



**UNIVERSITI PUTRA MALAYSIA**

**MOLECULAR CHARACTERISATION OF BETA THALASSAEMIA  
IN PATIENTS FROM SABAH, MALAYSIA**

**TEH LAI KUAN**

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BERILMU BERBAKTI

**MOLECULAR CHARACTERISATION OF BETA THALASSAEMIA  
IN PATIENTS FROM SABAH, MALAYSIA**



By

**TEH LAI KUAN**

**Thesis Submitted to the School of Graduate Studies,  
Universiti Putra Malaysia, in Fulfilment of the Requirements for the  
Doctor of Philosophy**

**September 2014**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Doctor of Philosophy

**MOLECULAR CHARACTERISATION OF BETA THALASSAEMIA IN PATIENTS FROM SABAH, MALAYSIA**

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**TEH LAI KUAN**

**September 2014**

**Chairman : Professor Elizabeth George, PhD**  
**Faculty : Medicine and Health Sciences**

Sabah has the largest number of  $\beta$ -thalassaemia major ( $\beta$ -TM) patients in Malaysia with estimated over 1000 cases of transfusion dependent  $\beta$ -TM patients. However, complete molecular characterisation of thalassaemia major patients has not been done. The objective for this study is to characterise the molecular spectrum in Sabah population through  $\beta$ - and  $\alpha$ -globin gene genotyping, identifying *XmnI*  $\text{G}\gamma$ -polymorphism, haplotyping for  $\beta$ -globin gene cluster and to develop an ideal diagnostic algorithm and tools which is suitable for this population.

In this study, 252  $\beta$ -TM patients (Group I) and 165 carriers (Group II) were recruited from ten different hospitals in Sabah. Filipino  $\beta^0$ -deletion was the predominant mutation identified in the Kadazandusun, Rungus, Murut, Sungai and Bajau. A total of 219 (86.9%)  $\beta$ -TM patients were identified as homozygous Filipino  $\beta^0$ -deletion. HbE and Hb Malay were found as the most common Hb variants to co-inherit with Filipino  $\beta^0$ -deletion. Some common mutations in West Malaysia were found to co-exist with Filipino  $\beta^0$ -deletion. This can be due to intermarriage between different ethnic groups. Carriers showed the frequency of Filipino  $\beta^0$ -deletion at 95.2% (n=157). Only seven (4.2%) carriers were found with point mutations commonly seen in West Malaysia.

High frequency of co-inheritance of  $-\alpha^{3.7}$  deletion was found in the Sabah  $\beta$ -thalassaemia population. Co-inheritance of heterozygous  $-\alpha^{3.7}$  deletion was found in 67 (26.6%)  $\beta$ -TM patients and 42 (25.3%) carriers. Co-inheritance of homozygous  $-\alpha^{3.7}$  deletion was found in seven (2.8%)  $\beta$ -TM patients and six (3.6%) carriers. This may be related to the natural selection and protection for survival from severe malaria (*Plasmodium Falciparum*). Only type I of  $-\alpha^{3.7}$  deletion was observed in this study population, indicating that the population has a single origin.

*XmnI*  $\text{G}\gamma$  polymorphism was reported with higher  $\text{G}\gamma$ -globin gene expression. Clinical presentation will be ameliorated in homozygous states. In this study, *XmnI* (-/-) genotype was found in 237 (94%)  $\beta$ -TM patients and 156 (94.4%) carriers, indicating low existence of this polymorphism as an ameliorating factor.

In haplotyping analysis, seven haplotype patterns were inferred in 417 samples consisting of 252  $\beta$ -TM patients and 165 carriers. Hp I (+ - - -) was the predominant

pattern demonstrated in 98.14% of the population. This suggested a unicentric origin and an apparent single origin with low genetic diversity. This is the first report to demonstrate Hp I in the Sabah population with Filipino  $\beta^0$ -deletion.

Two new diagnostic tools, Taqman and HRM analysis were developed using real-time detection for Filipino  $\beta^0$ -deletion. Taqman analysis was found more ideal as a diagnostic tool by having high specificity and sensitivity although it is more expensive. An added advantage is that there is no requirement for post-PCR processing. Multiplex ligation-dependent probe amplification (MLPA) analysis is an efficient technique for the screening of large deletions which can be included in the diagnosis algorithm provided technical expertise and necessary funding are available.

This study reveals a notable regional specificity of the  $\beta$ - and  $\alpha$ -thalassaemia mutations, which are Filipino  $\beta^0$ -deletion and  $-\alpha^{3.7}$  deletion. *XmnI* polymorphism is uncommon in this study population. From the haplotype analysis and type of  $-\alpha^{3.7}$  deletion, the findings suggested that the Sabah population with  $\beta$ -thalassaemia may belong to the same stock with similar origin. Taqman analysis is more ideal as a diagnostic tool. The findings from this study are informative for molecular diagnosis in the Sabah population with  $\beta$ -thalassaemia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**ASAS MOLEKUL BAGI PESAKIT BETA TALASEMIA DARI SABAH,  
MALAYSIA**

Oleh

**TEH LAI KUAN**

**September 2014**

**Pengerusi : Profesor Elizabeth George, PhD**  
**Fakulti : Perubatan dan Sains Kesihatan**

Sabah mempunyai bilangan pesakit  $\beta$ -talasemia utama ( $\beta$ -TM) yang paling banyak di Malaysia dengan anggaran melebihi 1000 kes pesakit  $\beta$ -TM bergantung kepada pemindahan darah. Walau bagaimanapun, pencirian molekul lengkap pesakit talasemia utama tidak dilakukan. Objektif kajian ini adalah untuk mencirikan spektrum molekul penduduk Sabah melalui  $\beta$ - dan  $\alpha$ -globin gen genotip, mengenal pasti XmnI  $G\gamma$ -polymorphism, haplotyping untuk kelompok  $\beta$ -globin gen dan untuk membangunkan algoritma diagnostik yang ideal dan alat-alat yang sesuai untuk populasi ini.

Dalam kajian ini, 252 pesakit  $\beta$ -TM (Kumpulan I) dan 165 pembawa (Kumpulan II) telah dikumpul dari sepuluh hospital yang lain di Sabah. Filipino  $\beta^0$ -deletion adalah mutasi yang utama yang didapati dalam Kadazandusun, Rungus, Murut, Sungai dan Bajau. Seramai 219 (86.9%) pesakit  $\beta$ -TM telah dikenal pasti sebagai homozigus Filipino  $\beta^0$ -deletion. HbE dan Hb Malay didapati sebagai Hb varian yang paling biasa bersama dengan Filipino  $\beta^0$ -deletion. Sebahagian mutasi yang biasa di Semenanjung Malaysia telah didapati wujud bersama dengan Filipino  $\beta^0$ -deletion. Ini disebabkan oleh perkahwinan campur antara kumpulan-kumpulan etnik yang berbeza. Pembawa menunjukkan Filipino  $\beta^0$ -deletion dengan kekerapan sebanyak 95.2% ( $n = 157$ ). Hanya tujuh (4.2%) pembawa ditemui dengan mutasi titik yang biasa dijumpai di Semenanjung Malaysia.

Kekerapan yang tinggi bersama warisan bagi  $-\alpha^{3.7}$  deletion bagi penduduk  $\beta$ -talasemia di Sabah. Bersama warisan bagi heterozigot  $-\alpha^{3.7}$  deletion didapati sebanyak 67 (26.6%) pesakit  $\beta$ -TM dan 42 (25.3%) pembawa. Bersama warisan bagi homozigus  $-\alpha^{3.7}$  deletion didapati sebanyak tujuh (2.8%) pesakit  $\beta$ -TM dan enam (3.6%) pembawa. Ini mungkin berkaitan dengan pemilihan semula jadi dan perlindungan daripada penyakit malaria (*Plasmodium falciparum*). Hanya  $-\alpha^{3.7}$  deletion bentuk I didapati dalam populasi kajian ini, ini menunjukkan bahawa penduduk mempunyai asal usul yang tunggal.

XmnI  $G\gamma$  polymorphism dilaporkan dengan ekspresi  $G\gamma$ -globin gen yang lebih tinggi. Persembahan klinikal akan dikurangkan apabila dalam bentuk homozigus Dalam kajian ini, XmnI (-/-) genotip didapati dalam 237 (94%) pesakit  $\beta$ -TM dan 156

(94.4%) pembawa, menunjukkan kewujudan polymorphism ini sebagai faktor pembaiki adalah rendah.

Dalam analisis haplotyping, tujuh corak haplotaip telah disimpulkan dalam 417 sampel yang terdiri daripada 252 pesakit  $\beta$ -TM dan 165 pembawa. Hp I (+ - - -) adalah corak yang utama ditunjukkan dalam 98,14% daripada penduduk. Ini mencadangkan asal usul yang tunggal dan jelas dengan dengan kepelbagaian genetik yang rendah. Ini adalah laporan pertama menunjukkan Hp I bagi penduduk Sabah dengan Filipino  $\beta^0$ -deletion.

Dua diagnostik alat baru, Taqman dan HRM analisis telah dicipta dengan menggunakan pengesanan real-time untuk Filipino  $\beta^0$ -deletion. Analisis Taqman didapati lebih sesuai sebagai alat diagnostik dengan mempunyai kekhususan dan sensitif walaupun ia lebih mahal. Selain itu, ia tidak memerlukan pemprosesan pasca PCR. Analisis Multiplex ligation-dependent probe amplification (MLPA) adalah teknik berkesan untuk tayangan pemotongan besar yang boleh dimasukkan dalam algoritma diagnosis kalau mempunyai kepakaran teknikal dan pembiayaan yang cukup.

Kajian ini mendedahkan khusus serantau yang ketara bagi mutasi  $\beta$ - dan  $\alpha$ -talasemia, iaitu Filipino  $\beta^0$ -deletion dan  $-\alpha^{3.7}$  deletion. *XmnI* polymorphism adalah jarang dijumpai bagi populasi kajian ini. Daripada analisis haplotaip dan bentuk  $-\alpha^{3.7}$  deletion, ia mencadangkan bahawa penduduk Sabah dengan  $\beta$ -talasemia mungkin tergolong dalam asal-usul yang sama. Analisis Taqman lebih sesuai sebagai alat diagnostik. Hasil kajian ini adalah berguna untuk diagnosis molekul penduduk Sabah dengan  $\beta$ -talasemia.



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I certify that a Thesis Examination Committee has met on 29 September 2014 to conduct the final examination of Teh Lai Kuan on her thesis entitled "Molecular Characterisation of Beta Thalassaemia in Patients from Sabah, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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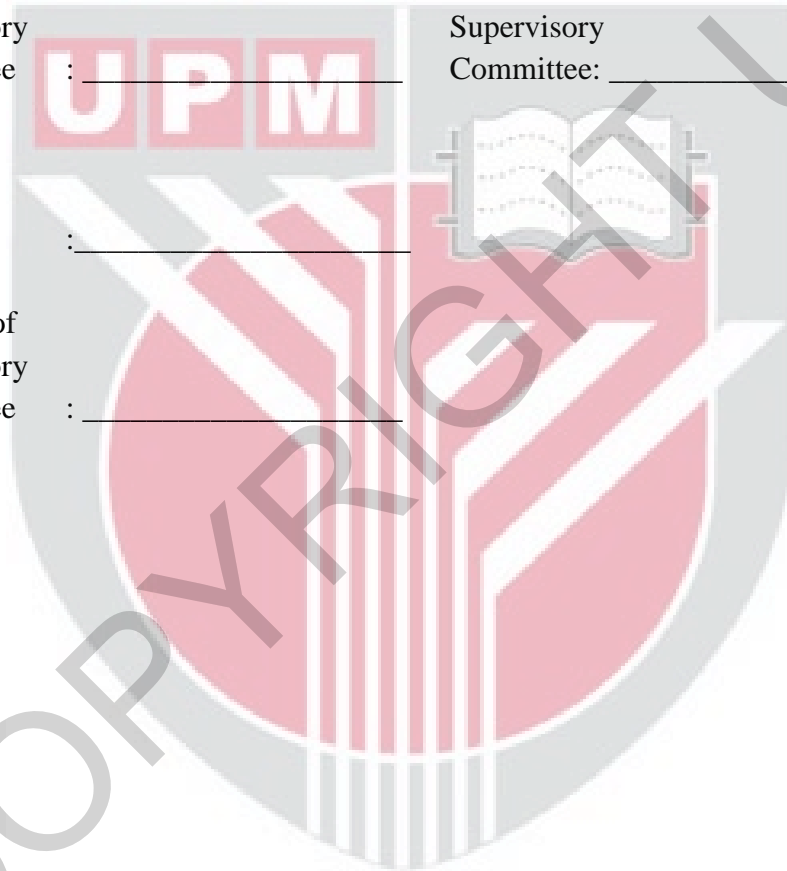
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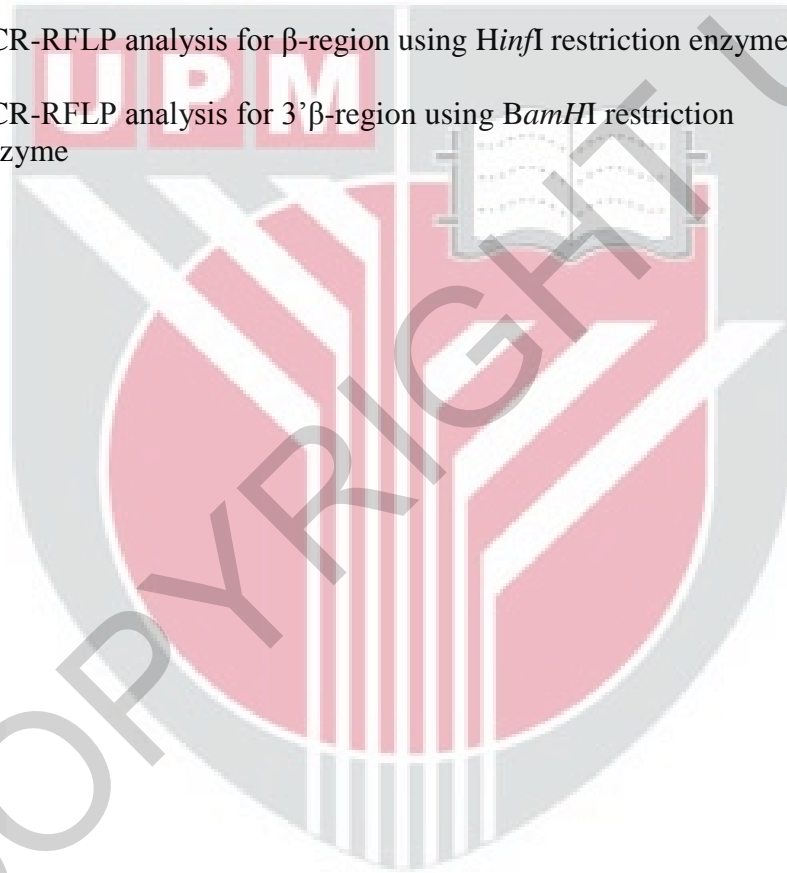
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## LIST OF ABBREVIATIONS

$\alpha$	alpha
$\beta$	beta
$\delta$	delta
$\epsilon$	epsilon
$\gamma$	gamma
$\zeta$	zeta
$\psi$	pseudo
$\mu$	micro
$\mu\text{l}$	microlitre
$\mu\text{g}$	microgram
nm	nanometer
ml	millilitre
bp	base pair
kb	kilobase
Hb	haemoglobin
RBC	red blood cell
LCR	locus control region
HS	hypersensitive site
MCV	mean corpuscular volume
MCH	mean corpuscular haemoglobin
RDW	red cell distribution width
DNA	deoxyribonucleic acid
TAE	tris-acetate- ethylenediaminetetraacetic acid
TM	thalassaemia major

ARMS	amplification refractory mutations system
PCR	polymerase chain reaction
RDBH	reverse dot blot hybridisation
MLPA	multiplex ligation-dependent probe amplification
RFLP	restriction fragment length polymorphism
RE	restriction enzyme
HRM	high resolution melting
DQ	dosage quotient
OD	optical density
Het	heterozygous
Hom	homozygous
Cd	codon
IVS	intervening
HW	Hardy-Weinberg
LD	Linkage disequilibrium
FAM	6-carboxyfluorescein
VIC	2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein



## CHAPTER 1

### INTRODUCTION

#### 1.1 Research Background

Malaysia has a huge diversity and multiracial population of over 28.3 million, which approximately 80% are from peninsular Malaysia and 20% from Sabah and Sarawak (Population by Age Group, Department of Statistics Malaysia, 2010). In East Malaysia, 70% of Sabah's and 50% of Sarawak's population are indigenous. In Sabah, the population was 3,117,405 in the year 2010 (Population Distribution and Basic Demographic Characteristic Report 2010) and divided into 35 officially recognised ethnic groups. The racial groups are heterogenous. The biggest indigenous ethnic group in Sabah is Kadazandusun, followed by Bajau and Murut.

Beta thalassaemia is the commonest inherited disease in Malaysia that affects about 4.5% of the Malaysian population (George *et al.*, 1992; Tan *et al.*, 2004). Whereas, in Sabah, the estimated prevalence rate of  $\beta$ -thalassaemia can be up to 10% (Malaysian Thalassaemia Registry, 2009 August). In  $\beta$ -thalassaemia patients, there is reduced production or absence of  $\beta$ -chains from the  $\beta$ -globin gene on chromosome 11. The resulting imbalanced globin chain production lead to reduced haemoglobin synthesis and anaemia, while the severity depends on the affected genes by different mutations or different combination of mutations inherited (Bowden, 2001). Partners who are  $\beta$ -thalassaemia carriers have 25% risk of getting a baby with  $\beta$ -thalassaemia major ( $\beta$ -TM) in each pregnancy which is transfusion dependent and requires iron chelation for life (George, 2001; Bowden, 2001).

There are over 250 mutations that result in  $\beta$ -thalassaemia (Thein, 2005b). Each ethnic group has four to five common mutations that form more than 95% of the mutations seen (George, 2001). In the last 15 years, the molecular epidemiology of  $\beta$ -globin mutations has been well documented in the main racial groups in Peninsular Malaysia (West Malaysia) (Malays by George *et al.*, 1992 and Chinese by George-Kodiseri *et al.*, 1990 and George *et al.*, 1993). In Sabah, there are estimated over 1000 cases of transfusion dependent  $\beta$ -TM. Hitherto, the spectrum of  $\beta$ -thalassaemia mutations in the various indigenous populations in East Malaysia is still not clear with only three studies done by Thong *et al.* in year 1999 and 2005; Tan *et al.* in year 2010 and the main mutation noted was the Filipino  $\beta^0$ -deletion.

Besides high prevalence of  $\beta$ -thalassaemia among indigenous population in Sabah, Tan *et al.* (2010) found high prevalence of  $\alpha$ -thalassaemia among the Kadazandusun in Sabah, especially the single  $\alpha$ -globin gene deletion ( $-\alpha^{3.7}$ ), in 33.6% (42/125). In West Malaysia, studies about co-inheritance of  $\alpha$ -thalassaemia with  $\beta$ -thalassaemia or HbE patients have been carried out by Wee *et al.* (2008), Tan *et al.* (2009) and Teh *et al.* (2009). Variable clinical heterogeneity was demonstrated depending on the involved co-inheritance

mutations (Wee *et al.*, 2008; Tan *et al.*, 2009 and Teh *et al.*, 2009). It is essential to characterise the molecular basis of thalassaemia in this region to provide better healthcare for the indigenous population of Sabah.

*Xmn*I  $\gamma$ -polymorphism (rs782144) with transition of C to T at the -158 bp from cap site 5' $\gamma$ -globin gene is correlated with higher  $\gamma$ -globin gene expression and results in elevation of HbF level, especially under erythropoietic stress. It has been found as an ameliorating factor for the phenotype of  $\beta$ -thalassaemia patients when in homozygous state (Thein, 2005a). Therefore, it is important to identify this polymorphism to provide more information to explain patient's clinical phenotype.

The origin and migration pattern of  $\beta$ -thalassaemia patients has not been identified in the indigenous population. It can be determined through haplotyping. Haplotype is the combination of allelic states lying along a chromosome constructed from a set of SNPs or linked markers that are genetically stable and inherited as a group. According to Lee *et al.* (2002), haplotyping can elucidate the molecular background of the  $\beta$ -globin gene clusters by comparing the haplotypes among the  $\beta$ -thalassaemia patients or by family linkage study. According to Gupta *et al.* (2008), analysis of polymorphic markers are important in haplotype-genotype and phenotype association for  $\beta$ -thalassaemia. Falchi *et al.* (2005) and Gupta *et al.* (2008) also found haplotype analysis as an important tool for tracing the spread of  $\beta$ -thalassaemia mutations to different regions and its origin. Haplotyping study is included in this study as a valuable tool to relate human genetic variation to  $\beta$ -thalassaemia and for understanding human evolutionary history.

PCR techniques [amplification refractory mutation system (ARMS-PCR), reverse dot blot hybridisation (RDBH) and Gap-PCR] used in thalassaemia mutation detection allow only end point detection and post PCR-processing procedures such as gel electrophoresis, gel labelling or colour development are required. All these techniques are labour-intensive, time consuming and tedious when a few mutations are needed to be identified for each patient. Therefore, it is essential to develop a new, sensitive, quick and accurate technique to overcome this problem. In this study, a real-time platform will be utilised in development new methods, which allow monitoring the PCR amplification progress in real-time by the fluorescence signals in each amplification reaction cycle.

## 1.2 General Objective

Beta thalassaemia is common in Sabah with the estimated prevalence rate up to 10%. However, there is limited information of the complete spectrum of thalassaemia mutations in Sabah population with the molecular epidemiology of the mutations not well documented. The general objective in this study was:

- To determine the molecular basis of thalassaemia mutations in transfusion dependent beta thalassaemia in Sabah

### 1.3 Specific Objectives

Co-inheritance of  $\alpha$ -thalassaemia and *XmnI* polymorphism as an ameliorating factor in  $\beta$ -TM patients has been commonly reported in West Malaysia. However, there is no study done before in Sabah to identify co-inheritance of  $\alpha$ -thalassaemia and *XmnI* polymorphism. The following was carried out to meet these objectives:

1. To characterise the spectrum of  $\beta$ -thalassaemia mutations and co-inheritance of  $\alpha$ -thalassaemia in  $\beta$ -thalassaemia major patients and carriers.
2. To identify *XmnI*  $\text{G}\gamma$ -polymorphism in  $\beta$ -thalassaemia major patients and carriers.

In Sabah, a specific mutation, Filipino  $\beta^0$ -deletion has been reported. Therefore, to relate the human genetic variation and the evolutionary history to this specific  $\beta$ -thalassaemia mutation, haplotyping was carried out. The following was carried out to meet this objective:

3. To determine the haplotype of the  $\beta$ -globin gene and its origin of the mutations identified.

Conventional methods used in detection of  $\beta$ -thalassaemia mutation are tedious which allowed only end-point analysis and required post PCR-processing. It is also not sensitive in low DNA concentration detection. Therefore, a new diagnostic tool without post-PCR processing and sensitive in low DNA concentration detection was developed for clinical diagnosis purposes. The following was carried out to meet this objective:

4. To develop a novel diagnostic tool and to design a simple and accurate algorithm incorporating PCR approach to identify  $\beta$ -thalassaemia mutations in Sabah population.

#### 1.4 Significance of study

The molecular basis of  $\beta$ -thalassaemia patients among Sabah population was characterised in this study. Co-inheritance of  $\alpha$ -thalassaemia mutations and  $XmnI$   $G\gamma$ -polymorphism among Sabah population were delineated in this study. The information from this study will lead to the development of informative diagnostic protocol. The origin and historical background of these mutations were identified through the association of  $\beta$ -globin gene cluster haplotypes with  $\beta$ -thalassaemia mutations among population of indigenous people in Sabah. This study generated a platform to design an algorithm for molecular diagnosis, which is feasible and accurate in a laboratory to be set up in Sabah where thalassaemia is a public health problem.





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