CLINICO-HAEMATOLOGICAL PROFILING OF TRANSFUSION DEPENDENT AND NON TRANSFUSION DEPENDENT BETA THALASSAEMIA SYNDROME IN KEDAH

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

Symbol/Abbreviation	Meaning
%	percentage
^{0}C	degree celcius
µ	Micro
ARMS-PCR	Amplification Refractory Mutation System-Polymerase Chain Reaction
bp	Base pair
CE	Capillary Electrophoresis
DC	Direct current
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
et al	et alia (and others)
FBC	Full Blood Count
FBP	Full Blood Picture
fl	Femtolitre
Glu	Glutamate
GH-IGF-1	Growth Hormone-Insulin like growth factor 1
Hb A	Haemoglobin A
Hb A_2	Haemoglobin A_2
Hb E	Haemoglobin E

Symbol/Abbreviation	Meaning
Hb F	Haemoglobin F
Hb H	Haemoglobin H
Hct	Haematocrit
HPLC	High Performance Liquid Chromatography
HUSM	Hospital Universiti Sains Malaysia
HSB	Hospital Sultanah Bahiyah
IMR	Institute Medical Research
kb	Kilobase
KKM	Kementerian Kesihatan Malaysia
Lys	Lysine
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
NTDT	Non transfusion Dependent Thalassaemia
PCR	Polymerase chain reaction
pg	pictograms
RBC	Red Blood Cell
RT	Room Temperature
RDW	Red cell distribution width
SLS	Sodium Lauryl Sulphate
SNP	Single nucleotide polymorphism

Symbol/Abbreviation	Meaning
TDT	Transfusion dependent thalassaemia
TI	Thalassaemia Intermedia
TM	Thalassaemia Major
TT	Thalassaemia Trait
UTR	Untranslated region
α	alpha
β	beta
β/β	Normal functional beta globin chain
β^{+}	Beta plus
β^{+}/β	Heterozygous β^{+}
$\beta^{+}/\ \beta^{+}$	Homozygous beta plus
β^{0}	Beta Zero
β^{+}/β	Heterozygous beta zero
$\beta^{0/}\ \beta^{0}$	Homozygous beta zero
$\beta^{+}/\ \beta^{0}$	Compound heterozygous of beta plus and beta zero
β^{E}	Haemoglobin E
$\beta^{E}/\ \beta$	Heterozygous Haemoglobin E
$\beta^{E}/\ \beta^{+}$	Compound heterozygous of Haemoglobin E and beta plus
$\beta^{E}/\ \beta^{0}$	Compound heterozygous of Haemoglobin E and beta zero
γ	Gamma
ε	Epsilon

PROFIL KLINIKAL DAN HEMATOLOGI DI KALANGAN PESAKIT BETA TALASEMIA YANG MEMERLUKAN TRANFUSI DARAH SECARA BERKALA DAN TIDAK BERKALA DI NEGERI KEDAH

ABSTRAK

Pengenalan: Beta (β) talasemia adalah penyakit berkaitan hemoglobin yang disebabkan oleh pengurangan atau ketiadaan β globin. Individu dengan β talasemia boleh menjadi heterozigot, heterozigot kompaun, atau homozigot serta boleh mempunyai interaksi dengan hemoglobinopati lain. Kedah mempunyai kadar pesakit talasemia yang tinggi dengan kadar 20.25 di dalam 100 000 penduduk. Mutasi yang berbeza dalam gen globin β menyebabkan kepelbagaian di dalam ciri-ciri klinikal pesakit β talasemia. Oleh itu, pengenalpastian genotip adalah penting untuk kaunseling. Objektif: Untuk menentukan profil klinikal, hematologi dan β mutasi pesakit β talasemia di Kedah. Kaedah: 100 pesakit β talasemia dikenalpasti melalui sistem maklumat hospital. Rekod sejarah beserta laporan makmal direkodkan. Untuk kajian mutasi β talasemia, pengesanan terhadap 20 jenis mutasi dijalankan melalui kaedah M-ARMS. Keputusan kajian dan perbincangan: Daripada 100 pesakit yang dikaji, 66% daripada mereka adalah pesakit yang bergantung kepada transfusi darah secara berkala (TDT) manakala 34% adalah tidak bergantung kepada transfusi darah secara berkala (NTDT). Dalam kajian ini, pesakit TDT didiagnos pada purata usia yang lebih muda 2.75 (2.28) tahun berbanding dengan pesakit NTDT, iaitu pada purata umur 19.87 (17.87) tahun. Pesakit TDT juga didapati mepunyai lebih kadar tumbesaran yang terencat dan kadar pembesaran limpa yang lebih signifikan berbanding dengan pesakit β talasemia NTDT. Perbezaan yang ketara antara parameter hematologi dalam kedua-dua kumpulan pesakit telah didapati di dalam kajian ini.

Kebanyakan pesakit di dalam kedua-dua kumpulan adalah di kalangan pesakit talasemia HbE/ β talasemia. Di kalangan pesakit TDT, mutasi yang paling kerap didapati adalah CD26 (G> A) /IVS1-5 (G> C) (28.8%), diikuti dengan CD26 (G> A) / IVS1-1 (G> T) (13.6%) dan CD26 (G> A) / CD41 / 42 (-TTCT) (13.6%). Manakala, di dalam kumpulan pesakit NTDT, mutasi yang lazim dikesan ialah IVS1-1 (G> T) dan CD19 trans kepada Hb E (CD26 (G> A) masing-masing sebanyak 32.4% dan 29.4%. Mutasi CD26 (G> A) / IVS1-1 (G> T), didapati dengan jumlah yang hampir sama untuk kedua-dua jenis kumpulan pesakit. Oleh itu, menunjukkan terdapat pengaruh pengubah suai genetik lain yang menyebabkan kepelbagaian di dalam ciri-ciri klinikal pesakit. Pesakit dengan tahap Hb A yang lebih tinggi semasa diagnosis mempunyai lebih rendah risiko untuk bergantung kepada transfusi darah (adjusted OR 0.93, 95% CI: 0.90,0.97; p <0.001), manakala tahap Hb F yang lebih tinggi mempunyai lebih risiko untuk bergantung kepada tranfusi secara berkala (adjusted OR 1.04, 95% CI:1.01,1.07, p = 0.007). Kesimpulan: Terdapat kepelbagaian di dalam jenis mutasi, profil hematologi dan klinikal didapati di kalangan pesakit β talasemia di Kedah.

(400 perkataan)

CLINICO-HAEMATOLOGICAL PROFILING OF TRANSFUSION DEPENDENT AND NON TRANSFUSION DEPENDENT BETA THALASSAEMIA SYNDROME IN KEDAH

ABSTRACT

Introduction: β thalassaemia is a group of haemoglobin diseases caused by a reduction or absence in the synthesis of β globin chains. Affected individuals can be heterozygous, compound heterozygous, or homozygous for β thalassaemia, or even have interactions with other haemoglobinopathies. Kedah has a high prevalence of thalassaemia patients with 20.25/100000 populations. Different mutations have been identified in the β globin (*HBB*) gene generating diverse clinical phenotypes in β thalassaemia. The identification of the genotype is important for proper counselling to patients and their families. Objectives: To determine the clinical, haematology and β mutation profiling of β thalassaemia patients in Kedah. Methods: 100 patients with β thalassaemia were traced through hospital information system. A detailed clinical and laboratory records of patients were retrieved. For β thalassaemia genotyping, 20 different mutations were tested by MARMS technique. Result and Discussions: Out of 100 patients, 66% were transfusion dependent (TDT) and 34% of the studied patients were non transfusion dependent (NTDT). In this study the TDT patients had significantly lower mean age of diagnosis with 2.75 (2.28) years as compared to 19.87(17.87) years in NTDT (p <0.001). There was statistically significant presence of short stature and a larger mean size of spleen in patients with TDT as compared to NTDT. The significant differences between haematological parameters in both groups of patient were well observed in this study. Both NTDT and TDT group of patients were among compound heterozygous Hb E/ β

thalassaemia with 85.3% and 75.8% respectively. In TDT patients, the commonest mutations observed was CD26(G>A)/IVS1-5(G>C) (28.8%), followed with CD26(G>A)/IVS1-1(G>T) (13.6%) and CD26(G>A)/CD41/42(-TTCT) (13.6%). In the NTDT group, the common mutations observed were IVS1-1(G>T) and CD19 trans to Hb E (CD26 (G>A) with 32.4% and 29.4%, respectively. It was noted in this study, diversity of phenotypes in patients with mutation CD26(G>A)/IVS1-1(G>T), thus indicating other genetic modifiers to be explored in these patients. An increment of 1 % of Hb A level at diagnosis have reduced odds of transfusion dependency by 7% (adjusted OR 0.93,95% CI: 0.90, 0.97; p<0.001) when adjusted for Hb F. Whereas, an increase in 1% of Hb F level, had 1.04 times higher odds to be transfusion dependent (adjusted OR 1.04,95% CI: 1.01, 1.07, p = 0.007) when adjusted for Hb A level. Conclusion: There are diversity of type of β mutation. haematological and clinical parameters among β thalassaemia patients in Kedah.

(399 words)

CHAPTER 1

INTRODUCTION

CHAPTER 1 : INTRODUCTION

1.1 General Introduction

Thalassaemia is the most common single-gene haemoglobinopathy worldwide, and together with sickle cell disease, it is estimated that over 5% of the world's population are carriers (Yamsri *et al.*, 2015). It has been estimated that around 300 000 to 400 000 babies born with severe form of this disease each year (Weatherall, 2012).

Thalassaemia is the commonest genetic disorder in Malaysia where 3–4.5% of the Malays and Chinese are carriers (G Elizabeth and Ann, 2010). There were 4768 registered thalassaemia patients in Malaysia reported in 2010 and between 600000 and 1000000 carriers of the thalassaemia trait (Yatim *et al.*, 2014). There are an estimated number of 120 – 350 babies born with thalassaemia major each year. Kedah has a high prevalence of thalassaemia patients (4th highest state), with 20.25/100000 populations (Ibrahim, 2012). Among the thalassaemia patients in Malaysia, β thalassaemia major comprises of 44.7% whereas Hb E/β thalassaemia and β thalassaemia intermedia was 31.6% and 9.8% respectively, with 75% of the them were among transfusion dependent patients. This has become a major public health problem, with the government spending big amount of money for the management of the patient (G Elizabeth and Ann, 2010). According to E George et al, in Malaysia, management of patients with transfusion dependent thalassaemia has become a heavy burden for health authorities with less than 20% of patients received adequate iron chelation therapy and the majority of them are destined to decease in the second or third decade of life as result of complications of multiple organ failure secondary to iron overload (George, 2001).

β thalassaemia is a group of haemoglobin diseases caused by either reduction (β^+ thalassaemia) or absence (β^0 thalassaemia) in the production of β globin chains. The affected persons can be either heterozygous (β^+/β or β^0/β), compound heterozygous ($\beta+/\beta^0$), or homozygous for beta-thalassaemia (β^+/β^+ or β^0/β^0), or even have interactions with other haemoglobinopathies (β^+ or β^0 with other Hb variant). Their phenotypes include hypochromic and microcytic anaemia, elevated Hb A_2 levels, and various syndromes as a result of the combination of β^0 and β^+ alleles. The pathophysiology of β thalassaemia comprises of ineffective erythropoiesis, peripheral haemolysis due to the presence of precipitation of alpha chain, and subsequent chronic anaemia and its long term sequelaes.

The clinical presentation of β thalassaemia displays a broad clinical variation or phenotypes, ranging from a complete absence of transfusions at one end of the spectrum to regular transfusions at the other end. With regard of clinical phenotype, β thalassaemia can be divided into three main categories: thalassaemia major (TM), thalassaemia trait (TT) and thalassaemia intermedia (TI). TM is the most severe form which requires transfusions early from infancy age for survival and also known as transfusion dependent thalassaemia (TDT), whereas TT is frequently asymptomatic. TI is a term used to represent an intermediate severity of clinical condition in between TT and TM, which incorporates a broad phenotypic spectrum ranging from mild anaemia to more severe anaemia and these patients only require occasional blood transfusions or never transfused. They are also being categorized as non transfusion dependent thalassaemia (NTDT), together with Hb E/ β thalassaemia, and α thalassaemia (Hb H disease) (Musallam *et al.*, 2013).

The different phenotypes of β thalassaemia i.e NTDT and TDT can be attributed to some genetic and environmental modifiers (Nadkarni *et al.*, 2015). The inheritance of a mild β thalassaemia mutation which results in a residual β chain output, reduction of α chain synthesis due to co-inheritance of α thalassaemia or any genetic interaction that leads to an increased in the synthesis of foetal haemoglobin (Hb F) are among the known genetic modifiers causing amelioration of the clinical severity by causing reduction in the imbalance of α and non-α chains.

There are more than 800 different mutations been identified and reported in the β globin (*HBB*) gene that are responsible for the β thalassaemia syndrome (Giardine *et al.*, 2013). Majority of β thalassaemia are caused by point mutations, and only rarely by large deletion mutations. These mutations and deletions create a diversity of clinical phenotypes. The identification and recognition of the genotype is crucial to provide a proper counselling to patients and their families. Characterization of these mutations should aid the planning of prenatal diagnosis program for β thalassaemia. In Malaysia the most common beta mutations reported are CD41/42 (–TTCT), CD26 (G>A) Hb E, IVS1–1 (G>T), and IVS1–5 (G>C). Among the Malays, CD26 (G>A) Hb E, CD41/42 (–TTCT), IVS1–1 (G–T), and IVS1–5 (G–C) were the most common mutations, whereas CD41/42 (–TTCT) and IVS2–654 (C–T) were most common among the Chinese (Hassan *et al.*, 2013).

Hb E (β^E) is one of β globin variant, occurring due to the point mutation (G>A) in codon 26 of the β globin gene (*HBB*), which induced alternative splicing and resulting in decreased β globin E chains, and considered as β^+. It is frequently seen in Southeast Asia countries, ranging from 10-60% for a country like Thailand, and differ from region to

region (Fucharoen *et al.*, 2011). Hb E syndrome is a disorder with very heterogenous clinical presentation ranging from symptomless to severe clinical manifestation. Combination of Hb E with β thalassaemia resulting in Hb E/β thalassaemia, which is the most serious form of Hb E syndrome with heterogeneity in the clinical phenotypes; from mild anaemia to severe transfusion dependent thalassaemia major. In Peninsular Malaysia, studies done among transfusion dependent thalassaemia, the commonest genotype noted to be compound Hb E/β thalassaemia as compared to Sabah in which β thalassaemia major constitutes majority of the transfusion dependent thalassaemia (Teh *et al.*, 2014).

The diagnosis of β thalassaemia in Hospital Sultanah Bahiyah (HSB) is based on parameters acquired from the clinical findings, blood picture and haemoglobin analysis. Haemoglobin analysis were performed by using Capillary Electrophoresis (CE) method, supplemented with other second method i.e. High Performance Liquid Chromatography (HPLC) or alkaline gel electrophoresis. Further molecular characterization or β globin gene analysis is suggested for patients with borderline Hb A_2, delta beta thalassaemia/Hereditary Persistence Haemoglobin F, suspected β variants, β thalassaemia intermedia and major as well as cases with Hb E/β thalassaemia. The molecular study is performed in the reference laboratory i.e. Institute Medical Research (IMR). Regrettably not all patients with diagnosis of beta thalassaemia major/intermedia/ Hb E/β thalassaemia are supported by the molecular analysis.

With the current aggressive structured school based screening programme which started in 2016 by the Ministry of Health, it is important that a clear molecular pathology of the disease can be ascertained and appropriate management can be done. Thus by knowing

the clinical, haematological as well as the different mutations in both transfusion and non-transfusion dependent thalassaemia may help in predicting the severity of the disease as well as establishing the appropriate screening program for thalassaemia syndrome.

1.2 **Problem Statements & Study Rationale**

Kedah, being a Northern state in Malaysia and bordered with Southern Thailand, has a relatively high incidence of both β thalassaemia and haemoglobinopathy (Hb E) that has been considered a known public health problem. The molecular aspect of β thalassaemia mutation in Kedah state has never been analysed before in relation to the different clinical phenotypes (TDT and NTDT).

The aims of this study are to determine the clinical features, haematology as well as β mutation profiles of β thalassaemia patients in Kedah state. The patients will be classified into 2 groups which are transfusion dependent and non- transfusion dependent by using certain criteria which is the frequency of blood transfusions per year. It is also aimed to characterize the haematology and β mutation profiles among the two groups and trying to see the association of the type of molecular characteristic affecting the disease severity. It is also hoped that by knowing the certain haematological parameters, it can help the clinicians in predicting the severity of the phenotype in a newly diagnosed β thalassaemia patients and thus helps in managing them accordingly.

There are more than 800 different mutations have been reported and identified in the β globin (*HBB*) gene which are responsible for the development of the β thalassaemia (Giardine *et al.*, 2013). By identifying the commonest β thalassaemia mutations in a

certain population, it will assist in a simpler subsequent diagnostic approach, more cost effective and more rapid by concentrating on the small number alleles which are predominant in different ethnic, rather than focusing on a wide range of rare uncommon alleles. As the occurrences of different β thalassaemia alleles vary significantly with geographic location and ethnicity (Yatim *et al.*, 2014), this study is performed to characterise β thalassaemia mutations at the molecular level among patients from Kedah, a northern state of Peninsular Malaysia as being near Thailand border, might have different beta gene mutation compared to study done in other settings in Malaysia.

It is also hoped that by knowing any genotype and phenotype associations, it can be utilised for improvement of management protocols, i.e. blood transfusions and iron chelation as well as also provides the potential for molecular therapies and improve genetic counselling in Malaysia.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2 : LITERATURE REVIEW

2.1 **Structure and Genetics of Haemoglobin**

Haemoglobin is a major protein molecule in RBC, playing a very crucial role in oxygen transportation from the lungs to all tissues in the body. It is comprised of two pairs of polypeptide chain i.e. a pair of α globin chain and β globin chain, with one haem molecule will be inserted to each pair, which are crucial for the accommodation of oxygen transportation (Bain, 2011).

There are different haemoglobins present during embryo life, foetus and adulthood, each adapted according to the particular oxygen requirement. Hb Portland, Hb Gower 1 and Hb Gower 2 are the haemoglobins present during embryonic life, whereas Hb F predominates in the foetus. Hb A ($\alpha_2\beta_2$), constitutes over 95% of the total haemoglobin in adult, with a minor proportion of adult haemoglobin is constituted by Hb A_2 ($\alpha_2\delta_2$) and Hb F($\alpha_2\gamma_2$). The difference in the types of haemoglobin is due to the adaptation for the physiological requirement that occur during the development. Foetal haemoglobin ($\alpha_2\gamma_2$) has a higher affinity for the oxygen as compared to the adult type, thus facilitates the oxygen transfer via the placenta from the maternal to the foetal circulation (A Victor Hoffbrand, 2016). Yolk sac is the main site of production of haemoglobin at the initial stage of embryonal period, followed by liver and spleen during 10^{th} to 12^{th} week of gestation, and later bone marrow will gradually take place as the primary site for the production of haemoglobin.

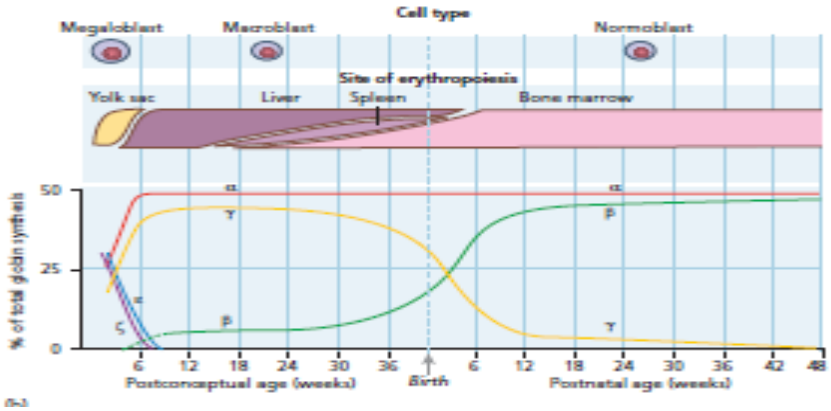
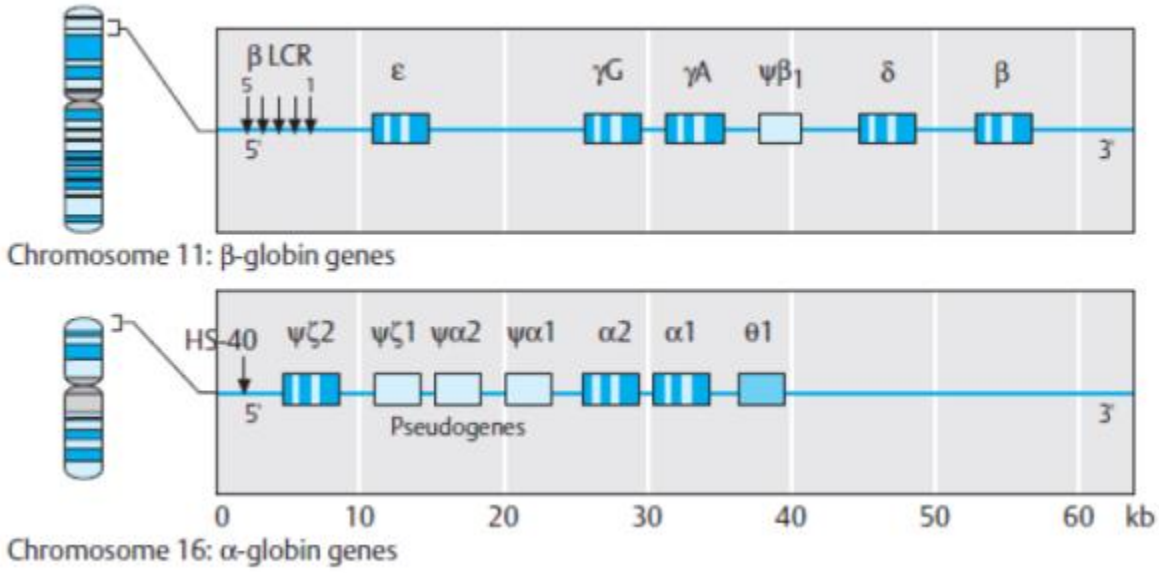


Figure 2.1: The timelines of expression of human globin gene (adapted from Postgraduate Haematology 7th edition, 2016)

Each of the α-like and β-like globin is encoded by genetically distinct loci, with α-like clusters are on the tip of chromosome 16p whereas the β-like globin gene is on chromosome 11p15.5. The genes are arranged along the chromosomes according to the sequence in which they are expressed during the development: 5′-ε-Gγ-Aγ-ψβ-δ-β-3′ and 5′-ζ-ψζ-ψα2-ψα1-α2-α1–3′. The ψβ, ψζ and ψα-genes are pseudogenes, in which they have sequences that bear a resemblance to the β, ζ or α-genes, but contain inactivating mutations that render them non-expressed (A Victor Hoffbrand, 2016). The globin genes have either one or more of the non-coding inserts, which is called intervening sequences (IVS) or also known as introns which intersecting the coding sequences or exons (A Victor Hoffbrand, 2016).

The β globin genes have three exons which are interrupted by the two introns of 122-130 and 850-900bp. This β genomic sequence encoded for 146 amino acids with intron 1 interferes the sequence between codons 30 and 31, whereas intron 2 between codons 104 and 105. Meanwhile, the α globin genes encoded for 141 amino acids and contain smaller introns between codons 30 and 31 and between codons 99 and 100 (A Victor Hoffbrand, 2016) (Figure 2-2)

a



b)

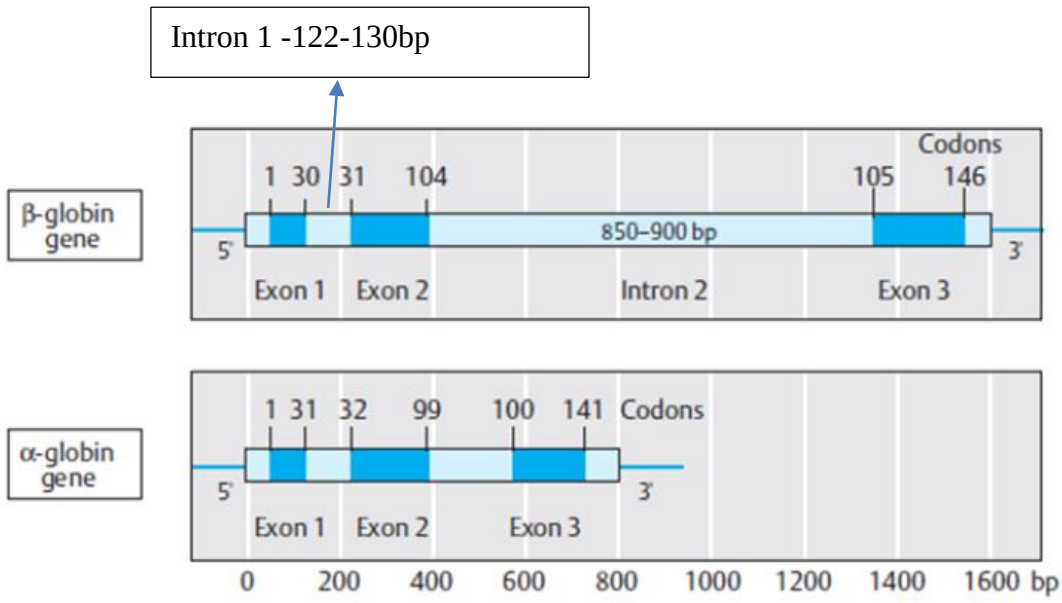


Figure 2.2: (a) and (b) showed the structure of α-globin gene and β globin gene. Adapted from (Passarge,2007)

The gene expression control occurs at multiple levels, but commonly at the transcriptional level. Other gene regulations also occurred during as well as after translational period. Besides the primary *cis* determinants of individual globin gene expression within each α and β globin complex, which are situated in the immediate vicinity and within each gene, there are also other local regulatory elements which better known as enhancers, situated at different distances from each individual gene.(A Victor Hoffbrand, 2016). The local *cis*-acting sequences which control globin gene expression include the promoter region, splicing donor and acceptors, as well as poly-A addition sites (A Victor Hoffbrand, 2016). The promoter, which is located in the 5′ flanking region, includes nucleotide homology blocks that are found in analogous positions in many other species (Thein, 2013). The three positive *cis*-acting elements comprise of the TATA box (position −28 to −31, i.e.

between 28 and 31 bases upstream from the mRNA ‘cap’ site), a CCAAT box (position −72 to −76), and a CACCC motif which may either be duplicated or inverted (position −80 to −140). The transcription factors will recognise these promoter elements and are responsible for the transcription initiation (Thein, 2013) (Figure 2-3).

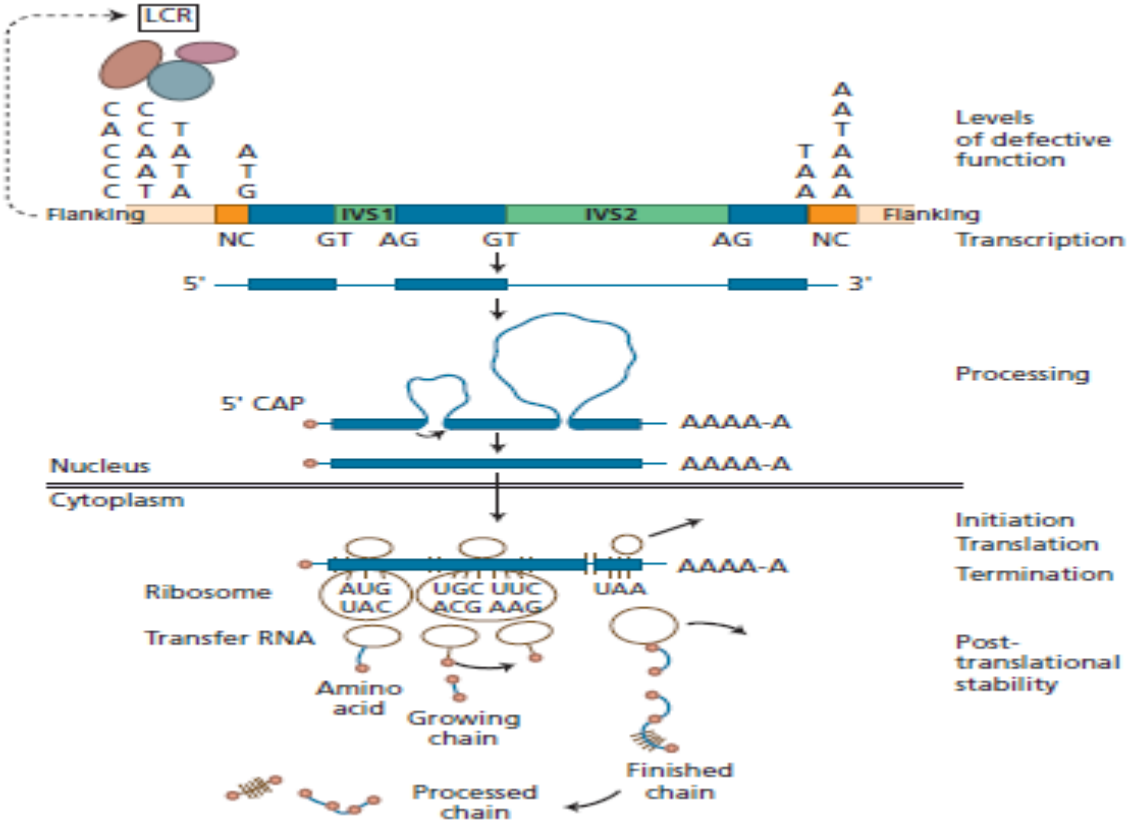


Figure 2.3 : A schematic presentation of prototype globin gene and the genetic control of globin chain synthesis (adapted from Postgraduate Haematology Seventh Edition,(A Victor Hoffbrand, 2016))

2.2 **Introduction of Thalassaemia/Haemoglobinopathy**

The thalassaemia is a heterogeneous group of haemoglobin disorder, which is characterized by the reduction in the synthesis (quantitative disorder) of one or more globin chains. Types of thalassaemia can be divided in accordance to the affected globin chains, namely α, β, δβ, δ or γ with the most common type of thalassaemia are α and β thalassaemia (A Victor Hoffbrand, 2016).

Haemoglobinopathy are characterized by the qualitative changes of the haemoglobin produced, which may include unstable haemoglobin, decreased in oxygen affinity etc. Among the commonest types of haemoglobinopathy includes Hb S, Hb E, Hb C, etc, with different prevalent in between regions (Rees *et al.*, 1998).

Thalassaemia is the commonest single gene disorders in the world, with about 4% of the human populations worldwide carry a gene for either thalassaemia or haemoglobinopathy (Ahmed, 2017). Among the earliest thalassaemia case reported was back in 1938 from Indian subcontinent (Weatherall, 2012).

Mutations in the globin genes may lead to the either reduction in the production of the protein or to the alteration in the sequence of amino acid of the protein, or mixture of two (A Victor Hoffbrand, 2016). Quantitative defects result in thalassaemia syndromes. The types of thalassaemia can be segregated in accordance to the affected type of globin chain, which are α, β, δβ, γδβ, δ, or γ. However, the most common types of thalassaemia reported are α and β. The qualitative changes or also referred as haemoglobin variants, cause a varied range of problems, among all are sickle cell disease, unstable haemoglobins,

decreased or increased oxygen affinity as well as methaemoglobinaemia (A Victor Hoffbrand, 2016). Some of the mutations may result in the mixture of quantitative and qualitative defects; resulting in a haemoglobin variant that is produced in a reduced amount, with the most common example is Hb E (A Victor Hoffbrand, 2016).

Alpha (α) thalassaemia is caused by either absence or reduced in production of the α globin chain. Majority of α thalassaemia is due to gene deletions and rarely caused by point mutations. It has been reported to be prevailing in many countries in South East Asia, with gene frequencies reported to be 16-30% in Thailand, 5% in Philippines, 2.6-11% in Indonesia, 4.3% in Brunei and 4.1% in Malaysia (Ahmad *et al.*, 2013). Alpha thalassaemia can be regarded on as a spectrum of conditions reflecting gene dosage effects, which result from the loss of function of different numbers of α globin genes (Kleanthous and Phylactides, 2008). They are categorised into two classes: α^{o} thalassaemia, in which both α globin genes are inactivated, and α^{+} thalassaemia, in which only one of the two α globin genes on chromosome 16 is defective or inactivated. The spectrum of diverse clinical disorders of α thalassaemia correlates well with the number of affected α globin genes (Kleanthous and Phylactides, 2008).

β thalassaemia on the other hand is due to the reduction or absence of the production of β globin chain. Differ from α thalassaemia, point mutation and small insertion/deletions of one or two bases on the β globin gene are the main causes for the β thalassaemia, with only a small group are caused by gene deletion.

Most thalassaemia is inherited in a Mendelian recessive way. Heterozygotes are mostly asymptomatic, although frequently they can be identified through a simple haematological analysis. More severely manifested patients are either homozygotes for α or β thalassaemia or compound heterozygotes for different molecular forms of α or β thalassaemia or for one or other form of thalassaemia and a gene for a haemoglobin variant (Brancaleoni *et al.*, 2016).

Clinically, the thalassaemia is categorized based on the severity into major, intermediate as well as minor forms. Thalassaemia major is a severe and transfusion dependent disorder whereas thalassaemia minor is the asymptomatic trait or carrier state(A Victor Hoffbrand, 2016). Thalassaemia intermedia embrace a wide spectrum of clinical severities intermediate between thalassaemia major and trait. Thalassaemia intermedia, which also known as non transfusion dependent thalassaemia (NTDT) remains as a clinical definition and comprises of β thalassaemia intermedia, Hb H disease and the Hb E/β thalassaemia (A Victor Hoffbrand, 2016; Danjou *et al.*, 2011).

Table 2.1: Classification of thalassaemia

Type of thalassaemia	**Chain or chains synthesised at reduced rate**	**Haemoglobin/s synthesised at reduced rate**
Alpha:α	α	A, A_2 and F
Beta:β	B	A
Gamma:γ	γ	F
Delta:δ	δ	A_2
Delta-beta:δβ	δ and β	A and A_2

2.3 **β Thalassaemia**

2.3.1 **Epidemiology**

β thalassaemia is a group of genetic disorder that results in either reduced or absent production of β globin protein. It is due to mutation in the β globin gene (*HBB*) on chromosome 11. Up to the present time, there are more than 800 mutations and haemoglobin variants have been identified (Giardine *et al.,* 2013), but according to the population studies, indicate that only about 40 mutations account for 90% of the β thalassaemia worldwide (Flint *et al.*, 1998).

Based on WHO report in 2008, β thalassaemia is the second most prevalent of haemoglobin disorder after sickle cell disease. Globally, it is estimated to be 1.5% (80-90 million) of people throughout the world is β thalassaemia carrier and about 60000 infants with carrier status are born annually (Kyrri *et al.*, 2013); (Galanello and Origa, 2010). Flint J *et al* in 1998 has found that incidence of β thalassaemia is highest in Mediterranean, North coast of Africa, South American, Central Asia, Middle east, India as well as Southern China. The carrier rate is higher in Cyprus (14%), followed by Sardinia and South East Asia (Flint *et al.*, 1998).

In Malaysia, the approximated carrier rate for β thalassaemia is between 3.5-4% (G Elizabeth and Ann, 2010). Kedah is one of the state in Malaysia with high prevalent of thalassaemia (4th highest state), with 20.25/100000 populations (Ibrahim, 2012).

2.3.2 **Genetic basis of β thalassaemia**

β globin is encoded by a gene found in a cluster with the other β like genes on the short arm of chromosome 11. The genomic sequence codes for 146 amino acids with the

transcribed region is confined in 3 exons which are separated by the two introns or intervening sequence (IVS). Exon 1 and 3 encode for the non-haem binding regions of the β globin chain whereas the residues that involved in haem binding and αβ dimer formation are encoded by exon 2 (Thein, 2005).

β thalassaemia results from the quantitative reduction of β globin chain synthesis which may lead to either reduced or absence of Hb A (α2β2) level. This is frequently caused by diverse mutations occurred on β globin gene (*HBB*). Most of the defects are the consequences of point mutations or a small deletion which results in the reduction or absence of β globin chain synthesis. This mutation or deletions may involve all the steps from the transcription of the DNA, processing of the mRNA transcript, translational or post-translational stability of the globin gene product. Majority of the mutations are point mutation with either small insertion, deletion, or single base substitution which involve the 5' and 3' flanking untranslated regions (UTR) sequences or at promoter region, exon, intron, intron-exon boundaries, and polyadenylation sites of *HBB* gene (Hanafi *et al.*, 2017). The difference in the mutations may either result in completely inactivated β gene with absence of β globin production (β^0-thalassaemia) or may allow some production of β globin results in β^+- or β^{++}-thalassaemia, either marked or mild reduction in the β-chains output, respectively (A Victor Hoffbrand, 2016; Danjou *et al.*, 2011). The mild β thalassaemia (β^{++}) alleles are associated with a mild change in heterozygotes and disorders of intermediate severity among homozygotes (Thein, 2004). Meanwhile, interactions with the other severe alleles are less predictable in view of the broader range

of β globin output, and may range from transfusion dependence to intermediate forms of β thalassaemia (Camaschella *et al.*, 1995; Thein, 2004).

There are also mutations known as the 'silent' β thalassaemia, where the deficit in β chain production is very minimal. The carriers may have either minimally reduced or even normal red cell indices and their Hb A_2 levels are within normal range (Thein, 2004). These 'silent' mutations are typically identified in the compound heterozygous states with other severe β thalassaemia allele, resulting in thalassaemia intermedia phenotypes or presented with a typical phenotype of β thalassaemia trait in homozygotes (Thein, 2013). This 'silent' β thalassaemia alleles are very uncommon, except for the -101 C–T, which contribute for a great number of the milder phenotypes of β thalassaemia in the Mediterranean region (Maragoudaki *et al.*, 1999; Thein, 2004).

Patients with genotype β^0/β^0 normally manifested with severe clinical presentation and are known as thalassaemia major whereas patients with genotype of $\beta^+/\ \beta^0$ or $\beta^+/\ \beta^+$ usually have diverse clinical severities and known as thalassaemia intermedia. Individual with $\beta/\ \beta^0$ or $\beta/\ \beta^+$ is known as thalassaemia trait and usually with no clinical significance (Thein, 2005).

The mutations of the β globin gene vary in between the regions. In Turkey, CD8 (-AA), IVS1-6(T>C) and IVS 2-1(G>A) are the commonest reported, whereas in Egypt (IVS1-1, IVS1-110 and IVS1-6 were the commonest reported mutations (Fettah *et al.*, 2013). In South East Asia, studies done in Thailand showed that the deletion at CD41/42 (-TCTT)

was the most frequent (48%) mutations. Other mutations found in order of decreasing frequency were CD17 (A>T) (30%), -28 (A>G) (6%) IVS1-1(G>T) (6%), A -87 (C>A) (4%), IVS2-654 (C>T) (2%), CD71/72 (+A) (2%) and CD35 (C>A) (2%), respectively (Mirasena *et al.*, 2013).

In Malaysia the commonest β mutations reported are CD41/42 (–TTCT), CD 26 (G>A) Hb E, IVS1–1 (G>T), and IVS1–5 (G>C). Among the Malays, CD26 (G>A) Hb E, CD 41/42 (–TTCT), IVS1–1 (G>T), and IVS1–5 (G>C) were the commonest mutations, whereas CD41/42 (–TTCT) and IVS2–654 (C–T) were most common among the Chinese (G Elizabeth and Ann, 2010). In a study done in Penang by Nur Fatihah Mohd Yatim et al, where they molecularly characterise 20 different β thalassaemia mutations in 40 unrelated Malays, the highest prevalence of beta thalassaemia alleles among Malays from Penang is β^E mutation (Yatim *et al.*, 2014). Figure 2.4 illustrates the schematic presentation of mutations in the *HBB* gene.

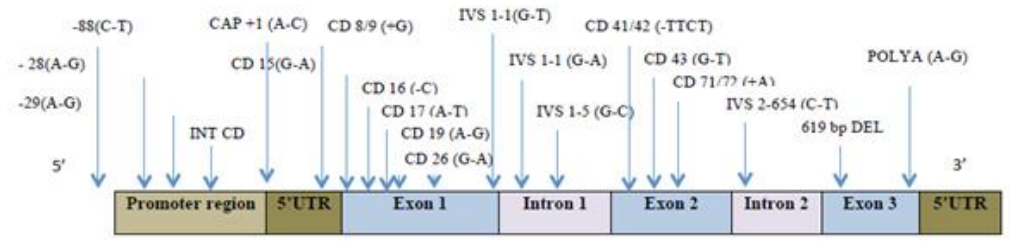


Figure 2.4: Schematic representation of some *HBB* mutations. (Adapted from Hassan et al, 2013)

2.4 **Haemoglobin E (Hb E)**

Hb E ($\alpha_2\beta_2^{26(Glu\text{-}Lys)}$ is one of the structural haemoglobin variant due to substitution of the glutamine by lysine at codon 26 of β globin gene due to the point mutation. It was described for the first time in 1950s by Chernoff and his colleague (Moiz *et al.*, 2012). The frequency of the disease differs in lines with different ethnicity and geographical area. South East Asia (SEA) regions is reported to have a highest prevalent of Hb E, ranging from 5-10% and may reached up to 50% in countries like Thailand and Cambodia (Ruengthanoo *et al.*, 2017). In Malaysia, Hb E (β^E) is among the most common β mutation found especially in Malay populations (G Elizabeth and Ann, 2010). It is believed that Hb E gives protection against malarial infection, and thus may explained the high prevalence in South East Asia countries (Moiz *et al.*, 2012).

The substitution of the base at codon 26 of β globin gene, GAG>AAG at exon 1, leads to alteration of Glutamic acid to Lysine. This abnormal gene (β^E-globin gene, HBB:c.79 G>A) produces a structurally abnormal Hb consisting of $\alpha 2\beta^E 2$-globin chains. In addition to that, the abnormal sequence also activates a cryptic 5' splice site which leads to abnormal pre-mRNA splicing. The normal donor splice site will compete with the new cryptic splice site and consequently results in the reduction of the level of correctly spliced β^E-globin mRNA while the aberrant splicing leads to a 16 nucleotide deletion of the 3' end in exon I , creating a new in frame stop (Figure 2-5) (Tubsuwan *et al.*, 2011). As a result, Hb E is produced at a reduced rate and the β^E-globin gene results in symptoms similar to a mild form of β-thalassaemia (Tubsuwan *et al.*, 2011). Thus, the phenotype

for Hb E is behaved as β^{+}. Heterozygous as well as homozygous Hb E is typically symptomless with microcytic hypochromic mild anaemia.

In view of high frequency of different β thalassaemia alleles as well as different forms of alpha thalassaemia in SEA regions, coinheritance of Hb E/ β and Hb E/α, occurs very frequently and with complex series of clinical phenotypes (Fucharoen *et al.*, 2011).

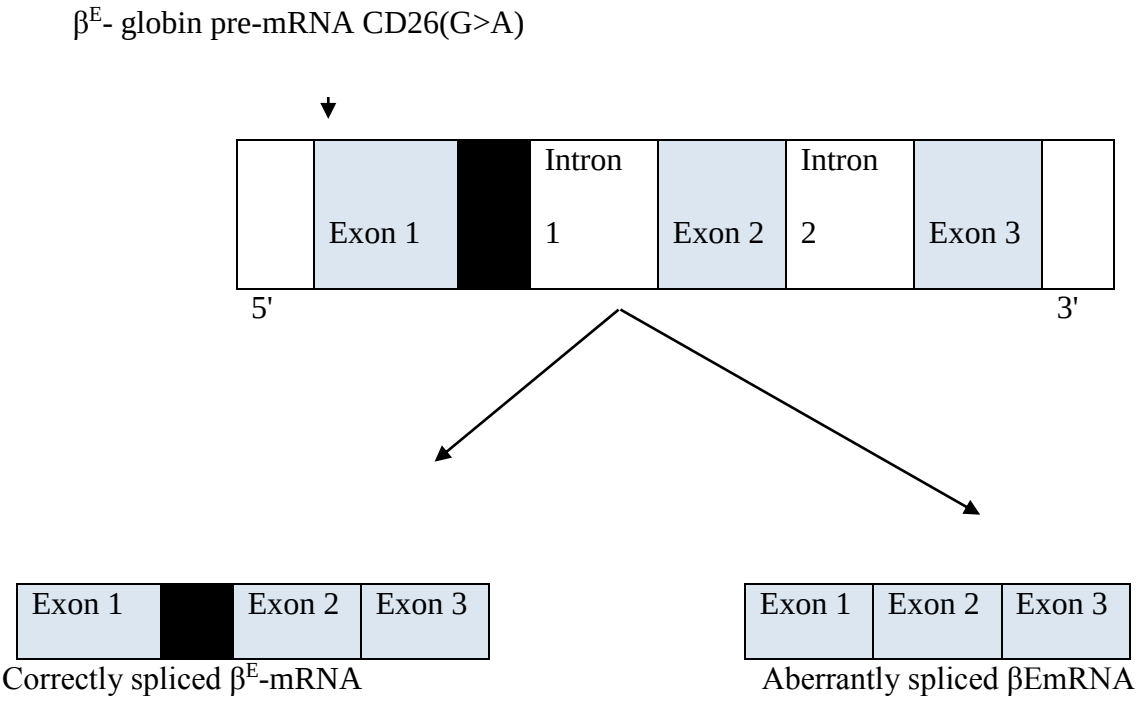


Figure 2.5: Illustration of aberrant splicing of β^{E}-globin mRNA. Black box denotes 16 nucleotides at the 3' exon 1 deleted by the aberrant splicing. Adapted with modification from (Tubsuwan *et al.*, 2011)

2.5 **Haemoglobin Malay (Hb Malay (β^{MALAY})**

Hb Malay is one of the β variant that was described firstly in 1989 in Malaysia, as a result of an investigation of anaemia in a 22-year-old Malay gentleman who was homozygous for this variant. This Hb variant is caused by AAC > AGC mutation at codon 19 of the β globin gene which results in the exchange of serine for asparagine. This mutation produces a cryptic RNA splice site in exon 1 of the β globin gene which leads to an abnormal RNA processing. Thus, this mutation not only yields variant haemoglobin but also results in a mild β^{+} thalassemia phenotype (Amran *et al.*, 2018).

A study done by IMR showed the prevalence of Hb Malay in Malaysia population was 5.5% with the majority of the cases were among Malays, 127/132 (96.2%), followed by Dusun, 2/132 (1.5%), Chinese, 1/132 (0.8%), Bajau, 1/132 (0.8%) and Orang Asli, 1/132 (0.8%) (Yusoff *et al.*, 2018). Majority of them were heterozygous Hb Malay (83/132) with 27/132 were compound heterozygous Hb Malay/Hb E, 8/132 were compound heterozygous Hb Malay/β^{+} thalassaemia, 7/132 were compound heterozygous Hb Malay/β^{0} thalassaemia 4/132 followed with other combinations with other thalassemia/haemoglobinopathy(Yusoff *et al.*, 2018).

Both high performance liquid chromatography (HPLC) for haemoglobin variant as well as capillary zone electrophoresis (CE) cannot discriminate between Hb A and Hb Malay as it is co-migrated (Yusoff *et al.*, 2018). Thus, the definitive diagnosis of Hb Malay can solely be made by molecular analysis (Yusoff *et al.*, 2018).

In the heterozygous state, Hb Malay can be missed as normal study or borderline HbA2, as it is co-eluted with Hb A. Simple heterozygotes for Hb Malay have mild microcytosis and elevated/borderline Hb A2 levels, identical to the other β-thalassemia carriers (Ma *et*

al., 2000)**.** Individuals with either homozygous for Hb Malay or compound heterozygous for Hb Malay and Hb E have Hb levels around 90–100 g/l, significant microcytosis with MCV of approximately 60fl, and elevated Hb F levels from 12 to 32% (Ma *et al.*, 2000). There were case reports and case series regarding diversity in phenotypes in homozygous Hb Malay as well as compound heterozygosity of Hb Malay with other β^{+}/β^{0} or with HbE (β^{E}). Fucharoen in 2001, had described 2 cases of thalassaemia intermedia in two adolescents with homozygous Hb Malay ($\beta^{MALAY/}$ β^{MALAY}) and compound heterozygous Hb Malay and HbE (β^{MALAY}/ β^{E}) (Fucharoen *et al.*, 2001). A case series of 12 Thai patients with compound heterozygosity for Hb Malay and either β^{+} (IVS I-5 G→C) or β^{0} thalassemia mutations (CD 17 (A→C), CD 41/42(-C), and 3.4-kb deletion) were reported back in 1997. These patients presented with severe anaemia with Hb levels as low as 42 g/l, presence of hepatosplenomegaly, and required regular blood transfusions, similar with β thalassaemia major patients. Most of them also had raised Hb F levels, up to 46% or more. In 1991, a 4-year old Thai girl with compound heterozygosity for Hb Malay and a β^{0} thalassaemia mutation, codon 41 (-C), was noted to be severely anaemic with a Hb of 24 g/l. Another patient with Hb Malay/codon 41 (-C) had been described with Hb 52 g/l, MCV of 84 fl, and an Hb F level of 3.2% (Laosombat *et al.*, 1997). In these patients, since Hb Malay is indistinguishable from Hb A on electrophoresis, it is possible that these patients might be misdiagnosed as simply heterozygous carriers of β thalassaemia mutation based on Hb analysis alone. In Malaysia, a total of 12 cases of confirmed heterozygous, homozygous or compound heterozygous of Hb Malay were studied and noted 3/12 patient with β^{MALAY}/β^{+} presented with moderate anaemia and splenomegaly and required irregular blood transfusion during pregnancy or during acute illness (Amran