THE SIGNIFICANCE OF BORDERLINE HAEMOGLOBIN A_2 LEVEL AMONG KELANTAN POPULATION

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CE	Capillary Electrophoresis
DC	Direct Current
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
E&F	England and Fraser
FBC	Full blood count
FBP	Full blood picture
FIL	Philippines
Hb	Haemoglobin
HbA_2	Haemoglobin A_2
HbA	Haemoglobin A
HbE	Haemoglobin E
HbF	Haemoglobin F
HBB	Hb Subunit Beta
Hct	Haematocrit
HPLC	High Performance Liquid Chromatography
HRPZ II	Hospital Raja Perempuan Zainab II
HS	Hypersensitive Site
HUSM	Hospital Universiti Sains Malaysia
LCR	Locus Control Region
MARMS	Multiplex Amplification Refractory Mutation System
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MED	Mediterranean

PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
RDW	Red Cell Distribution Width
SEA	South East Asia
TDT	Transfusion Dependent Thalassaemia
α	Alpha
β	Beta
δ	Delta
γ	Gamma
ζ	Zeta
ε	Epsilon

KEPENTINGAN PARAS HEMOGLOBIN A_2 SEMPADAN DI DALAM POPULASI DI KELANTAN

ABSTRAK

Kira-kira 3-5% daripada rakyat Malaysia adalah pembawa talasemia. Diagnosis talasemia yang dibuat bersandarkan kepada parameter hematologi hanyalah sebagai andaian sahaja. Analisis DNA diperlukan untuk pengesahan. Hemoglobin A_2 (HbA_2) lebih daripada 4.0% adalah penanda menunjukkan yang seseorang itu adalah pembawa β-talasemia. Walau bagaimanapun, apabila parameter hematologi tidak disokong oleh kenaikan paras hemoglobin A_2, status mereka sebagai pembawa talasemia sukar dikenalpasti. Kajian keratan rentas ini dijalankan untuk menentukan bilangan sampel di HRPZ II yang mempunyai paras HbA_2 sempadan 3.0% hingga 3.9% dari 1 Januari 2015 sehingga 31 Mei 2016.

HRPZ II menerima 11,790 sampel untuk pemeriksaan talasemia menggunakan kapilari elektroforesis dari seluruh Kelantan. Golongan yang mempunyai HbA_2 antara 3.0 dan 3.9% telah dipilih dan kemudian profil hematologi mereka (Hb, MCV, MCH, RBC, RDW, platelet) dianalisa dan kemudian dipilih secara persampelan rawak untuk menjalani ujian molecular bagi mengesan mutasi gen β dan kehilangan gen α. Mutasi pada gen β dikesan dengan kaedah molekular MARMS-PCR manakala kehilangan pada gen α dikesan dengan kaedah Multiplex Gap PCR.

Daripada 11,790 sampel, seramai 405 (3.4%) didapati mempunyai HbA_2 antara 3.0 dan 3.9%. Seramai 117 (28.9%) sampel dipilih secara persampelan rawak. Daripada 117 sampel, 45 (38.5%) menunjukkan keputusan positif ujian molekular sebagai pembawa talasemia di mana 36 (30.8%) menunjukkan mutasi pada gen β, 8 (6.8%) menunjukkan kehilangan gen α dan 1 sampel (0.9%) menunjukkan kewujudan bersama mutasi pada gen α dan β. Daripada

37 sampel dengan mutasi pada gen β, 32 (86.5%) mempunyai satu mutasi gen manakala 5 (13.5%) dengan dua mutasi gen.

Mutasi gen β paling kerap dikesan ialah CD 19 (A-G) (45.9%), diikuti oleh IVS 1-1 (G-A) (24.3%), Poly A (13.5%) dan CAP 1 (A-C) (2.7%). Terdapat perkaitan yang signifikan antara tahap HbA_2 yang berbeza dan hasil ujian molekular. Seramai 10 orang (27.0%) menunjukkan keputusan positif mutasi gen β walaupun HbA_2 hanya 3.0%.

Daripada kajian ini, terdapat sebilangan golongan yang mempunyai HbA_2 3.0% hingga 3.9% dikesan sebagai pembawa talasemia dari ujian PCR. Kajian ini diharap dapat membuka ruang untuk penilitian semula tahap HbA_2 untuk menjayakan pengesanan program β-talasemia.

THE SIGNIFICANCE OF BORDERLINE HAEMOGLOBIN A_2 LEVEL AMONG KELANTAN POPULATION

ABSTRACT

About 3-5% of Malaysians are thalassaemia carriers. Thalassaemia diagnosis based on haematological parameters is presumptive thus DNA analysis becomes necessary. Haemoglobin A_2 (HbA_2) level higher than 4.0% is a classic marker of β-thalassaemia carriers. However, when haematological parameters are suggestive of thalassaemia phenotype but not supported by HbA_2 increment, this finding may put the diagnosis into dilemma. This cross sectional study was conducted to determine the proportion of borderline HbA_2 level among samples taken for haemoglobin (Hb) analysis in HRPZ II from January 2015 until March 2016.

HRPZ II received total of 11,790 samples for thalassaemia screening by CE from all over the state. Samples with HbA_2 between 3.0% and 3.9% were selected and their haematological profiles (Hb, MCV, MCH, RBC, RDW and platelets) were analysed and then randomly selected for molecular testing (PCR) for β-gene mutation and α-gene deletion. MARMS-PCR was used for β-globin gene mutation detection while α-globin genes deletion detected by Multiplex Gap-PCR method.

From 11,790 samples, 405 (3.4%) samples were found to have HbA_2 between 3.0 and 3.9%. Out of that, 117 (28.9%) samples were selected by simple random sampling to proceed with PCR. From 117 samples, 45 (38.5%) showed positive molecular result in which 36 (30.8%) showed β-globin gene mutations, 8 (6.8%) showed α-globin gene deletions and 1 sample (0.9%) showed coexistence of α- and β-globin gene mutations. Out of these 37 samples with β-globin gene mutations, 32 (86.5%) had single gene defect while 5 (13.5%) had two gene mutations.

The commonest of single β-gene mutation detected were Cd 19 (A-G) (45.9%), followed by IVS 1-1 (G-A) (24.3%), Poly A (13.5%) and CAP +1 (A-C) (2.7%). There was statistically significant association between HbA_2 levels and positive molecular result. Interestingly, this study also showed 10 (27.0%) positive β-gene mutation even though the HbA_2 level was normal at only 3.0%.

There was significant number of people with borderline HbA_2 and confirmed as thalassaemia carriers by PCR. This study revealed an important finding to consider revision of HbA_2 range and haematological parameters that should prompt for DNA analysis in case of borderline HbA_2 for the success of thalassaemia screening programme in our population.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

The thalassaemia is a heterogeneous group of genetic disorders with defective synthesis of one or more globin chains. It was originally confined to the tropics and subtropics region with high incidence rates. Beta thalassaemia (β-thalassaemia) is commonly found in Mediterranean basin, Middle East and Asia while alpha thalassaemia (α-thalassaemia) predominates in South East Asia. However due to the increasing global migration, this haemoglobin (Hb) disorder can also be seen in the areas where they were not originally endemic (Ministry Of Health Malaysia, 2016).

In Thailand and other Southeast Asian countries, thalassaemia is very common with 20–30% of the population having the α-thalassaemia trait, 3–9% having β-thalassaemia trait whereas 20–30% having the HbE trait (Srivorakun *et al.*, 2011). In Malaysia, thalassaemia is one of the common genetic abnormalities with 4.5% of Malaysians are carriers of β- thalassaemia (George, 2001).

Until November 2015, according to the thalassaemia registry, total number of transfusion dependent thalassaemia (TDT) patients in Malaysia was 6646. Sabah presented with the highest TDT cases which is more than 1600 patients while Kelantan, the place where this study was conducted has more than 250 TDT patients (Ministry Of Health Malaysia, 2016).

Thalassaemia gives financial burden to this country. It was estimated that RM 3 million needed for treatment of thalassaemia major patient until they reach 30 years old. Therefore, prevention of this disease is more cost effective. Developing a prevention programme is important in reducing the birth of blood transfusion dependent thalassaemia considering the

cost needed for the optimal care of affected patients and also socio economic burden to patients, family members and government (Ministry Of Health Malaysia, 2016).

The thalassaemia and haemoglobinopathy screening is based on the detection of red cell indices, Haemoglobin A_2 (HbA_2) value, Haemoglobin F (HbF) and Hb variant values. Even though diagnosis of β-thalassaemia can be made base on haematological parameters, the diagnosis is only presumptive. Thus in these cases, molecular test by Polymerase Chain Reaction (PCR) becomes necessary (Giambona *et al.*, 2009).

HbA_2 determination plays an important role in the screening programs for β-thalassaemia in which if the value is more than 4.0% with normal HbA and HbF, the person will be diagnosed as β-thalassaemia carrier. Individuals with "grey zone" or borderline HbA_2 are difficult to be classified base on haematological parameters alone (Giambona *et al.*, 2008). The individuals who have borderline HbA_2 values are not rare, ranging from 2.2% to 16.7% among population in the countries where thalassaemia is commonly seen (Lou *et al.*, 2014). If PCR test was not done among borderline HbA_2 samples, significant numbers of thalassaemia carriers will be missed with probability of birth of an affected offspring. Various factors can contribute for normalization of HbA_2 that can interfere with carrier detection such as mild β-thalassaemia mutations and coinheritance of other molecular defect such as α- or δ-thalassaemia (Colaco *et al.*, 2012).

Borderline HbA_2 level in this study was justified at the level between 3.0% - 3.9% even though few previous studies done had classified borderline HbA_2 level between 3.3% to 3.7% (Mosca *et al*., 2008; Lou *et al*., 2014) and 3.1% to 3.9% (Giambona *et al*.,2008; Sharma *et al*.,2015). While previous researchers used High Performance Liquid

Chromatography (HPLC) to quantify HbA_2, this study used capillary electrophoresis (CE) method. Thus, HbA_2 level between 3.0% and 3.9% were taken as borderline values in this study as HbA_2 level by CE was significantly lower than HPLC (Alauddin Hafiza MBBS *et al.*, 2012; Borbely *et al.*, 2013).

The aim of this study was to determine the proportion of borderline HbA_2 value and its significance in diagnosing β-thalassaemia among Kelantan populations. Samples with borderline HbA_2 were randomly selected for molecular test with Multiplex Amplification Refractory System (MARMS) PCR for β-thalassaemia detection and Multiplex Gap-PCR for α-thalassaemia gene deletion. The haematological profiles will be studied together with the spectrum of thalassaemia mutations. To perform molecular test to all samples with borderline HbA_2 is not permissible due to logistic reason and financial constraint. But neglecting and labelling the samples as normal might cause at risk couple to give birth to an affected offspring, which can give rise to bigger nuisance for the nation. In addition, false assurance from the test results may have legal implications.

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 The haemoglobin

Each red blood cell (RBC) contains approximately 300 million molecules of Hb. Hb consists of haem and globin components. Haem is vital to transport oxygen to the tissues while globin will protect haem from oxidation, provide the solubility to the molecule and gives variation to the oxygen affinity. Two pairs of globin chains formed one molecule of Hb, each of which encompasses an iron containing porphyrin designated haem. These highly specialised molecules act as oxygen transporter from the lungs to the tissues (Bain, 2006).

From the time of fertilization until adulthood period, there are four major types of Hb which started with 'embryogenic Hb', represent $\zeta_2\varepsilon_2$ (Hb Gower 1), $\alpha_2\varepsilon_2$ (Hb Gower 2), $\zeta_2\gamma_2$ (Hb Portland 1) and $\zeta_2\beta_2$ (Hb Portland 2). These kinds of Hb are detected during 3rd to the 10th week of gestation. Towards foetal period, the predominant oxygen carrier is 'foetal' Hb or HbF that consist of $\alpha_2\gamma_2$ molecule. Shortly after birth, adult Hb (HbA) that consists of $\alpha_2\beta_2$ molecule will replace HbF.

There are also other trait adult Hb components that consist of $\alpha_2\delta_2$ which are HbA_2. Under normal condition, the red cells of an adult human contain approximately 97-98% of HbA, 2-3% of HbA_2 and traces of HbF (Federation, 2014). Figure 2.1 illustrates different types of Hb from embryonic period until first year of life.

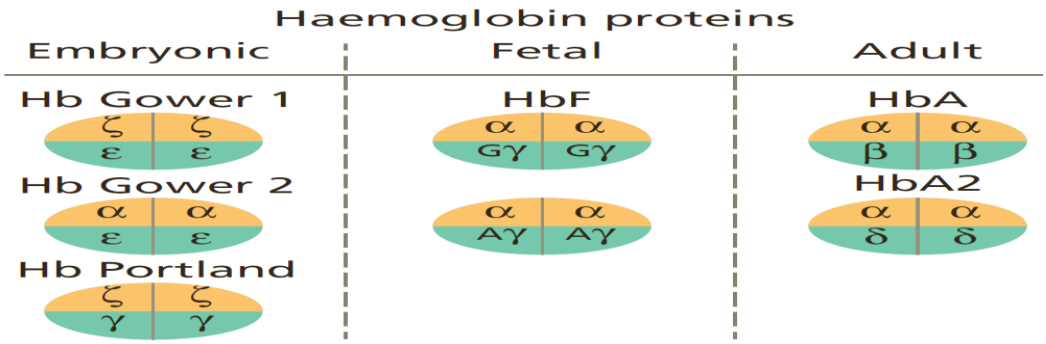

Figure 2.1: Different expression of human globin chain in different stages of life (adapted from (A. Victor Hoffbrand, 2016)).

Each of α- and β-like globin chains are encoded by genetically different loci. The α-like cluster is located on the tip of short arm of chromosome 16 while β-like cluster is located on chromosome 11p15.5. The β-genomic sequence codes for 146 amino acids, has three exons (coding regions) that interrupted with two introns. Intron 1 interrupts the sequence between codons 30 and 31 while intron 2 between codons 104 and 105. The α-globin genes which encode for 141 amino acids have introns between codons 30 and 31 and also 99 and 100.

The appropriate genes of α- and β-gene clusters are expressed accordingly to maintain the normal production of α-and β-like globin chains to produce normal Hb level and type. The regulation of globin gene expression is mediated at several levels. Most will be regulated during transcriptional level, but with some adjustment during and after translation level (A. Victor Hoffbrand, 2016). Figure 2.2 showed the genomic structure of α-like and β-like globin clusters on chromosome 16 and chromosome 11.

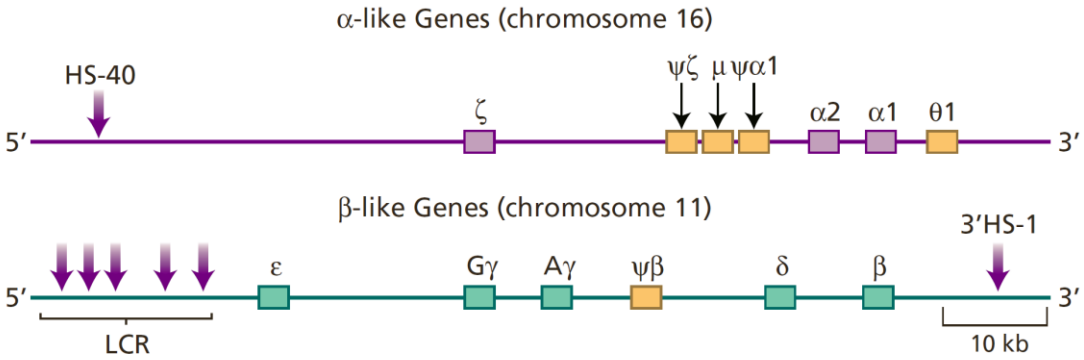

Figure 2.2: Genomic structure of α-like and β-like globin clusters (adapted from (A. Victor Hoffbrand, 2016)).

2.2 Global epidemiology and population genetics of thalassaemia

Cooley and Lee in 1925 first described a form of severe anaemia that occurred early in life with presence of splenomegaly and skeletal changes. George H. Whipple and William L. Bradford had published a comprehensive pathologic finding of this disease in 1932 with the phrase or 'thalassic' (sea) anaemia due to Mediterranean background of early patients. The phrases were later became thalassaemia (Marshall A. Litchman, 2006).

Thalassaemia were originally endemic in 60% of 229 countries, potentially affects 75% of births. Around 1.1% of couples worldwide are at risk for having children with a Hb disorder and 2.7 per 1000 conceptions are affected (Modell and Darlison, 2008). There are 4.5% (270 million) thalassaemia carriers worldwide with 60,000 thalassaemia major patients are born every year (Ministry Of Health Malaysia, 2016).

In many parts of the world, thalassaemia contributes to major health problem. The thalassaemia syndromes are a group of inherited disorders manifested with anaemia. During those days when thalassaemia was discovered, these conditions were frequently encountered

among people from the Italian, Greek coasts and nearby islands. The prevalence of thalassaemia in the regions known as 'thalassaemia belt' was around 2.5 to 15%. Thalassaemia belt' extends along the shores of the Mediterranean and throughout the Arabian Peninsula, Turkey, Iran, India and South East Asia (Ronald Hoffman, 2013) .

Five to ten percent of the population in South Italy and Greece were heterozygous for β-thalassaemia whereas in Thailand, the gene frequency for the various forms of thalassaemia was approximately around 25% (Gary Moore, 2010). Thalassaemia is commonly seen in countries with high population of immigrants (Marshall A. Litchman, 2006). The Asian, Indian, and Middle Eastern regions account for 95% of thalassaemia births worldwide. The rapid growth in the thalassaemia population from these regions has changed the clinical picture of thalassaemia worldwide. Today's epidemiology of thalassaemia is strikingly different from that of the past involving ethnicities, phenotypes, and treatments (Vichinsky, 2005).

This disorder is characterized by quantitative defects in the synthesis of one or more of globin chain subunits of the Hb tetramer. The individual thalassaemia syndromes are named according to the globin chain whose synthesis is adversely affected, either reduced or absent production of one or more globin chains (Ronald Hoffman, 2013) . The quantitative defect of the globin chain results in serious abnormality in the Hb molecule, which leads to poor oxygen transport. Patient will subsequently manifest with sign and symptoms of anaemia and ineffective erythropoiesis (Gary Moore, 2010). In each of the high frequency areas for the β-thalassaemia, a few common mutations and varying numbers of rare mutations are seen. The mutation pattern is different in each of these regions (Marshall A. Litchman, 2006).

Anaemia is the common characteristic of thalassaemia but this hereditary Hb disorder has wide spectrum of clinical severity. Clinically, thalassaemia becomes a significant disorder when the patient has severe anaemia that needs regular transfusion. These include β-thalassaemia major/intermedia, compound HbE/β- thalassaemia and some types of non-deletional α-thalassaemia (Old *et al.*, 2013).

The thalassaemia syndromes especially the transfusion dependent type are serious burden to the health services worldwide, not only because of requirement of multiple blood transfusions for thalassaemia major patients but also includes the milder type of thalassaemia, known as thalassaemia intermedia or non-transfusion dependent thalassaemia. This type of thalassaemia requires thorough and serial monitoring since complication may expect to arise at any time. The need for lifelong monitoring with the manifestation of complications affecting major organs such as endocrine glands, heart and liver creates the necessity to organise expert services and also the need for major resources in managing essential drugs and donated blood for transfusions. By implementing the holistic approach, thalassaemia patients are expected to survive without compromising their quality of life (Old *et al.*, 2013).

2.3 Thalassaemia screening in Malaysia

Ministry of Health Malaysia has estimated that 350 of β-thalassaemia major and 120 of Hb Bart hydrops fetalis patients were delivered every year in this country. This number was calculated by Hardy-Weinberg equation (Ministry Of Health Malaysia, 2016). Hardy-Weinberg equation is a simple mathematical expression formulated by mathematics professor from Cambridge University, Godfrey Harold Hardy and a physician from Stuttgart, Germany, Wilhelm Weinberg in 1908. This equation relates allele with genotype frequencies in a population, predicted the stability of their relationship from one generation

to the next with the assumption that allele and genotype frequencies do not change over the cause of many generations (Shukla, 2009).

Survival rate of thalassaemia major patients depend on the socioeconomic status. Those born in high-income countries survive with a chronic disorder while children who born in low-income countries mostly die before the age of 5 years old (Modell and Darlison, 2008). Thalassaemia is a burden to this country in terms of financial allocation and its effect to the productivity of the country. It was estimated that RM 3 million needed for treatment of thalassaemia major patient until they reach 30 years old. Therefore, prevention of this disease is more cost effective (Ministry Of Health Malaysia, 2016). The estimated costs needed for treatment of a thalassaemia patient are listed in Table 2.1.

Table 2.1: The cost for treatment of a thalassaemia patient

Treatment description	Estimated cost
Blood test every 6 months	RM 500
1 blood filter	RM70
1 unit of packed cells	RM 200
1 desferal infusion pump	RM 2500
1 Thalaset needle	RM 10
MRI T2	RM 1200

Adapted from (Ministry Of Health Malaysia, 2016)

Iron chelation therapy is mandatory for every transfusion dependent thalassaemia patient to avoid complication of iron overload. It is a major contribution of the high cost for treatment of thalassaemia patient. The algorithms for iron chelation in transfusion dependent thalassaemia are described in Figure 2.3.

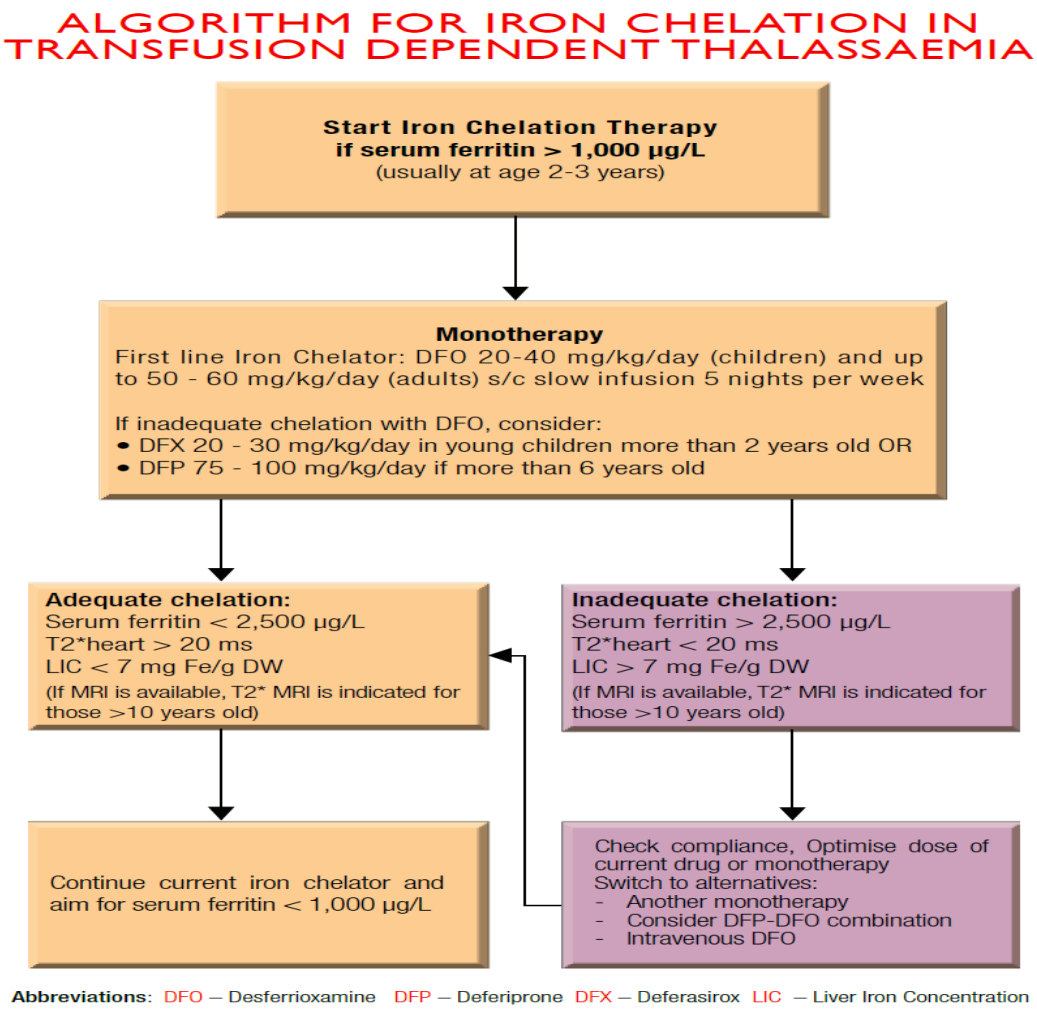

Figure 2.3: The algorithms for iron chelation in transfusion dependent thalassaemia (adapted from (Ministry of Health Malaysia, November 2009)).

The algorithm shows that iron chelation therapy usually started as early as 2 years of age, with the frequency of 5 nights per week. Owing to the necessity of iron chelators, the costs of these medications are listed in Table 2.2.

Table 2.2: The costs of iron chelating agents

Iron Chelators	Cost
Deferiprone (tablet) 9tablets/day	RM 29
Deferiprone (syrup) 45mls/day	RM 54
Desferrioxamine 2g/ day	RM 43
Deferiserox (Exjade) 1.5g/day	RM 274

Adapted from (Ministry Of Health Malaysia, 2016)

Multi organ toxicity due to iron overload that often observed as a complication in transfusion dependent thalassaemia patients were growth impairment, pubertal delay, cardiac and lung dysfunction and hypothyroidism.

By listing the cost needed to treat one thalassaemia patient, one will clearly realize how important for Malaysian government to start the thalassaemia screening programme in the country in which 1 in 10 of their people carry these thalassaemia genes. Early prevention is recommended as it could be beneficial to the person with a family history of this Hb disorder as the mode of inheritance is autosomal recessive. Since the late 1970s, genetic counselling and prenatal diagnosis have been introduced among the population at risk in the Mediterranean region where thalassaemia is endemic. The screening programs had been very effective since an intensive education were given to couples to make a correct decision before conceive (Rashid *et al.*, 2012).

Hence on 25th of August 2004, Malaysia cabinet has endorsed national programme in Malaysia designated as 'Thalassaemia Prevention and Control Programme' which had listed 4 major components:

1. Public awareness and health education
2. Comprehensive management of patients
3. Population screening and laboratory diagnosis
4. Thalassaemia registry

Without this screening programme, the birth rate of affected offspring will be more or less remains the same. The targeted group for thalassaemia screening were Form 4 students, volunteers, family and cascade screening (Ministry of Health Malaysia 2016). Selection of a test for population wide screening programmes involves a practical approach, weighing the

test specificity and sensitivity that is not only economical but also easy to handle. In country such as Malaysia who involves in screening programme for thalassaemia, initial screening with haematological indices is needed in order to select samples with some criteria that need to proceed with Hb analysis and if needed, with confirmatory PCR test. Thus, it is essential for the screening to have high specificity with high negative predictive vales to ensure that the programme does not miss thalassaemia carriers (Nadarajan *et al.*, 2010).

While family and cascade screening will usually suggested by clinicians or haematopathologist upon reviewing Hb analysis result that possibly a thalassaemia carrier, screening for form 4 students had encountered difficulties to pursue. The pioneer project was started in 2005-2006 in Penang, Sabah and Malacca. Pioneer project in Penang which involved 7,281 form 4 students from 37 schools however had gained only 42% written consent from parents on first attempt and 59% on second attempt owing to lack of promotions (Ministry of Health Malaysia 2016).

Bahagian Pembangunan Kesihatan Keluarga on 22nd of May 2015 had presented a proposal to Deputy Health Minister I for thalassaemia screening among form 4 students. Hence from 2016, the screening programme for form 4 students has been started with aim for 95% reduction of affected off springs by year of 2038 (Ministry of Health Malaysia 2016).

Hospital Raja Perempuan Zainab II as one of the centre for thalassaemia screening programme in Malaysia which received all Hb analysis samples in Kelantan state was also involved in this project. Ministry of Health had designed an algorithm for voluntary and cascade screening as shown in Figure 2.4.

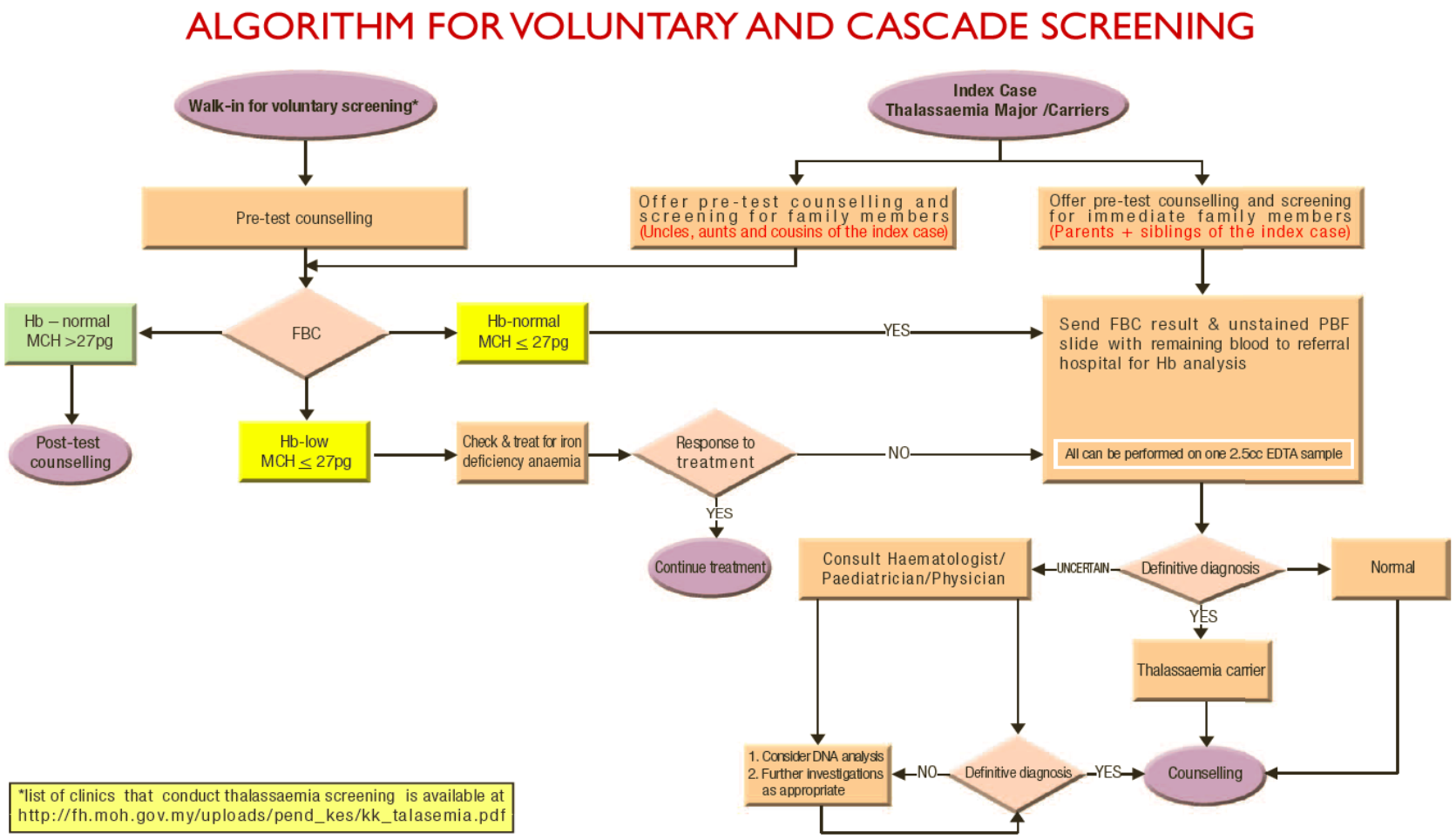

Figure 2.4: The algorithms for voluntary and cascade screening in thalassaemia **(**adapted from (Ministry of Health Malaysia, November 2009)).

The objectives of screening programme for thalassaemia in Malaysia were to detect β-thalassaemia carrier and to reduce the likelihood of birth of affected offspring from marriage of thalassaemia carrier parents. Screening is usually done base on haematological parameters including red cell indices and Hb variant values. HbA_2 determination plays a key role in making diagnosis of β-thalassaemia in which HbA_2 level more than 4.0% is a classic marker of β-thalassaemia carriers (Mosca *et al.*, 2009).

To achieve the objective of this screening programme, starting from 2008, 192 of government health clinics were equipped with haematological analyser while main hospitals were provided with HPLC machine. The numbers of haematological analysers available in health clinic were increased to more than 400 machines in 2012 (Ministry Of Health Malaysia, 2016).

2.4 The β-thalassaemia

The inherited disorder of Hb, the thalassaemia and the structural Hb variant were the first human single defective gene disorder to be studied in a systematic manner at molecular level with great discovery about their phenotype-genotype relationship (Gribben, 2010). B-thalassaemia is prevalent in Mediterranean countries, the Middle East, Central Asia, India Southern China and the Far East as well as countries along the north coast of Africa and in South America. The highest carrier frequency is reported in Cyprus (14%) followed by Sardinia (10.3%) and Southeast Asia. The high gene frequency of β-thalassaemia in the region mentioned is most likely related to the selective pressure from *Plasmodium falciparum* infection (Flint *et al.*, 1998).

Thalassaemia and haemoglobinopathy are the commonest single gene disorders known. This syndrome is so common in some regions that the majority of the population carries at least one genetic abnormality that may affect Hb structure. The prevalence of this disease is attributed with the protection against malaria. The distributions of malaria and common haemoglobinopathies largely overlap and the relationship between thalassaemia or haemoglobinopathy with protection against *Plasmodium falciparum* infection have been confirmed by microepidimiological surveys. However, it is later found that in some areas, malaria and haemoglobinopathies are not coincidentally happened together which might be as a result of sporadic mutations in local populations (Flint *et al.*, 1998).

The β-thalassaemias are a group of conditions resulting from reduced rate of synthesis of β-globin. It is extremely heterogeneous at the molecular level. More than 200 different mutations present on chromosome 11 have been found in association with the β-thalassaemia phenotype which result in reduced or absent synthesis of β-globin chains. These mutations maybe located within the gene complex or on nearby promoter or enhancer region (Gary Moore, 2010). The majority of these mutations are single nucleotide substitutions, deletions, or insertion of nucleotides and leading to frame shift mutation. Clinical severity of β- thalassaemia is related to the extent of imbalance between α- and non α-globin chains (Srisupundit *et al.*, 2013).

Several β-thalassaemia mutations are responsible for the majority of β-thalassaemia among Malays and Chinese in Malaysia. Among Malaysian Chinese, there are five common β-globin mutations: Cd 41/42 (–TTCT), IVS2-654 (C-T), –28 (A-G), Cd 17 (A-T) and Cd 71/72 (+A) (Mary Anne Tan *et al.*, 2006). Among Malays in this country, the common mutations seen are codon 26 (HbE), IVS 1-1 and IVS 1-5 (George *et al.*, 1992).

Considered as autosomal recessive inheritance, molecular analysis of β-thalassaemia demonstrated a notable heterogeneity. Each of the populations in high prevalence area has own unique group of mutations owing to the local selection due to malaria. The large majority of β-thalassaemia mutations are point mutations which can involve any step in globin chain production either transcription, translation or post translational stability of the globin gene product (A. Victor Hoffbrand, 2016).

2.4.1 Characteristic of β-thalassaemia

β-thalassaemia major patient usually presented at 6 months to two years of life with failure to thrive, feeding problems, diarrhoea, irritability, progressive pallor, progressive abdominal distension and recurrent fever. Thalassaemia major patient is usually transfusion dependent. The sign and symptoms of thalassaemia in developing countries due to in adequate blood transfusion are growth retardation, pallor, jaundice, genu valgus, leg ulcers, hepatosplenomegaly and skeletal changes (Galanello and Origa, 2010). Thalassaemia intermedia sometimes will present later at life and may or may not require blood transfusion depending on the severity. Thalassaemia trait is clinically asymptomatic but sometimes presented with mild degree of anaemia. As thalassaemia is an autosomal recessive disorder, there is 25% risk at each pregnancy of having children with thalassaemia major if both parents are thalassaemia carriers (Galanello and Origa, 2010).

2.4.2 Pathophysiology of β-thalassaemia

Molecular defect in β-thalassaemia leads to reduce or absent β-chain production. Imbalance in globin chain production results in a relative excess of unbound α-globin chains that precipitate in erythroid precursors in the bone marrow forming intracellular inclusions. The degree of globin chain imbalance is determined by the nature of the mutation in short arm of

chromosome 11 where β-globin chain is located. When excess α-globin chain degraded, the end product particularly haem and iron produce harmful effect on red cell membrane proteins and lipids that caused red cells become less deformable and has shortened survival. Destruction of the erythroid precursor is called as ineffective erythropoiesis (A. Victor Hoffbrand, 2016).

Insoluble α-globin chain induce membrane damage to the peripheral RBCs that result in peripheral haemolysis and leads to anaemia. Hence, anaemia in β-thalassaemia results from ineffective erythropoiesis and haemolysis. Prolonged and severe anaemia is compensated by extramedullary erythropoiesis and results in hepatosplenomegaly. β-thalassaemia stimulates erythropoietin production that results in bone marrow expansion and may lead to serious deformities of skull and long bones. Some adult red cell precursors that produce variable amount of gamma (γ) chains can combine with excess α-chains to form HbF that provide protection against deleterious effect of α-chain precipitation (A. Victor Hoffbrand, 2016).

In transfusion dependent thalassaemia, steady accumulation of iron in the liver, endocrine glands and myocardium might occur as each unit of blood contains 200-250mg of iron. Thus, TDT patient may die following complication of iron overload. Figure 2.5 showed pathophysiology of β-thalassaemia (A. Victor Hoffbrand, 2016).

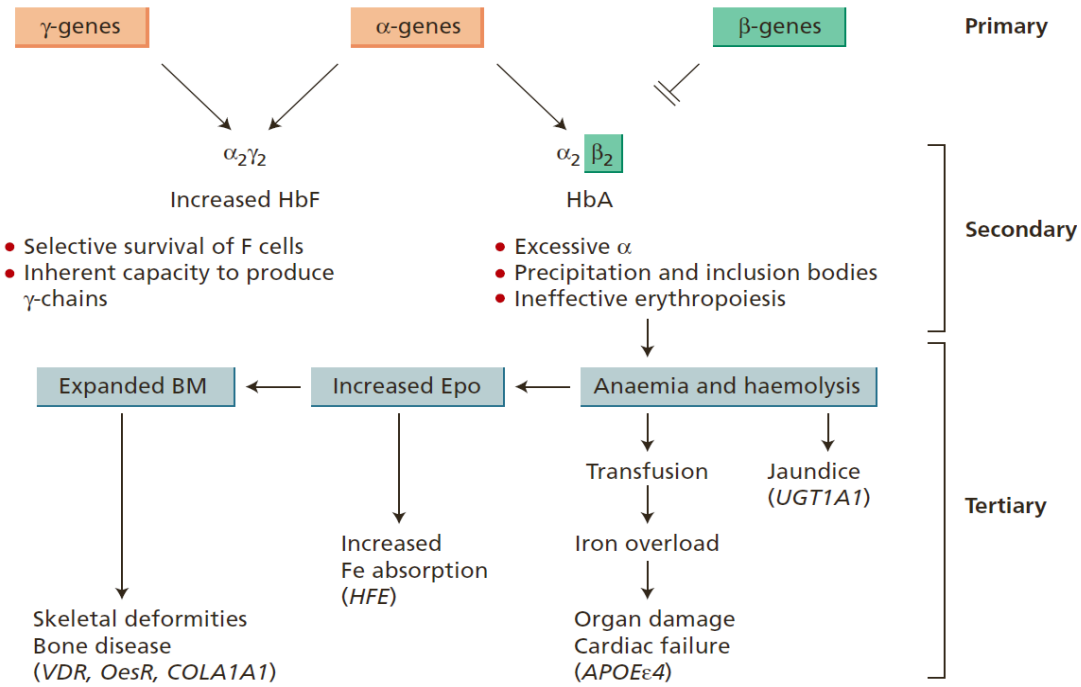

Figure 2.5: The pathophysiology of β-thalassaemia (adapted from (A. Victor Hoffbrand, 2016)).

2.4.3 Molecular genetics of β-thalassaemia

The β-globin gene is located in the short arm of chromosome 11. Besides β-globin chain, δ-globin gene, embryonic ζ-gene, fetal A-γ and G-γ genes and pseudogenes ψ*B1* also share the same region. 1.6kb haemoglobin subunit beta (HBB) gene containing three exons and both 5' and 3' untranslated regions (UTR) is regulated by an adjacent 5 promoter in which TATA, CAAT and duplicated CACCC boxes are situated. 50kb from β-globin chain is a major regulatory region that contains strong enhancer. This region known as locus control region (LCR) contains four (HS-1, HS-2, HS-3 and HS-4) erythroid specific DNAse hypersensitive sites (HSs) which act as hallmark of DNA-protein interaction (Cao and Galanello, 2010).

Combination of several DNA motifs interacting with transcription factors to constitute each HS site. The most important one are GATA-1, nuclear factor erythroid 2, eryhroid Kruppel-

like factor and friend of GATA 1. The significance of this LCR is to control β-like globin gene expression has been studied in which if HS sites are totally or partially remove, it will result in the inactivation of β-globin gene. The arrangements of these genes are shown in Figure 2.6.

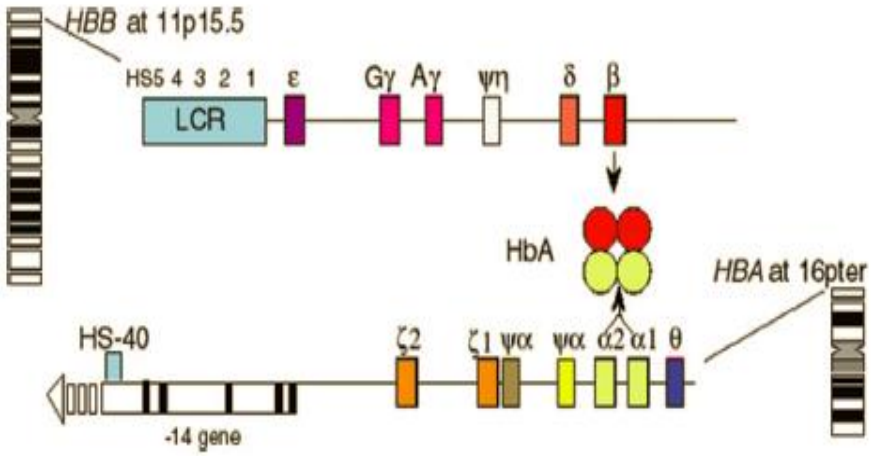

Figure 2.6: Location of the chromosomes with the structure of α and β gene clusters (adapted from (Cao and Galanello, 2010)).

From more than 200 disease-causing mutations of β-thalassaemia that have been identified, majority of the mutations are single nucleotide substitutions or deletions, or insertions of oligonucleotides leading to frameshift (Cao and Galanello, 2010).

There are three different categories of point mutations affecting the β-globin expressions which consist of:

1) Promoter and 5' UTR mutations that lead to defective β-gene transcription
2) Mutations affecting mRNA processing which includes splice junction and consensus sequence mutations, polyadenylation, and other untranslated regions mutation.
3) Mutations resulting in abnormal mRNA translation that consist of initiation codon mutations, nonsense and frameshift (Cao and Galanello, 2010).

Study done by Saripah *et al*. among transfusion dependent thalassaemia patient in Kelantan showed that Cd 26 (G>A) HbE was the commonest (84.78%) β chain mutation defect detected by MARMS PCR method (Hanafi *et al.*, 2014). Another study done among β-thalassaemia carrier and patients in Malaysia showed high frequency of carriers with Cd 26 (G–A), IVS1–5 (G–C), IVS 1–1 (G–T), and Cd 41/42 (–TTCT) among the Malays (Hassan *et al.*, 2013).

β° is characterized by complete absence of β chain production as a consequence of deletion, initiation codon, nonsense, frameshift, and splicing mutations. β^{+} is characterized by reduced production of β chain production as a result of mutations in the promoter area (either the CACCC or TATA box), the polyadenylation signal, and the 5_ or 3_ UTR or by splicing abnormalities (Cao and Galanello, 2010).

β^{+} mutation is further characterized into severe, mild and silent base on reduction of β-globin chain. The silent mutations have normal haematological findings and defined only by a mildly imbalanced alpha/beta globin chain synthesis ratio. Silent mutations result from mutations of the distal CACCC box, the 5_ UTR, the polyadenylation signal and some splicing defects. The mild mutations show moderate thalassaemia-like haematological features and imbalanced globin chain synthesis and are produced by mutations in the proximal CACCC box, TATA box, 5_ UTR, or exon 1, causing alternative splicing (Hb Malay, HbE, and codon 24 T3A), or in the consensus splicing sequence, 3_ UTR, and poly-A site (Cao and Galanello, 2010).

Mutations activating a cryptic splicing site in exon 1, at codons 19, 26, and 27, are associated with a mild or silent phenotype, and result in the production of the abnormal Hb Malay, HbE and Hb Knossos. A few β^{0}-thalassaemias display a mild phenotype such as

Cd6-A and Cd 8-AA (Cao and Galanello, 2010). List of mild and silent HBB gene mutations are shown in Table 2.3.

Table 2.3: Mild and silent HBB gene mutations causing β- thalassaemia.

Mutation type or location	Mild β^+	Silent
Transcriptional mutants in the proximal CACC box	-90 C>T -88 C>T -88 C>A -87 C>T -87 C>G -87 C>A -86 C>T -86 C>T	-101 C>T -92 C>T
TATA box	-31 A>G -30 T>A -29 A>G	
5' UTR	+22 G>A +10 -T +33 C>G	 +1' A>C
Alternative splicing	Cd 19 A>C (Hb Malay) Cd 24 T>A	Cd27 G>T (Hb Knossos)
Consensus splicing	IVS 1-6 T>C	
Intron		IVS2-844 C>G
3' UTR		+6 C>G
Poly-A site	AACAAA AATGAA	AATAAG
Mild β° frameshift	Cd 6-AA Cd 8-AA	

(Adapted from (Cao and Galanello, 2010)

The presumptive diagnostic feature of heterozygous β-thalassaemia is the hypochromic microcytic RBC with increased HbA_2 level. Normal HbA_2 β-thalassaemia refers to the forms in which the blood picture is characteristic of heterozygous β-thalassaemia except for the fact that HbA_2 is normal (Cao and Galanello, 2010).

2.5 The alpha thalassaemia (α-thalassaemia)

In view of high prevalence of α-thalassaemia in Southeast Asia region, co-inheritance of α with β-thalassaemia was postulated to be one of the causes of normal or borderline HbA_2 level (Saleh-Gohari *et al.*, 2015). Thus, the molecular test for α-thalassaemia by Multiplex Gap-PCR method was done.

The molecular pathology of the α-thalassaemia is more complicated than β-thalassaemia in view of presence of two globin genes per haploid genomes (Gribben, 2010). The α-thalassaemia is characterized by the decrease or complete suppression of α-globin polypeptide chains, resulting from over 128 different deletions or point mutations of the α-globin genes. The α-globin genes are located at the short arm of chromosome 16 (16p13.3). In normal individual, there are four functional α-genes, genotyped as αα/αα (Harteveld and Higgs, 2010).

Malaysia and its neighbouring Southeast Asian countries have a high prevalence of α-thalassaemia. The carrier rate for α-thalassaemia of the South East Asian type among Malaysian Chinese was around 4.5% (Rahimah *et al.*, 2012). α-thalassaemia is an inherited autosomal recessive disorder caused by a complete absence or reduces production of α-globin peptides due to a deletion or mutation of one or more of the four α-globin gene (two on each copy of chromosome 16). Deletion of one or both α-globin genes are the commonest form of α thalassaemia. Rarer non deletional α-thalassaemia resulting from point mutations is commoner in Southeast Asia region than any other part of the world (Harteveld and Higgs, 2010). In order to determine genotypes of the thalassaemia carrier, the accurate determination of the carrier phenotype is necessary. This can be done through haematological indices in full blood count (FBC).