7 fL and MCH = 22.6 ± 4.2 pg were relatively low compared to the normal range. The mean for Hb F was 4.6 ± 9.6% and with observed range of 1.1 to 68.1%. Hb A2 displayed a mean value of 2.5 ± 0.6%, which fell within the standard normal range (Table 2).

Table 1: Demographic data of patients (n=54)

Characteristics	Demographic	Frequency	Percentage (%)
Sex	Male	15	27.8
	Female	39	72.2
Age group	1-20	31	57.4
	21-40	19	35.2
	41-60	2	3.7
	61 and above	2	3.7

Table 2: Summary of the hematological profiles of recruited patients

Red Cell indices (Unit)	Range	Mean ± SD	Median
Hb (g/dL)	4.2-8.9	10.16 ± 2.3	10.5
MCV (fL)	48.5-95.4	72.44 ± 11.7	71.8
MCH (pg)	14.7-31.7	22.6 ± 4.2	22.6
Hb F (%)	1.1-68.1	4.6 ± 9.6	2.3
Hb A2 (%)	1.1-3.9	2.5 ± 0.6	2.6

Abbreviations: Hb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; g/dL, gram per deciliter; fL, femtoliter; pg, picogram; %: percentage.

The results indicated that in all 54 tested samples, an internal control band size of 304 base pair (bp) has been successfully amplified. However, out of these only one patient (patient P38) was found positive with Thai ($\delta\beta$)^o-thalassemia type deletions when subjected to the multiplex Gap-PCR consisted of four target deletions; $\delta\beta$ -thalassemia, HPFH-6, Siriraj J and HbLepore as shown in Fig 1.

Following this, MLPA was carried as a second line of screening on 12 randomly selected samples. Our results revealed that one patient (patient P2) was positive with double deletions within the region of the β -globin gene cluster. These deletions occur at gamma-globin gene 1 (HBG1) and gamma-globin gene 2 (HBG2) in exon 3 as shown in Fig. 2.

Discussion

The main aim of this study was to screen for selected β -globin gene cluster deletions in a cohort of hematology patients. Briefly, a total of 54 patients with high Hb F (>1%) and with normal Hb A2 (<4%) were subjected to molecular analyses, namely multiplex Gap-PCR and MLPA. In this study, the chosen inclusion criteria have been shown to be commonly associated with the β -globin gene cluster deletions, with the suggested diagnoses such as $G\gamma(A\gamma\delta\beta)$ ^o-thal, (Siriraj J Deletion~118 kb del), Thai ($\delta\beta$)^o-thal (~12.5 kb deletion), HPFH-6 and Hb Lepore.²⁰

Analyses of the hematological data on the study subjects findings were in agreements with previous published studies eg: Fucharoen et al. (2002) reported similar low mean values of MCV and MCH observed in HPFH-6 and $\delta\beta$ -thal Thai patients, together with high Hb F (>5%), but with normal Hb A2 (2.2%).

Following this, all patients DNA samples, together with appropriate number of controls were subjected to multiplex Gap-PCR screening for four different types of the β -globin gene cluster deletions namely $G\gamma(A\gamma\delta\beta)$ ^o-thal (Siriraj J Deletion~118 kb del), Thai ($\delta\beta$)^o-thal (~12.5 kb deletion), HPFH-6 and HbLepore. These mutations have been shown to

have high prevalence rate among the Southeast Asia populations.^{19,25}

Our initial work was focusing on optimizing relevant parameters via multiple rounds of singleplex PCR, which act as a basic step for multiplex Gap-PCR. These include assessing the specificity of the individual primer, optimizing the annealing temperature (T_m), adjusting the concentration of MgCl₂ and the amount of DNA template. Once the crucial parameters have been optimized, extensive molecular screening for all samples against the target deletions were performed. Overall, the screening of patient samples using multiplex Gap-PCR showed only one patient was found to be positive with Thai ($\delta\beta$)^o-thalassemia deletions. Low positive rate result in this study may be due to only limited types of deletions were screened. Nevertheless, technically the amplification of the multiplex Gap-PCR was considered successful since all included controls were working optimally. Non-template control (NTC) as well as normal/healthy individual control samples produced no visible band indicating that there was no contamination occurred or the control samples were free from any of the mutations screened.

Hypothetically Hb F level for homozygous $\delta\beta$ -thalassemia may achieve 100% and clinically it exhibits the features of thalassemia intermedia rather than the thalassemia major. In contrast, the phenotype of heterozygotes $\delta\beta$ -thalassemia may resemble the β -thalassemia trait, with 5-20% of Hb F levels, but with almost normal Hb A2. To date, there are different types of $\delta\beta$ -thalassemia that have been reported in different ethnic groups and regions of Southeast Asian countries, India, Africa, Central America, and the Middle East.²⁶⁻²⁷

To date, there are approximately 12 types of deletions in the β -globin gene cluster that have been well characterized within the Southeast Asian community, which are the β ^o-thal 105 bp deletion, the 619 bp deletion, the 3.5 kb deletion, the novel $G\gamma(A\gamma\delta\beta)$ ^o-thal, Siriraj J deletion (~ 118 kb) and the Southeast Asian (SEA) deletion (~27 Kb). This list also include the Filipino deletion (~45 Kb), HbLepore (~7.4 Kb deletion), the Thai ($\delta\beta$)^o-thal deletion (~12.5 Kb), the Chinese $G\gamma(A\gamma\delta\beta)$ ^o-thal deletion (~100 Kb) and the Asian Indian deletion-inversion $G\gamma(A\gamma\delta\beta)$ ^o-thal, as

well as HPFH-6 and Vietnamese HPFH (also known as a HPFH-7 in HbVar) deletions. The reported cases of the β -globin deletion from the Southeast Asian countries such as Malaysia are exponentially increasing due to the migration and multiracial ethnics of its population.¹⁹

Following the multiplex gap-PCR testing, the molecular screening was performed on 12 randomly selected samples using the MLPA technique. The results revealed that another one patient (patient P2) was found to have double deletions within the β -globin gene cluster, detected at HBG1 ($G\gamma$ -gene) and HBG2 ($A\gamma$ -gene) in exon-3 region. This 23-years-old female patient depicted a slight increase in her Hb F (3.6%), but has a normal Hb A2 (2.3%) as shown by the hematological profile. Detail MLPA analysis showed that the final calculated probe ratio for HBG1 and HBG2 was 0.60, which indicated a heterozygous deletion.

In principle, during a developmental growth phase, the expression of γ -gene should be declined as the expression of the β -globin gene begins.²⁸ However, any deletion that occurs at the γ -gene may cause the expression of this gene to be distorted. Thein and Wood (2009) postulated that high Hb F levels might be due to the deletion of regulatory sequences affecting γ -gene expression.²⁹ For P2 patient, the deletion was detected at the specific part of the γ -gene, but not on the entire region itself. Therefore, other γ -gene at different exons could be continuously developed or expressed in this globin. Perhaps, this could possibly explain the slight increment of Hb F observed in this patient (Hb F = 3.6%). This condition has also been clearly discussed by others. Interestingly Lee *et al.* (2010)³⁰, has also observed similar type of deletion in one their male patient (Hb F: 5.9%, Hb A2: 2.5%) that depicted half dosages for two probes targeted for exon 3 of HBG2 and a genomic region between HBG2 and HBG1 as revealed by MLPA. This condition is known as HBG2–HBG1 fusion, although the exact mechanism of this condition is still unknown. It was hypothesized that HBG2 and HBG1 may play a role in Hb F regulation, and combinations of specific polymorphisms could influence reactivation of HBG2 and HBG1 in adult life, resulted in moderately increased of Hb F. Furthermore, it was also noted that deletion at the β -globin gene cluster which is

associated with the $\delta\beta$ -thalassemia trait lead to the elevated level of Hb F (5 to 20%), while moderate increment of Hb F (< 10%) is commonly associated with the β -thalassemia trait.¹²

Overall both multiplex Gap-PCR and MLPA used in this study for the screening of the selected β -globin gene cluster deletions on a specific cohort of hematology patients were shown to be feasible. Nonetheless, there are several limitations in this study. The study can be improved by creating a larger study in term of; the number of subject enrolled, types of β -globin gene cluster deletions screened as well as to expand the study to include different ethnics in Malaysian populations. This study would provide a wider spectrum of deletions in the β -globin gene cluster which can be accurately established in the future. Clearly, the limited number of samples used in the current study definitely could not fairly represent the spectrum mutations among the Malaysian population. However, these data may act as basis for a larger research study in the future, in particular when the task seems to be more challenging in a multiethnic country like Malaysia.

Conclusion

In conclusion, this study highlighted the importance of screening and characterization of the β -globin gene cluster deletions using multiplex Gap-PCR and MLPA which helps in establishing their clinical relevance, thus providing a definitive diagnosis among the selected group of patients. Both techniques are found to be robust, easy to handle and reliable, hence suitable to be incorporated as one of the screening tools in a molecular diagnostic routine test panel.

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Conflict of interest: The authors have no conflict of interests.

Authors' contribution

Listed below the role of each author:

1. Yasmin Mohamad Redzuwan- sample collection, performed the MLPA, analyzed the data and wrote the paper
2. Siti Nor Assyuhada Mat Ghani - performed the Gap-PCR and analyzed the data
3. Rozanah Abdul Ghani - sample collection and analyzed the data
4. Yousef Saeed Mohammad Abu Za'ror - performed the research and contributed to data analysis
5. Rosnah Bahar - design the research and edited the manuscript
6. Maryam Azlan - advised on statistics and edited the manuscript
7. Zefarina Zulkaffi - design and fund the research, obtained ethical approvals, edited the manuscript and approval of final draft
8. Roziyati Mohamed Saleh - design, coordinated and fund the research, managed the DNA samples, obtained ethical approvals, advised on statistics edited the manuscript and approval of final draft.

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