HAEMATOLOGICAL PARAMETER EVALUATION IN DIFFERENT TYPES OF DELETIONAL ALPHA THALASSAEMIA AND IN COINHERITANCE OF DELETIONAL ALPHA THALASSAEMIA AND BETA THALASSAEMIA IN HOSPITAL UNIVERSITI SAINS MALAYSIA

DR SAMILAWATI BINTI MOHD ARIFIN

Dissertation Submitted In Partial Fulfillment Of The Requirements For The Degree Of Master Of Pathology (Haematology)

UNIVERSITI SAINS MALAYSIA

2018

ACKNOWLEDGEMENT

All praises to Allah S.W.T the Most Merciful and the Most Beneficent.

First of all, I would like to express my thankfulness to Allah S.W.T for His blessing and for giving me strength to complete my research successfully within the given time.

I wish to take this opportunity to express my appreciation to helpful and knowledgeable supervisor, Associate Prof. Dr Rosnah Bahar, whom I deeply indebted for her continuous supervision and care in the process of finishing this study. Without her excellent guidance, caring, patience as well as continuous encouragement, I would never be able to write this dissertation completely.

With great pleasure, I would like to acknowledge Dr Marini Ramli, my co-supervisor for her helps, ideas and continuous support.

Also, a million thanks to all medical laboratory technologists in haematology department of HUSM who involves direct or indirectly in this study process.

Most importantly, my special appreciation goes to my beloved husband, Akmal Azrin bin Ariffin, my son, Azad Syazwan as well as my daughters, Azra Syaziya and Azzahra Syaifa for their sacrifice, countless loves, understanding and patience throughout my study. Last but not least, I would like to express my deepest appreciation to my family members and friends, for their prayers, encouragement and endless supports.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

--/--	Complete four alpha genes deletion
--/αα	Heterozygous α^0
3D	Three dimensional
ARMS-PCR	Amplification Refractory Mutation System- Polymerase Chain Reaction
Aγ	A-gamma
bp	Base pair
CE	Capillary Electrophoresis
CV	Coefficient of variance
dl	Deciliter
DNA	Deoxyribonucleic acid
DPX	Dibutylphthalate Polystyrene Xylene
EDTA	Ethylenediaminetetraacetic acid
FBC	Full blood count
$--^{FIL}$	Philippine type α^0
fL	Femtoliters
G	Grams
Gγ	G-gamma
Hb Bart	Haemoglobin Bart

HbA	Haemoglobin A
HbA_2	Haemoglobin A_2
HBB	Beta globin gene
HbC	Haemoglobin C
HbCS	Haemoglobin constant spring
HbD	Haemoglobin D
HbE	Haemoglobin E
HbF	Haemoglobin F
HbG	Haemoglobin G
HbH	Haemoglobin H
HbO	Haemoglobin O
HbS	Haemoglobin S
Hct	Haematocrit
HPLC	High Performance Liquid Chromatography
HUSM	Hospital Universiti Sains Malaysia
IMR	Instituted Medical Research
kb	Kilobase
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration

MCV	Mean Corpuscular Volume
$--^{Med}$	Mediterranean type α^0
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
pg	Picograms
RBC	Red Blood Cell
RDW	Red cell distribution width
RM	Ringgit Malaysia
RNA	Ribonucleic acid
SEA	South East Asia
$--^{SEA}$	South East Asian type α^0
SLS	Sodium Lauryl Sulphate
$--^{THAI}$	Thailand type α^0
UTR	Untranslated Region
A	Alpha
-α/-α	Homozygous α^+
-α/αα	Heterozygous α^+
α^+	Alpha plus

α^0	Alpha zero
$\alpha_2\beta_2$	Haemoglobin A
$\alpha_2\gamma_2$	Haemoglobin F
$\alpha_2\delta_2$	Haemoglobin A_2
$\alpha_2\varepsilon_2$	Haemoglobin Gower 2
$\alpha\text{-}^{3.7}$	Rightward 3.7 kb gene deletion
$\alpha\text{-}^{4.2}$	Leftward 4.2 kb gene deletion
B	Beta
β/β	Normal functional beta globin chain
β^+	Beta plus
β^+/β	Heterozygous β^+
β^+/β^+	Homozygous β^+
β^0	Beta zero
β^0/β	Heterozygous β^0
β^0/β^+	Compound heterozygotes of β^0 and β^+
β^0/β^0	Homozygous β^0
Γ	Gamma
δβ	Delta/beta
E	Epsilon

εδβ	Epsilon/delta/beta
Z	Zeta
$\zeta_2\gamma_2$	Haemoglobin Portland
$\zeta_2\varepsilon_2$	Haemoglobin Gower 1

PENILAIAN TERHADAP PARAMETER HEMATOLOGI DALAM PELBAGAI KES PENGHAPUSAN ALFA THALASSEMIA DAN DALAM KES WARISAN BERSAMA ALFA DAN BETA THALASSEMIA DI HOSPITAL UNIVERSITI SAINS MALAYSIA.

ABSTRAK

Alfa thalassemia merupakan salah satu penyakit genetik yang biasa dijumpai di Malaysia dan merupakan satu isu kesihatan awam. Kaedah saringan yang efektif adalah elemen penting dalam Program Pencegahan dan Kawalan Thalassemia bagi mengesan semua kes-kes thalassemia secara efektif serta memaksimumkan penggunaan sumber.

Tujuan kajian ini adalah untuk mengenal pasti hubungkait antara parameter hematologi dalam kes-kes penghapusan alfa thalassemia dan juga warisan bersama alfa dan beta thalassemia terhadap parameter hematologic di kalangan pesakit-pesakit di HUSM.

Satu kajian keratan rentas dengan ulasan rekod retospektif pada 214 sampel yang dihantar ke unit Hematologi, HUSM telah dijalankan dengan kebenaran daripada Pengarah HUSM. Data dianalisis bagi melihat perkaitan antara indeks darah merah dalam pelbagai spektrum alfa thalassemia dan kes perwarisan bersama alfa dan beta thalassemia.

Tujuh puluh satu (71) kes telah dikesan mempunyai penghapusan alfa thalassemia. Jenis yang paling kerap dijumpai ialah jenis South-East Asian (SEA), iaitu 50.7%. Sebanyak 13.8% kes perwarisan bersama alfa dan beta thalassemia telah dikesan dengan jenis perwarisan bersama yang paling kerap dijumpai adalah antara SEA heterozygous dengan mutasi pada Codon 26 (HbE), iaitu 50%.

Terdapat perbezaan yang ketara pada nilai RBC, MCV dan MCH dikalangan subjek yang mempunyai masalah penghapusan alfa thalassemia berbanding dengan subjek yang mempunyai normal siasatan molecular (nilai $p < 0.001$). Subjek yang mempunyai penghapusan alfa thalassemia menunjukkan paras median MCH < 20.8 fL. Nilai MCV dan kiraan RBC memberi nilai tambahan dalam membezakan kumpulan ini dan membantu dalam diagnosis sekiranya teknik molekular tidak boleh didapati.

Parameter hematologi yang ketara termasuk kepekatan hemoglobin, MCV, MCH, RDW dan kiraan platelet diperhatikan diantara 3 spektrum penghapusan alfa thalassemia yang paling kerap dijumpai iaitu antara 3.7 heterozygous, SEA heterozygous dan 3.7 bersama SEA heterozygous. Parameter-parameter ini boleh digunakan sebagai saringan sebelum ujian molekular dijalankan untuk mengurangkan beban keseluruhan ujian diagnostik.

HAEMATOLOGICAL PARAMETER EVALUATION IN DIFFERENT TYPES OF DELETIONAL ALPHA-THALASSAEMIA AND IN COINHERITANCE OF DELETIONAL ALPHA-THALASSAEMIA AND BETA-THALASSAEMIA.

ABSTRACT

Alpha thalassaemia is a common genetic disorder in Malaysia and is a public health problem. Effective screening tool is an essential element in Thalassaemia Prevention and Control Programme to effectively detect all cases of thalassaemia and to maximize the resources.

The aim of this study was to evaluate the haematological parameters in various types of deletional alpha (α) thalassaemia and effect of coinheritance with beta (β) thalassaemia towards the haematological parameters among patients in Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia.

A cross-sectional study with retrospective record review on 214 samples sent to Molecular Unit, Hematology Unit, HUSM was conducted. A permission from Director of HUSM was obtained. The data was analyzed to study the red cell indices (haemoglobin concentration, red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), red cell distribution width (RDW) and platelet count in various spectrum of α thalassaemia and in coinheritance cases.

Seventy one (33.2%) cases were detected to have deletional α thalassaemia. The most common type detected was South-East Asian (SEA) type deletion (αα/--^{SEA}), α thalassaemia (50.7%). Coinheritance of α thalassaemia with β thalassaemia/haemoglobinopathy was seen in 13.8% of

cases with the most common coinheritance was seen between SEA heterozygous (αα/--^{SEA}) with codon 26 mutation (HbE).

There were significant differences of RBC count, MCV and MCH level in patients with deletional α thalassemia compared to group without α gene deletion (*p*-value < 0.001). Deletional α thalassaemia cases showed median MCH level of 20.8 fL. The MCV and RBC counts gave an added value in differentiating between the group and might aid in making the diagnosis if the molecular technique is not available.

Significant haematological differences include haemoglobin concentration, MCV, MCH, RDW and platelet count were observed among 3 commonest spectrum of deletional α thalassaemia which is between 3.7 heterozygous, SEA heterozygous and 3.7 heterozygous with SEA heterozygous. The parameters can be used as a good screening tool before persuing molecular testing in order to reduce the overall financial burden of diagnostic testings.

CHAPTER 1

GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

Thalassaemia is a hereditary disease in which there is reduce or absent in production of the globin chains of haemoglobin. It is an increasing global health problem. It has been estimated that approximately 7% of the world population are carriers of such disorders and that 300 000 – 400 000 babies with severe forms of this disease are born each year (Weatherall and Clegg, 2001). In South East Asia (SEA) region, thalassaemia and haemoglobinopathies are the most common genetic disorders which have a tremendous psychological, social, financial and health economic burden to the country. Alpha (α) thalassaemia, beta (β) thalassaemia, haemoglobin E (HbE) and haemoglobin constant spring (HbCS) are prevalent in this region.

In Malaysia, α thalassaemia is a public health concern, with a frequency of 51.2% (Ahmad *et al.*, 2013). According to Malaysian Thalassaemia Registry 2017, a total of 7220 patients with thalassaemia are registered with 2611 cases are β thalassaemia major, 2402 cases of compound heterozygous HbE/β thalassaemia, 733 cases of thalassaemia intermedia, 1022 cases of haemoglobin H (HbH) disease and 452 cases with other diagnosis. Out of this figure, 5013 cases are transfusion dependent thalassaemia, with diagnosis of β thalassaemia major and HbE/β thalassaemia (Malaysian Thalassemia Registry, May 2017).

The disease is a burden to our country as well as to the patients and family members. About RM 3 million are spent for treatment of thalassaemia major patient until they reach age of 30 years old. Prevention strategy to reduce this disease is important and is more cost effective. Reducing the birth of transfusion dependent thalassaemia patients by an effective screening strategy is important considering the cost needed for the optimal care of affected patients as well as socio economic burden to patients, family members and government.

Current algorithm for voluntary and cascade screening in Malaysia is including walk in and cascade screening. This screening strategy is mainly targeting the antenatal, pre-marital and adolescences group (secondary Form 4 students) which recently had been implemented. The haematological parameters is used as first laboratory screening tool before other diagnostic test including peripheral blood smear, haemoglobin analysis, Deoxyribonucleic acid (DNA) analysis is carried out for a definitive diagnosis (Ministry Of Health, 2009).

In α thalassaemia, the haematological parameters were correlated with the number of α globin genes affected (Akhavan-Niaki *et al.*, 2012; Clarke and Higgins, 2000). The degree of microcytosis and number and type of α gene deletions were correlated, with --/αα genotype associated with the lowest mean corpuscular volume (MCV), followed by the -α/-α genotype, and then the -α/αα genotype, while the red blood cells (RBC) counts showed the reverse trend (Wang *et al.*, 2000). However, currently, in our practice, the correlation between the number of gene deletion and the degree of microcytosis is not clearly seen. The findings might be due to the high prevalence of nutritional deficiencies in our country, which may not be similar with the findings in other countries. In an area where α thalassaemia is prevalent, an effective screening parameters to differentiate it from other condition is mandatory as a cost effective way to make the preventive programme a success. Other than that, an efficient screening parameters to discriminate the genotypes of α thalassaemia for a judicious selection of patients for molecular testing of α thalassaemia is helpful to reduce the unnecessary cost of diagnostic investigations.

Besides that, in cases coinheritance of thalassaemia, the haematological parameters as well as the amount of the variant haemoglobin presence may be altered. This may lead to misdiagnosis by routine investigations.

A safe haematological parameters cut-off value for thalassaemia screening in area of nutritional deficiencies as well as thalassaemia are highly prevalent need to be established, as the definitive diagnostic tools is still relatively expensive, time-consuming and not always available.

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 Normal human haemoglobin

Haemoglobin is a red cell pigment, functions as an oxygen carrier for carbon dioxide exchange at tissues level. Each red cell contains approximately 640 million haemoglobin molecules. A molecule of haemoglobin is made up from 4 haem groups linked to 4 globin chains and carries 4 oxygen molecules. The synthesis of various globin chains is started during embryonal life and undergo a major switch to adult haemoglobin 3 to 6 months after birth (Weatherall, 2004). It varies according to oxygen requirement in different stages of life. In embryonic life, the haemoglobins are composed of combination between α-like globin called zeta (ζ) globin with gamma (γ) globin producing haemoglobin Portland ($\zeta_2\gamma_2$) and ζ globin with epsilon (ε) globin producing haemoglobin Gower 1 ($\zeta_2\varepsilon_2$) and combination of α globin with ε globin producing haemoglobin Gower 2 ($\alpha_2\varepsilon_2$). In foetal life, haemoglobin F (HbF) ($\alpha_2\gamma_2$) is predominant. In adults, haemoglobin A (HbA) ($\alpha_2\beta_2$) comprises more than 95% of total haemoglobin while haemoglobin A_2 (HbA_2) ($\alpha_2\delta_2$) is present as minor component in red blood cells (Givens Bell, 1999). The different combination of the globin chains and type of haemoglobin produced from foetal life until adult life are showed in Figure 2.1 and Table 2.1. Under normal circumstances, the red cells of an adult human contains approximately 97-98% of HbA, 2-3% of HbA_2 and traces of HbF (Ministry Of Health, 2009).

Table 2.1. Haemoglobins normally present during adult, foetal and embryonic periods of life. Adapted from (Bain, 2008)

Haemoglobin species	Globin chains	Period when normally present
A	$\alpha_2\beta_2$	Major haemoglobin in adult life
A_2	$\alpha_2\delta_2$	Minor haemoglobin in adult life
F	$\alpha_2\,\gamma_2$	Minor haemoglobin in adult life, major haemoglobin in foetal life with a declining percentage through the neonatal period
Gower 1	$\zeta_2\varepsilon_2$	Significant haemoglobin during early intrauterine life
Gower 2	$\alpha_2\varepsilon_2$	Significant haemoglobin during early intrauterine life
Portland	$\zeta_2\,\gamma_2$	Significant haemoglobin during early intrauterine life

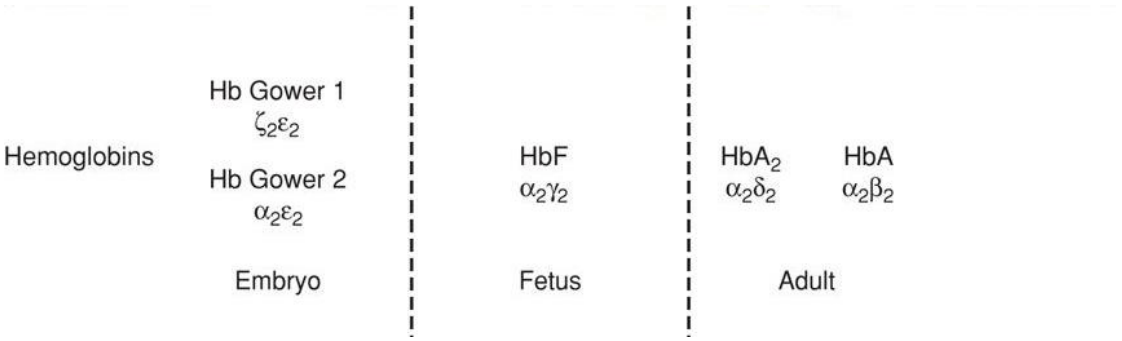

Figure 2.1. The schematic representation of different combination of α and β globin at different stages of life and haemoglobin types produced. Adapted from (Cunningham *et al.*, 2014)

2.1.1 Genetic control of human haemoglobin

Each type of globin chain is controlled by distinct genes. There are 2 genes controlling α and γ chain, while 1 gene for each other globin chains. The specific regions that control the production of this different globin chains have been determined (Weatherall and Clegg, 2008). The α-like and β-like globin chain is regulated by clusters of gene located at chromosome 16 and chromosome 11 respectively. The α-like globin gene is located close to telomere of chromosome 16 (16p13.3). The functional genes are arranged along the chromosome in the order of telomere-ζ-α_2-α_1-centromere surrounded by widely expressed genes as shown in Figure 2.2. Upstream of this α-gene cluster, there are 4 highly conserved non-coding sequences or multispecies conserved sequences (MCS) called MCS R1-R4. The only sequence shown to be essential for α globin gene expression is MCS R2, also known as HS-40 (Higgs *et al.*, 1998).

The key erythroid transcription factors and its various co-factors progressively bind to the upstream elements and the promoters of the α-like gene as the haematopoietic progenitor cells commit into erythroid lineages and begin to differentiate into mature red blood cells. RNA polymerase II is recruited to both the upstream elements and promoter of α-globin as transcription starts in early erythroid progenitors. The upstream elements and promoters of α-globin genes interact with each another via the formation of chromatin loops, which occurs at the same with the transcription process (Vernimmen *et al.*, 2007).

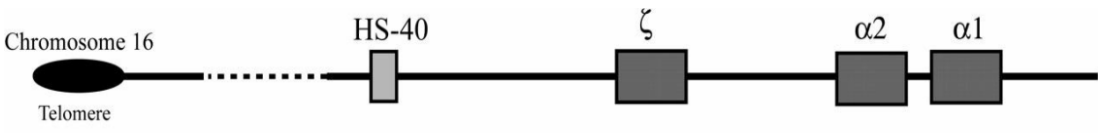

Figure 2.2. Schematic representation of the human α globin gene clusters. The α globin gene cluster is situated near the telomeric region, arranged in ζ, α2, α1 of the short arm of chromosome 16 (Steensma *et al.*, 2005).

The β-like gene cluster is located on the short arm of chromosome 11 (11p15.5). This gene cluster also contains embryonic ε, foetal G-gamma (Gγ) and A-gamma (Aγ) and pseudogenes which is arranged according to the developmental expression as shown in Figure 2.3. The gene expression is regulated by adjacent 5' promoter in which the TATA, CAAT, and duplicated CACCC boxes are located. The locus control region (LCR) is located in this region which contain hypersensitive site (HS) which is the hallmark for DNA-protein interaction and important for the control of β globin gene expression (Cao and Galanello, 2010).

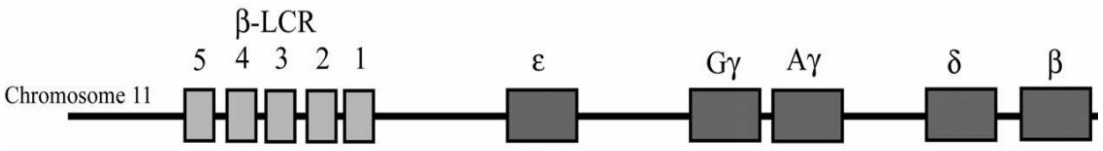

Figure 2.3. Schematic representation of the human β globin gene clusters. The β globin gene cluster is located on the short arm of chromosome 11 (Steensma *et al.*, 2005).

The appropriate α and β genes expression is regulated accordingly to maintain the normal production of α- and β-like globin chains for normal haemoglobin production. This regulatory mechanisms occur at several levels, mostly during transcription level but with some adjustment during and after translation level (A. Victor Hoffbrand, 2016).

2.2 Inherited haemoglobin disorders

There are 2 main groups of inherited haemoglobin disorders;

1) Structural haemoglobin variants (haemoglobinopathies)

2) Thalassaemia

The structural haemoglobin variants are mostly due to single amino acid substitution in the α or β chains (Weatherall and Clegg, 2008). There are more than 1000 different haemoglobinopathies have been identified (Steinberg *et al.*, 2009). However the most frequently found structural haemoglobin variants are Haemoglobin S (HbS), Haemoglobin C (HbC) and HbE variants. HbE is the commonest haemoglobin variant found globally (Rees *et al.*, 1998).

The thalassaemia is due to reduce or absent of the normal globin chain synthesis either due to mutation or deletion of the affected genes. It is broadly classified according to the affected globin chain that is ineffectively synthesized into α, β, delta/beta- (δβ) and εδβ- thalassaemia (Weatherall and Clegg, 2008).

2.3 Alpha thalassaemia

Alpha thalassaemia is caused by reduction or absent of α globin chains. It is most frequently caused by deletion of one or both α globin genes and less commonly by mutational defects (Galanello and Cao, 2011).

2.3.1 Pathophysiology of α thalassaemia

The α genes are located on the short arm of chromosome 16 (16p13.3). The molecular pathology of the α thalassaemia is more complicated than β thalassaemia as normal human received 2 copies of gene from each parent (Weatherall and Clegg, 2001). Normal individual carries 4 functional α globin genes, designated as αα/αα. Reduce in α globin chain synthesis either due to deletion or mutation of the globin genes leads to α thalassaemia. There are 2 main classes of α thalassaemia, the alpha zero (α^0) and alpha plus (α^+) (Higgs and Weatherall, 2009). The α^0 thalassaemia is a state where both α genes in one chromosome are deleted, either all or part of genes are missing. The heterozygotes state of α^0 is labelled as αα/--, while homozygotes is --/--. In α^+ thalassaemia, there is only one gene in one chromosome is lost or inactivated. The heterozygotes state is labelled as αα/-α while homozygotes is -α/-α. In some α^+ thalassaemia patients, the mutation leads to an inactivation of α gene rather than deletion, stated as $\alpha^T\alpha/\alpha\alpha$ in heterozygous and $\alpha^T\alpha/\alpha^T\alpha$ in homozygous individual (Harteveld and Higgs, 2010). In Malaysian populations, α thalassaemia is most commonly due to gene deletion leading to α^0 thalassaemia and α^+ thalassaemia (George, 1998).

2.3.2 Types of deletional α thalassaemia and its worldwide distribution

The α^0 thalassaemia is commonly resulting from deletion of 17.5 kb to 20 kb of both duplicated α globin genes leaving the ζ globin gene intact, known as South East Asian (SEA) α^0 ($--^{SEA}$). In the Thailand ($--^{THAI}$) and Philippine ($--^{FIL}$) α^0 thalassaemia, there is extensive deletion of α

globin gene involving the ζ globin gene. This α^0 thalassaemia is found at highest frequency in South East Asia, in which more than 10% of the population are carriers in some regions. It is also commonly found in the Eastern Mediterranean Island and mainland population but reported sporadically in Middle East and Indian subcontinent (Higgs and Weatherall, 2009).

The α^+ thalassaemia is caused by deletion of one globin gene in one chromosome. There are 2 common types of α^+ thalassaemia have been observed, the leftward 4.2 kb gene deletion (α-$^{4.2}$) and the rightward 3.7 kb gene deletion (α-$^{3.7}$). In α-$^{3.7}$ thalassaemia, there is reciprocal recombination between highly homologous regions called Z boxes resulting the specific deletion, leaving only one normal gene reserved. In α-$^{4.2}$ thalassaemia, there is recombination between mispaired homologous X boxes producing the characteristic deletion as shown in Figure 2.4 (Embury *et al.*, 1980).

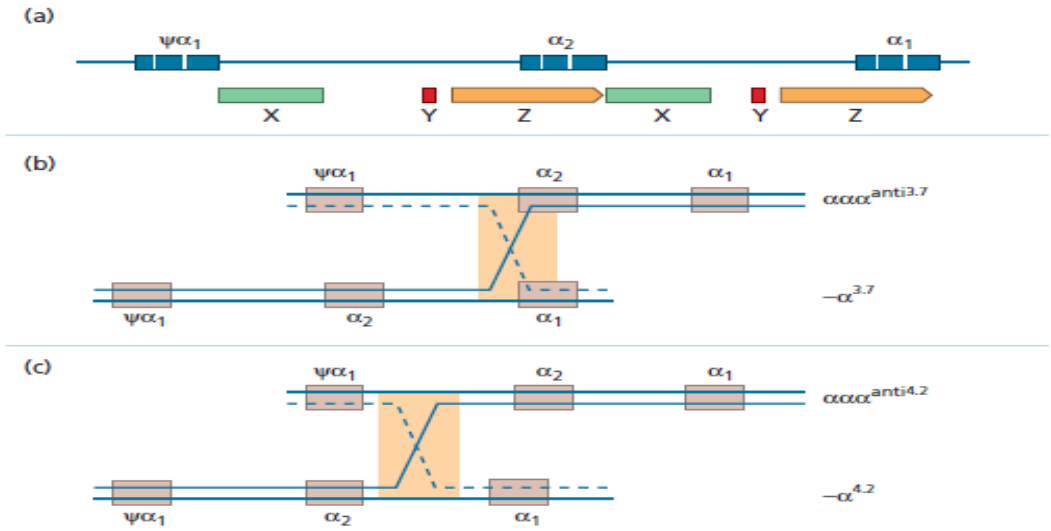

Figure 2.4. The molecular mechanism that underlie the deletion forms of α^+ thalassaemia
(a) normal cluster showing X, Y and Z homology boxes
(b) 3.7-kb deletion
(c) 4.2-kb deletion
Adapted from Weather and Clegg 2001 (A. Victor Hoffbrand, 2016)

The α^+ thalassaemia has a worldwide distribution with the most frequently found is the rightward 3.7 kb gene deletion. In Malaysian population, α thalassaemia is most commonly due to gene deletion leading to α^0 and α^+ thalassaemia (George, 1998). Population studies have indicated that the types and frequencies of the different α thalassaemia defects vary among different ethnic communities and tend to be geographically specific (Rahimah *et al.*, 2012).

2.3.3 Genotypic and phenotypic characteristics of α thalassaemia

The affected individual can have variable phenotypic expression depends on the number of the affected genes as well as the type of deletion or mutation involved (Leung *et al.*, 2008). The inheritance of α thalassaemia genes and its probable phenotype is represented in Figure 2.5. Individual with one gene deletion (-α/αα) or two genes deletion either heterozygous α^0 (--/αα) or homozygous α^+ (-α/-α) is usually asymptomatic. However, some might have borderline hypochromic microcytic red blood cells or anaemia (Higgs, 2001). Individuals with three genes deletion (--/-α) have thalassaemia intermedia picture characterised by moderate anaemia, marked hypochromic microcytic red cells and increased in spleen size (Chen *et al.*, 2000). When only one functional α globin gene is inherited, this results in HbH disease. This is usually the result of the compound heterozygous state for α^0 and α^+ thalassaemia (Ahmad *et al.*, 2013). Patient with HbH disease is presented with variable severity of chronic haemolytic anaemia. It is characterized by greatly reduced rate of synthesis of α chain, imbalance and excess of β chain production which lead to the formation of an abnormal haemoglobin with β chain tetramers referred to as HbH. This HbH is unstable and precipitates in erythroblasts and results in haemolysis and intramedullary cell death or also referred to as ineffective erythropoiesis (A. Victor Hoffbrand, 2016).

Complete four α genes deletion (--/--) leads to Haemoglobin Bart's hydrops foetalis which causes severe intrauterine anaemia, resulting death in late gestation or shortly after birth. This sometimes leads to maternal obstetric complications including pre-eclampsia, antepartum and postpartum haemorrhage as well as premature onset of labour (Liang *et al.*, 1985). This condition is not compatible with extra uterine life (Harteveld and Higgs, 2010).

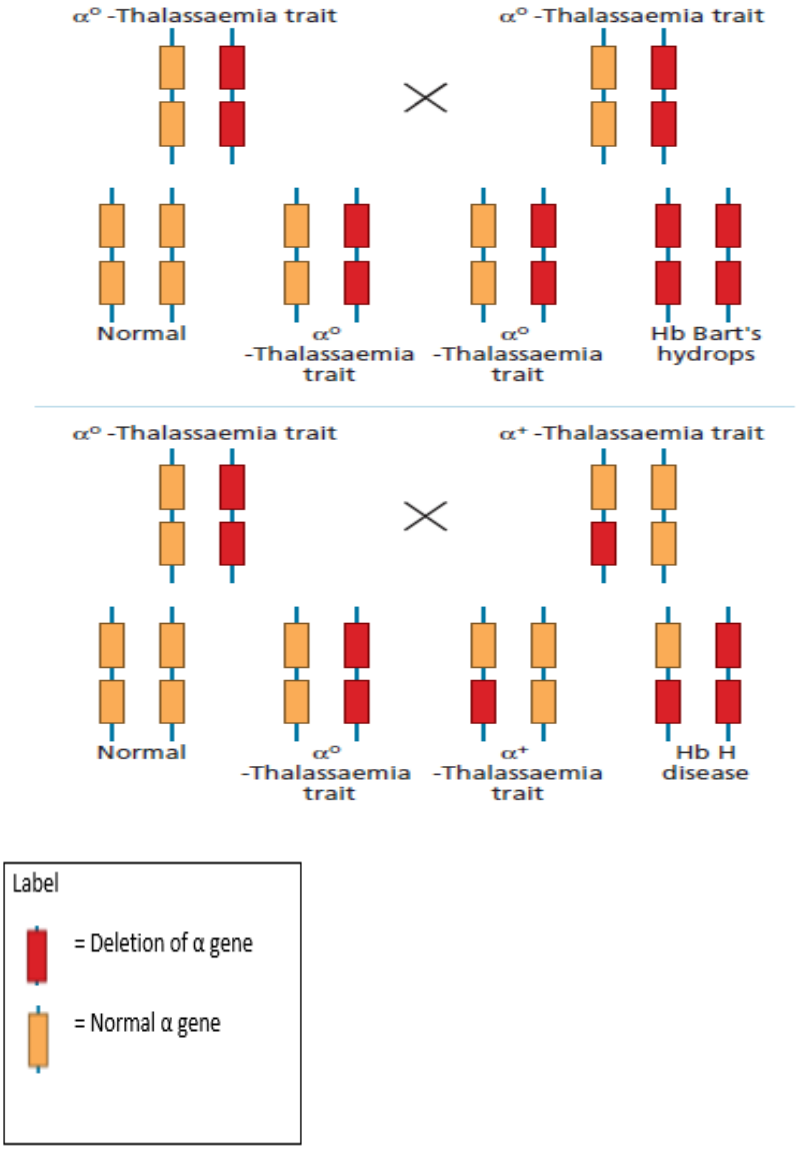

Figure 2.5. The genetics of α thalassaemia. Adapted from (A. Victor Hoffbrand, 2016).

2.4 Beta thalassaemia

Beta thalassaemia is caused by reduction or absent of the β globin chains. It has very variable clinical phenotypic based on the rate of imbalance between α and β globin chains (Cao and Galanello, 2010).

2.4.1 Pathophysiology of β thalassaemia

Beta globin gene (HBB) is located on the short arm of chromosome 11 (11p15). Normal individual carries 2 functional globin genes, termed as β/β. Reduce in the β globin chain synthesis leads to β thalassaemia. Beta thalassaemia is commonly caused by point mutation of the involved gene and rarely due to extensive deletions (Cao and Galanello, 2010). As with α thalassaemia, it is classified as beta zero (β^0) and beta plus (β^+) thalassaemia. The β^0 thalassaemia is characterised by total absence of the involved β gene while the β^+ thalassaemia is characterised by reduce in the amount of the globin chains produced (Musallam *et al.*, 2013). The heterozygotes state is labelled as β^0/β and β^+/β while homozygotes state is β^0/β^0 and β^+/β^+. The compound heterozygotes state is labelled as β^0/β^+ (Schrier *et al.*, 2014).

2.4.2 Types of β thalassaemia and its worldwide distribution

The β thalassaemia is a heterogeneous disease at molecular level with more than 200 mutations have been so far identified. Majority of cases are single nucleotide point mutation including insertion, substitution or deletion leading to a frameshift. Generally, the mutations are divided into 3 categories;

1) Mutations leading to defective β gene transcription (promoter and 5' Untranslated Region (UTR) mutations);

2) Mutations affecting messenger RNA (mRNA) processing (splice junction and consensus sequence mutations, polyadenylation, and other 3' UTR mutations); and

3) Mutations resulting in abnormal mRNA translation (nonsense, frameshift and initiation codon mutations) (Cao and Galanello, 2010).

Beta thalassaemia is inherited as autosomal recessive and is present with high prevalence in the Mediterranean, Middle-East, Central Asia, Indian subcontinent and Far East. The highest incidence of β thalassaemia are reported in Sardinia (19%), Cyprus (17%), and Greece (19%) (Weatherall and Clegg, 2001).

2.4.3 Genotypic and phenotypic characteristics of β thalassaemia

The β thalassaemia has variable clinical severity based on the degree of affected β globin chain synthesis. There are 3 recognised clinical and haematological conditions of β thalassaemia which are the β thalassaemia carrier, β thalassaemia intermedia and β thalassaemia major. The β thalassaemia carrier state is characterised by having hypochromic microcytic red blood cells indices with marked variation of size and shape, but clinically asymptomatic. It is resulted from heterozygosity for β thalassaemia (Weatherall and Clegg, 2001).

The homozygosity for β thalassaemia results in β thalassaemia intermedia or thalassaemia major. The β thalassemia major affected individual is usually presented with severe transfusion dependent anaemia within first 2 years of life (Danjou *et al.*, 2011) . Haematological parameters show a severe hypochromic microcytic anaemia and presence of nucleated red blood cells with anisocytosis and poikilocytosis on peripheral blood smear. The number of nucleated red blood cells is related to the degree of anaemia and is markedly increased after splenectomy (Cao and Galanello, 2010).

Individual with β thalassaemia intermedia shows a markedly heterogeneous clinical picture and genotypic expression, ranging from asymptomatic thalassaemia carrier to severe transfusion dependent state. The transfusion dependent individual generally present later in life (aged 2-6 years) in comparison to those with thalassaemia major (Higgs *et al.*, 2012). The affected individual has a moderate anaemia and shows a markedly heterogeneous haematological picture, ranging from that of β thalassaemia carrier state to that of thalassaemia major (Cao and Galanello, 2010).

The allelic heterogeneity at β globin locus gives rise to a variable clinical severity of β thalassaemia. It is mainly related to the extent of imbalance between α globin chains and non-α chains (Danjou *et al.*, 2011). The clinical phenotype is also modified by genetic factors mapping outside the globin gene clusters and not influencing the foetal haemoglobin including those affecting bilirubin, iron and bone metabolisms (Cao and Galanello, 2010).

2.5 Effect of coinheritance for thalassaemia

The coinheritance of β thalassaemia with α thalassaemia may change the haematological parameters and the phenotypic characteristic of the condition. This may lead to misdiagnosis during routine investigations.

A case reported by E. Kanavakis *et al* describing a case of coinheritance of severe form of β thalassaemia and α thalassaemia which interacted in synergistic manner to balance the phenotype of classic thalassaemia syndrome. The patient was first hospitalised at the age of 50 years old for 2 days with presenting symptoms of fatigue in the constitutional symptoms and apparent absence of other thalassaemia major or intermedia associated clinical manifestations

such as jaundice, growth retardation or bone deformities. Full blood counts demonstrated a severe anaemia (haemoglobin 5.4 g/dL) with extremely low red cell indices (MCV 48.7 fL and MCH 13.9 pg) and blood film examination found significant hypochromic microcytic red blood cells. No inclusion was detected with methyl violet incubation. Patient's DNA analysis showed coexistence of β thalassaemia major (IVSI-6 T>C/IVSI-I G>A) with HbH disease ($-\alpha^{3.7}/--^{Med}$). Overall, the case had a well-compensated anaemia with mild expression of the symptoms of thalassaemia such as ineffective erythropoiesis and oxidative stress. It was concluded that coinheritance of severe form of β thalassaemia and α thalassaemia interacted in a synergistic manner, which almost complete negated the symptoms of classic thalassaemia syndromes (Kanavakis *et al.*, 2004).

Another study by O. Sripichai *et al* at Mahidol University (Thailand) assessing the effect for α globin gene copy number in modifying β thalassaemia disease severity by studying 925 cases of β^0 thalassaemia and HbE patients aged 2 to 77 years old in Thailand. In this study, they found that 7.9% (73 of 925) of the cohort with haemoglobin level > 7.5 g/dl, MCV < 60 fl, and HbF < 30%, and out of this 73 cases, 55 cases (75.3%) were found to have α thalassaemia coinheritance. This study also clearly demonstrated that a depletion of α globin chains could ameliorate the effect of excess α globin chains in β^0/HbE patients (Sripichai *et al.*, 2008).

Melis *et al* concluded that the interaction of heterozygous β^0 thalassaemia with deletion of one or two α genes may produce normal red cell indices. They found that normal red cell indices in several β thalassaemia heterozygotes seem to represent the maximum expression of the ameliorating tendency due to the coinheritance of α thalassaemia. Therefore, it is advisable to incorporate testing for HbA_2 value in the first screening test set for β thalassaemia in the population where both α and β thalassaemia is prevalent as screening based on MCV/MCH value may miss a significant population of the carriers (Melis *et al.*, 1983).

2.6 Prevalence of thalassaemia

It is estimated that approximately 7% of the world population are thalassaemia carriers and about 300 000 to 500 00 babies were born with clinically identifiable and significant haemoglobin disorders each year with approximately 80% of affected children are born in countries classified as low income (Organization, 1985). Generally, this condition is most prevalent in tropical region, including Mediterranean Sea, the Middle East and South East Asia. However, this condition could now be encountered in most countries due to population migrations (Weatherall and Clegg, 2001).

2.6.1 Prevalence of thalassaemia in Malaysia

It is estimated that the Malaysian population in 2017 is at 32.0 million with 28.7 million are citizens and 3.3 million are non-citizens. Malays and other Bumiputera comprised of 68.8%, 23.2% are Chinese, 7.0% are Indians and other races make up 1.0% of the population (Department of Statistics Malaysia, 2017).

In Malaysia, α thalassaemia, HbE and β thalassaemia are prevalent. According to Malaysian Thalassaemia Registry 2017, a total of 7220 patients with thalassaemia are registered with 2611 cases are β thalassaemia major, 2402 cases of HbE/β thalassaemia, 733 cases of thalassaemia intermedia, 1022 cases of HbH disease and 452 cases with other diagnosis. Out of this figure,

5013 cases are transfusion dependent thalassaemia, mostly β thalassaemia major and HbE/β thalassaemia cases (Malaysian Thalassemia Registry, May 2017).

There are increasing trend of reported cases of thalassaemia in Malaysia over the years. It is most probably due to better case reporting, increase number of thalassaemia new birth with less mortality and increasing number of survivors because of better treatment. It is estimated that about 200 new cases per year are reported. Sabah gives the highest contribution for thalassaemia cases in Malaysia with 23.5% of total cases of thalassaemia, followed by Selangor (15%), Kedah (9.4%) and Kuala Lumpur (8%) (Malaysian Thalassemia Registry, May 2017).

Various ethnic groups exhibit unique phenotypes and genotypes characteristic of thalassaemia distribution. β thalassaemia major is more prevalent in Kadazan ethnic of Sabahan, while HbE-β thalassaemia is more commonly seen in Malays and HbH disease is seen commonly in Chinese (Malaysian Thalassemia Registry, 2010).

2.6.2 α thalassaemia in Malaysia

The α thalassaemia is highly prevalent in Malaysia with the prevalence is up to 51.2%. There are 8 different deletions and mutations demonstrated in our population, including 3 double gene deletions, $--^{SEA}$, $--^{THAI}$, and $--^{FIL}$; 2 single gene deletion, $-\alpha^{3.7}$ and $-\alpha^{4.2}$; and 3 non-deletional mutation, codon59G>A (Haemoglobin Adana), codon125T>C (Haemoglobin Quong Sze) and codon142 (Haemoglobin Constant Spring). The high incidence of $-\alpha^{3.7}$ was observed in Malays, Indians, Sabahans, Sarawakians and Aborigines. In Chinese population, $--^{SEA}$ was the most commonly detected, followed by the $-\alpha^{3.7}$ deletion (Ahmad *et al.*, 2013). The $-\alpha^{3.7}$ deletion is identified as the most common mutation to produce a genetic disorder and is found to be

prevalent in most tropical and subtropical population studied (Galanello and Cao, 2011; Harteveld and Higgs, 2010; Rahimi, 2013) .

A study was done in regular blood donors in Kelantan population showed that 9.25% of them were detected to have deletional α thalassaemia with the commonest spectrum was $-\alpha^{3.7}$ detected and found in high frequency among Malay compared to other races (Rosnah *et al.*, 2012).

2.6.3 β thalassaemia in Malaysia

It is estimated that about 4.5% of Malaysian are heterozygous carriers for β thalassaemia. The couples are at risk of producing a child with β thalassaemia major where affected births annually would be 2.1/1000 live births (George, 2001). In Malaysia, the spectrum of β thalassaemia mutations have been systematically delineated since 1984. There are 19 beta mutations characterised in Malaysian population with the commonest was codon 26 mutation commonly in Malays while codon 41/42 mutation was common in Chinese ethnicity (Sivalingam *et al.*, 2012). In Malays, there were three common β gene mutation identified which were HbE (codon 26 GAG → AAG), IVS1-5 G→C and IVS1-1 G→T, responsible for about 73.1% of β thalassaemia. In Chinese-Malaysian, there were five common β globin gene mutations found which were codon 41/42 (-TCTT), IVS2-654 (C→T), -28 (A→G), codon 17 (A→T) and codon 71/72 (+A) and these account for about 90% of β thalassaemia. Among the Kadazan-Dusun of Sabah population, the commonest mutation found was the 45kb Filipino deletion, a β^0 thalassaemia phenotype and accounted for over 90% of transfusion dependent thalassaemia cases (Elizabeth and Ann, 2010). Other β gene mutations found included codon 19, codon 30, codon 8/9, Cap+1, -28, -29 and codon 43 (Ahmad *et al.*, 2013).

2.7 Disease burden

Thalassaemia major, if left untreated, results in death in the first few years of life. Their effect on the burden of health care is only now being appreciated in many parts of the world. Improvements in hygiene, nutrition and control of infection reduce childhood mortality rates. These lead to babies with severe haemoglobin disorders to survive long enough and present for diagnosis and treatment (Weatherall and Clegg, 2001).

Thalassaemia is a burden to the country. It is estimated that approximately about RM 3 million is needed for treatment of thalassaemia major patients until they reach the age of 30 years old (Ministry Of Health Malaysia, 2016). The costs include tests for diagnosis and monitoring, treatment for the disease itself and its complications as well as investigation modalities for complications monitoring (Table 2.2).

Table 2.2. Estimated cost for monitoring, treatment and complications monitoring of thalassaemia. Adapted from (Ministry Of Health Malaysia, 2016)

Tests/ items 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

In transfusion dependent thalassaemia, iron chelation therapy is mandatory. The therapy is usually started at the age of 2 to 3 years (Figure 2.6). The cost of each type of iron chelators

differs (Table 2.3) and the frequency of its administration adds the cost burden in the thalassaemia management (Ministry Of Health, 2009).

Table 2.3. The cost for different types of iron chelation therapy. Adapted from (Ministry Of Health, 2009)

Iron Chelators	Unit price	Drug cost for a 30 kg patient/month
Deferiprone (Ferriprox)	500 mg = RM 3.32	75 mg/kg/day x 30 kg ~2.5 gm/day 1 week = 35 tablets 4 weeks = 140 tablets x RM 3.32 = RM 464.80
Deferiprone (Kelfer)	500 mg = RM 4.18	75 mg/kg/day x 30 kg ~2.5 gm/day 1 week = 35 tablets 4 weeks = 140 tablets x RM 4.18 = RM 585.20
Desferrioxamine (Desferal)	500 mg = RM 10.60	40 mg/kg/day x 30 kg ~ 1.5 gm/day 1 week = 18 vials 4 weeks = 72 vials x RM 10.60 = RM 763.20
Deferasirox (Exjade)	125 mg = RM 22.79 500 mg = RM 91.21	20 mg/kg/day x 30 kg ~ 500mg/day For 28 days = RM 91.21 x 28 tabs/500mg One month = RM2553.88

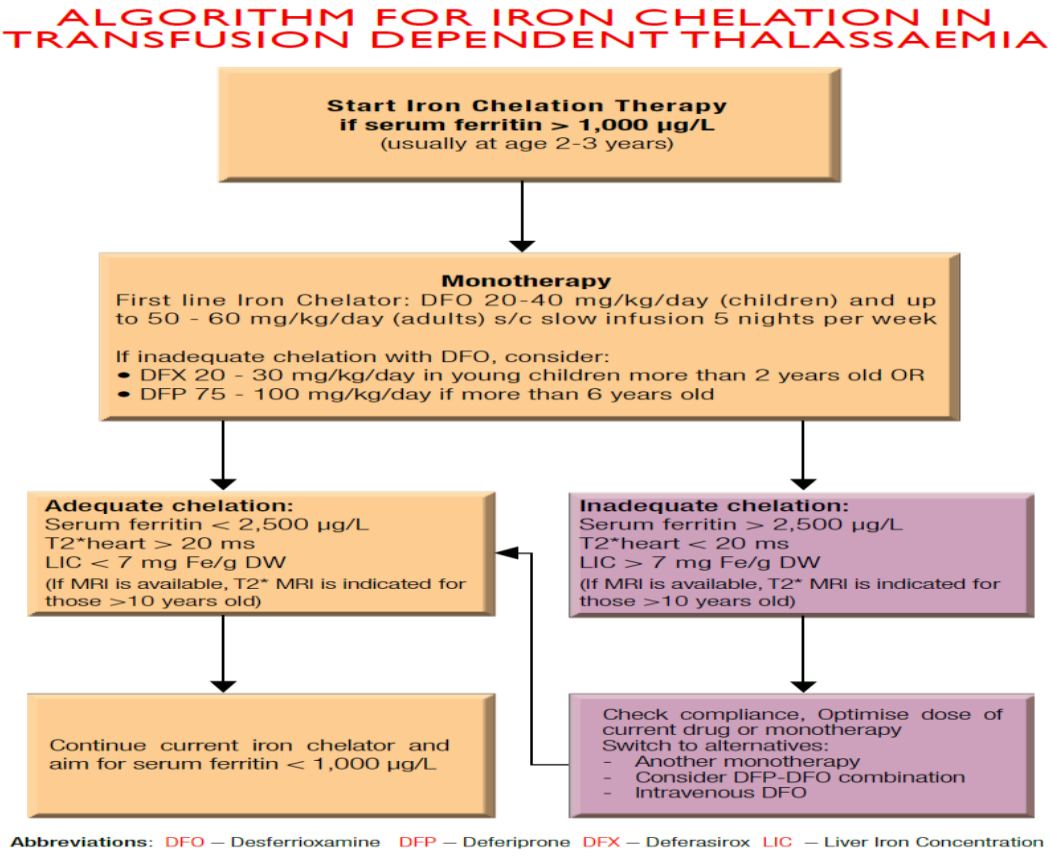

Figure 2.6. Algorithm for iron chelation in transfusion dependent thalassaemia. Adapted from (Ministry Of Health, 2009)

Multi organ toxicity often been observed as a complication in transfusion dependent thalassaemia patients including endocrine and cardiac problem. Examples of endocrine problem include short stature and growth failure, delayed puberty and hypogonadism, hypothyroidism, diabetes mellitus, osteoporosis/osteopenia, hypoparathyroidism, hypoadrenalism. Hepatitis B, Hepatitis C, HIV and bacterial infection are examples of infection that are implicated in these patients, thus affecting the patient's life as well as the country economics.

Therefore, it is vital for the health agencies and governments of countries where the haemoglobin disorders occur at a high frequency become aware of the future extent of this problem and to develop a programme for the control and management (Weatherall and Clegg, 2001).

2.8 Screening programme

Thalassaemia can be identified in the carrier state and most forms can be diagnosed in the foetus. Thus it is possible to offer counselling and prenatal diagnosis for parents who wish to terminate pregnancies carrying babies with severe forms of the disease. This approach has resulted in a major reduction in the birth of new cases in some of the Mediterranean islands and in other countries (Cao and Rosatelli, 1993). Diagnosis of thalassaemia and haemoglobinopathies can be made by combination of at least 2 different techniques and must be correlated with the clinical findings and background ethnicity of the patients (Bain, 2008).

2.8.1 Screening tests

Diagnostic laboratory investigations for thalassaemia and haemoglobinopathy include full blood count (FBC), haemoglobin electrophoresis at alkaline pH, sickling test and quantification of abnormal HbA_2 and HbF, as recommended by International Committee for Standardization in Haematology expert panel on thalassaemia and haemoglobinopathy (Clarke and Higgins, 2000).

2.8.1(a) Full blood count (FBC) and peripheral blood film

The first step for haemoglobin analysis is scrutiny for the red blood cell indices. The red blood cells indices are importance for both α and β thalassaemia carrier diagnosis. The value of MCV