DHPLC FOR SCREENING OF β-THALASSAEMIA GENE MUTATIONS

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DISCLAIMER

I, hereby declare that the dissertation is wholly prepared based on my own research. It does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university. To my knowledge, it does not contain any material previously written by another person where the reference is not made in the text.

Date:.....

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ABSTRACT

β thalassaemia is a heterogeneous group of disorder resulted from more than 200 mutations to date with most of the defects involve a few base pair changes. Denaturing high performance liquid chromatography (DHPLC) is a relatively new technique for mutation screening, which have been reported to have a high sensitivity rate but its application for β thalassaemia mutation screening is still very limited. DHPLC was used in this study to screen for β thalassaemia gene mutations in 25 participants; 19 known β thalassaemias (16 patients and 3 carriers) and 6 normal controls. Five sets of primers were designed to cover the β gene with consequential 45 amplicons have been analysed. DHPLC was only able to detect 6 of 28 mutation-containing amplicons resulting in its sensitivity of 21.4%. Twelve of the β thalassaemia patients were homozygous for the common β thalassaemia mutations causing these to be undetected. Only one compound heterozygous patient was successfully detected. The mutations in the 3 carriers also failed to be detected, most likely due to their locations at the end of the D A sequence and possibly due to the stabilized the heteroduplex molecules. The pre-DHPLC preparation steps were labour intensive, analysis was lengthy, optimization and standardization were difficult. The cost, on the other hand could potentially be advantageous in the long run. In conclusion, this study showed that DHPLC was labour intensive, was technically difficult in terms of optimization and standardization and was also insensitive for mutation screening in β thalassaemia but had an advantage of being relatively cheap for long term use.

ABSTRAK

β thalasemia adalah satu kumpulan penyakit disebabkan mutasi pada gen β thalassaemia dan lebih daripada 200 jenis mutasi telah ditemui setakat ini, dimana kebanyakan mutasi tersebut adalah disebabkan perubahan pada nucleotid. 'Denaturing high performance liquid chromatography' (DHPLC) adalah satu kaedah yang masih baru untuk saringan mutasi yang telah dilaporakan mempunyai paras sensitiviti yang tinggi. Namun kegunaannya dalam saringan mutasi β thalasemia masih lagi terhad. Kami telah menjalankan kajian ke atas 25 orang dimana 16 pesakit dan 3 pembawa β thalasemia yang dikenalpasti serta 6 individu normal untuk saringan mutasi pada β globin gen menggunakan kaedah DHPLC. Lima set primer untuk gen β thalasemia telah digunakan, menjadikan jumlah keseluruhan 'amplicon' yang diuji adalah sebanyak 45. Kajian ini mendapati bahawa DHPLC hanya dapat mengenalpasti 6 daripada 28 'amplicon' yang mempunyai mutasi, menjadikan sensitiviti teknik ini hanya 21.4%. Dua belas orang pesakit β thalasemia tersebut mempunyai mutasi 'homozygous', menyebabkan ia tidak dapat dikenalpasti dengan DHPLC. Hanya seorang pesakit yang mempunyai mutasi 'compound heterozygous' dapat dikenalpasti dengan DHPLC. Mutasi gen β thalasemia pada tiga orang pembawa juga tidak dapat dikenalpasti oleh DHPLC mungkin disebabkan lokasi mutasi tersebut berada terlalu dipenghujung jujukan (sequence) D A, atau mungkin kerana kehadiran struktur molekul 'heteroduplex' yang stabil. Dengan menggunakan DHPLC, kami juga mendapati penyediaan sampel mengambil masa yang panjang, analisa memakan masa yang lama serta optimisasi and standardisasi adalah sukar dilakukan. Walau bagaimanapun, kos jangka panjang penggunaan DHPLC mungkin akan lebih murah. Kesimpulannya, kami mendapati penyaringan mutasi gen β thalasemia menggunakan DHPLC adalah kaedah yang memerlukan tenaga kerja yang banyak pada peringkat penyediaan serta kaedah optimisasi dan standardisasinya sukar dan juga tidak sensitif, namun agak murah dari segi kos dalam jangkamasa panjang.

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LIST OF ABBREVIATIONS

ARMS	Amplification Refractory Mutation System
ASO	Allele-Specific Oligonucleotide
ASOH	Allele-Specific Oligonucleotide Hybridization
BCSH	British Committee for Standards in Haematology
DGGE	Denaturing Gradient Gel Electrophoresis
DHPLC	Denaturing High Performance Liquid chromatography
D A	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EKLF	Erythroid-Kruppel-Like Factor
Hb A	Haemoglobin A
Hb F	Haemoglobin F
Hb	Haemoglobin -
HPLC	High Performance Liquid chromatography
HUKM	"Hospital Universiti Kebangsaan Malaysia"
IP-RP	Iron-Pair Reverse-Phase
IVS	Intervening Sequence
MCH	Mean Cell Haemoglobin
MCV	Mean Cell Volume
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RTm	Recommended melting Temperatures
S P	Single Nucleotide Polymorphism
SSCP	Single-Stranded Conformation Polymorphism
TEAA	Triethylammonium Amine

UTR	Untranslated Region
α	alpha
β	beta
β-LCR	β-Locus-Controlling Region
γ	zeta
δ	delta
ε	epsilon
ζ	zeta

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CHAPTER ONE

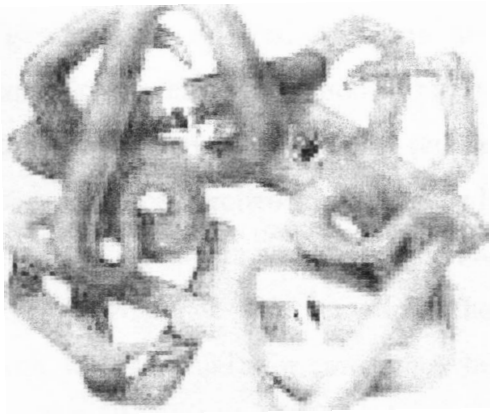
INTRODUCTION

1.1 Haemoglobin

Haemoglobin (Hb) molecule is a tetramer of two alpha-like and two beta-like globin chains. Each of the globin is covalently linked to an oxygen-binding haem group. In a healthy adult, about 95% of the haemoglobin is haemoglobin A (Hb A) with less than 4% haemoglobin A_2 (Hb A_2) and less than 2% haemoglobin F (Hb F). Hb F is the main haemoglobin during fetal life while three embryonic haemoglobins, Hb Gower 1, Hb Gower 2 and Hb Portland are synthesized before the 8th week of intrauterine life. The ζ and ε chains are the embryonic counterparts of the adult α and β and γ and δ chains respectively. During fetal development, orderly switch from ζ to α-chain and from ε- to γ-chain production occurs, followed by β- and δ-chain production after birth. These are all physiological haemoglobin that changes throughout a human life to adapt to differing physiological needs. In addition, there are minor haemoglobin components that are the result of post synthetic modifications that may take place *in vivo*, or *in vitro* during storage in the laboratory, and the most commonly encountered are HbA_{1c}, the product of Hb A reacting with glucose, Hb A_3, which possibly occur *in vitro* as a result glutathione binding to Hb, and the acetylated form of fetal haemoglobin, Hb F1 (Weatherall D., 2001).

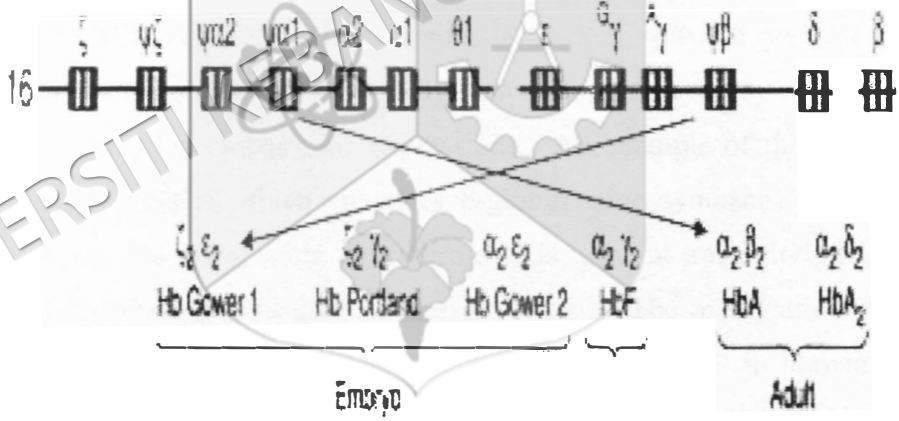
All normal human haemoglobins are tetramers of two pairs of unlike globin chains (Figure 1.1). Adult and fetal haemoglobins have α chains associated with β (Hb A, $\alpha_2\beta_2$), δ (Hb A_2, $\alpha_2\delta_2$) or γ chains (Hb F, $\alpha_2\gamma_2$), whereas in the embryo ζ chains combine with γ (Hb Portland, ζ2γ2) or ε chains (Hb Gower 1, ζ2ε2) and α and ε chains combine to form Hb Gower 2 (α2ε2) (1). The alpha globin chain is produced by of two sets of α-genes (termed α1 and α2) located within the α-gene cluster situated on the short arm of chromosome 16.

imilarly, the β-globin chain is formed by β-globin genes that are present within the β-globin gene cluster on chromosome 11p (Figure 1.2). The γ-genes are present in pairs, called $^{G}\gamma$ and $^{A}\gamma$ genes, which differ by a single amino acid.



(Reference : www.thalassaemia.org.cyimagesHem.jpg)

Figure 1.1 Three dimensional structure of haemoglobin structure composed of two α and two β globin chains as well as four haem rings at the centre of each globin molecule.



(Reference : www.thalassaemia.org.cyimagesHem.jpg)

Figure 1.2 β-globin gene cluster on chromosome 11 (left figure) is made up of ε, two γ, δ and β genes that are developmentally regulated. Also shown is α gene clusters (right).

Regulation of β-globin gene cluster expression occurs at various levels, with transcription being the most important level of regulation. Transcription of globin gene cluster is initiated by alterations in chromatin structure that render this part of the gene susceptible to exogenous nucleases. It is controlled by interaction between the genes and transcription factors that bind to both promoters and β-locus-controlling region (β-LCR) (Bain). The promoters of all the globin genes share remarkable homology but they also show unique sequences that may be responsible for conveying the developmental stage specificity of each promoter. The three regulatory elements within the promoter are identified as TATA, CAAT and CACCC boxes (Cao, 2002). For β-globin, the sequences within the boxes are CATAAAA, CCAAT and CACCC respectively. These lie in close proximity with the β-globin gene and are required for full β-globin gene expression. The β-LCR includes four erythroid-specific D ase sites, named HS1, HS2, HS3 and HS4. The HS are a hallmark of D A-protein interactions and of interesting modifications of the chromatin structure that facilitate the access of regulators and lower the threshold for activation of the linked genes (Cao, 2002). Their locations vary during different stages of development. In fetal life, these sites are associated with the promoter regions of all four globin genes. In adult erythroid cells, the sites associated with the γ-genes are absent (Williams). *Trans*-acting factors, encoded by genes on chromosomes other that 11 and 16, are also important for globin gene expressions. An example of these is erythroid-Kruppel-like factor (EKLF) which enhances β-globin gene synthesis. In addition to transcription factors that are specific to erythroid cells, general transcription factors also play a role in influencing globin gene expression (Bain). The methylation state of the genes also plays an important role in the ability to be expressed. In human and other animal tissues, the globin genes are extensively methylated in nonerythroid organs and are relatively undermethylated in haematopoietic tissues. Changes in chromatin configuration around the globin genes at different stages of development are reflected by alterations in their methylation state (Williams).

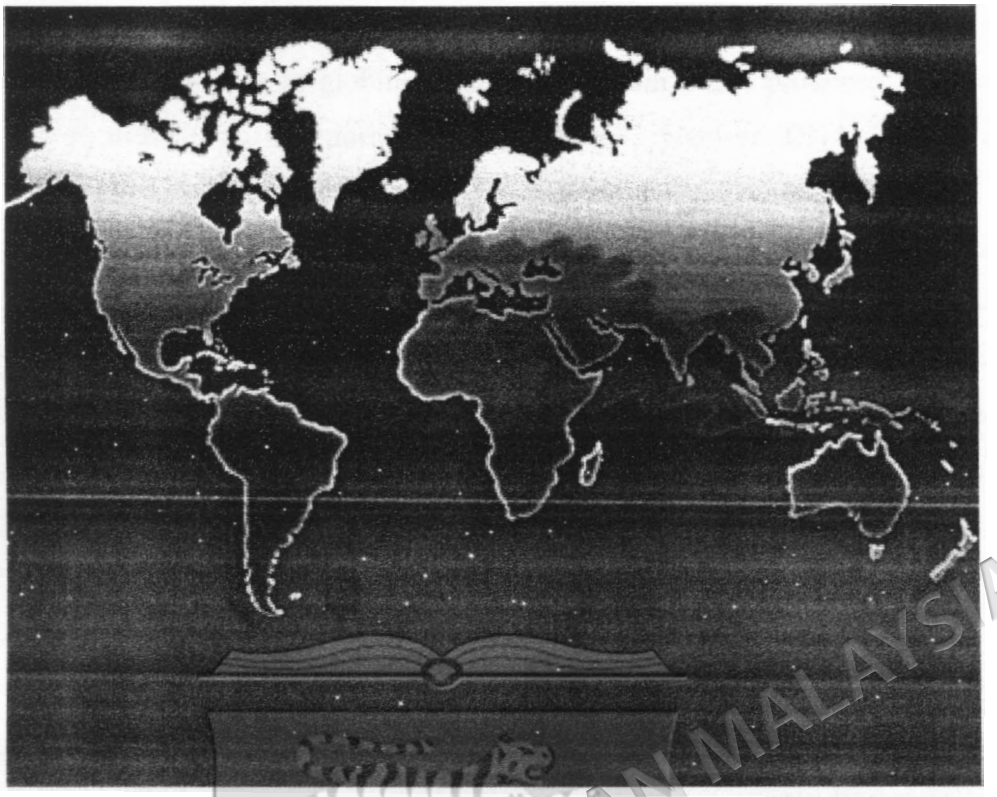
After transcription, the primary transcript undergoes a great deal of processing such as capping the 5` end and polyadenylation of the 3` end, both of which are for stabilization

of the transcript. Then, the intervening sequences are spliced from the primary transcript in a complex two-stage process that relies on certain critical sequences at the intron-exon junctions. From the nucleus, the mRNA moves into the cytoplasm where translation takes place in the polyribosomes. The control of globin chain synthesis is probably mainly at the level of transcription, with translational control being less important.

The content of haemoglobin tetramer changes to adapt to the physiological needs at different stages of human development. The haemoglobin switching is a process in which there is suppression of the γ-globin gene with complementary rise in the expression of the previously silent β-globin gene. Promoter sequence seems to be the regulator of this event as the γ- and β-globin genes are developmentally regulated in transgenic mice even in the absence of LCR. Several transcription factors that bind to the promoter elements have been implicated, one example of which is EKLF that activates the β-globin gene (Cao, 2002). This could explain the time of presentation of various types of thalassaemia corresponding to the type of globin chain affected and the severity of the mutation.

1.2 Thalassaemia

'Thalassaemia' originated from a Greek word means 'the sea'. It was first recognized by Dr T. Cooley in 1925 in children of Mediterranean origin. It is now known that thalassaemia affects many other different countries in the world, extending from the Mediterranean across the Middle East right down to Indian subcontinent, South East Asia and the Pacific islands. In fact, it is the most common monogenetic disease in human, affecting 4.5% of the world population. This is an example of positive selection pressure of the mutations that result in thalassaemia as a survival advantage against the fatal malaria infection (Figure 1.3).



(Reference : www.thalassaemia.org.cvimagesHem.jpg)

Figure 1.3 World distribution of thalassaemia.

Thalassaemia is essentially a group of inherited disorders that result in variable reduction in the production of globin chain. The globin chain produced, however is structurally equivalent to the normal globin chain. Nuclear DNA is subject to spontaneous mutation, ranging from point mutation to more extensive deletions. The different mutations can lead to production of structurally abnormal haemoglobin (variant haemoglobin), reduction of globin chain synthesis (thalassaemia) or the combination of both (thalassaemic haemoglobinopathy). A significantly reduced rate of synthesis of one type of globin chain leads to unbalanced chain synthesis, with an excess of a normal globin chain contributing to the pathological effects, causing either damage to the red cell precursors and ineffective erythropoiesis or damage to mature erythrocytes and haemolytic anaemia. The nomenclature of thalassaemias derived from which globin chain is affected. for example α- or β-thalassaemia when the α- or β-globin chain synthesis is reduced respectively. Thalassaemia can either be classified according to phenotype or genotype. The major types of thalassaemia, classified according to genotype, are shown in table 1.1 (Bain B., 2005).

Table 1.1 : The major type of thalassaemia, classified according to genotype

Type of thalassaemia	Chain or chains synthesized at a reduced rate	Haemoglobin(s) synthesized at a reduced rate
Alpha: α^0 or α^+	α	A, A_2 and F
Beta: β^0 or β^+	β	A
Gamma: γ	γ	F
Delta: δ^0 or δ^+	δ	A_2
Delta beta: $\delta\beta^0$ or $\delta\beta^+$	δ and β	A and A_2
^{A}Gamma delta beta: $^\beta\gamma\delta\beta^0$	$^A\gamma$, δ and β	A and A_2
Epsilon gamma delta beta*:		A, A_2 and F
$\varepsilon^G\gamma^A\gamma\delta\beta^0$	ε, $^G\gamma$, $^A\gamma$, δ and β	
Haemoglobin Lepore	δ and β	A and A_2

(Bain, 2006)

1.3 Beta thalassaemia

β-thalassaemia in itself is a heterogenous group of disorders affecting the β-globin gene with a highly variable clinical severity. It is divided into two broad categories: β^{o}-thalassaemia and β^{+}-thalassaemia. β^{o}-thalassaemia is most commonly a result of gene mutations, or less commonly deletions that lead to a total lack of β-globin chain synthesis. In homozygous or compound heterozygous states for the severe mutations, the individual does not have any haemoglobin A production. In β^{+}-thalassaemia, there is reduced but not absent expression of the abnormal β-gene so that even in homozygotes, there is still some production of haemoglobin A.

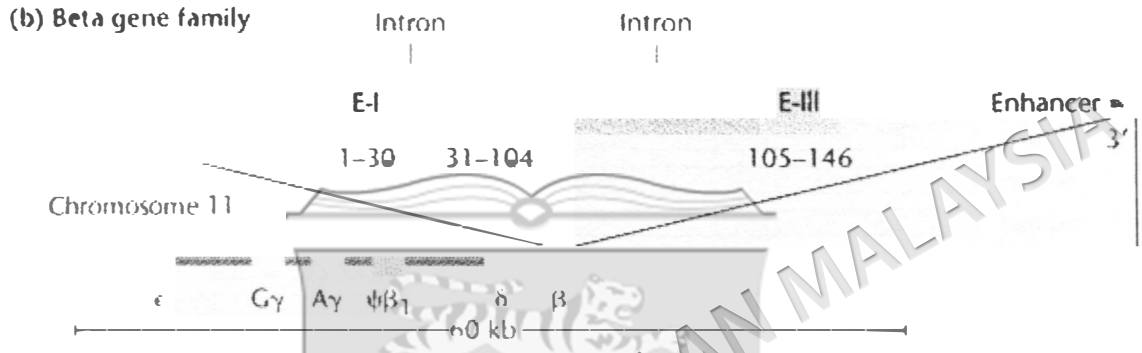
The general structure of β-globin gene is typical of the other globin loci (Efstratiadis et al, 1980). The genomic sequence, which codes for 146 amino acids, spans 1600 bp; the transcribed region is contained in three exons separated by two introns or intervening sequences (IVSs). Exon 2 encodes the residues involved in haem binding and αβ dimer formation, while exons 1 and 3 code for the non-haem-binding regions of the β globin chain. Many of the amino acids involved in globin subunit interactions required for the Bohr effect, and 2,3-diphosphoglycerate binding, are found in exon 3. Conserved sequences important for β globin gene expression are found in the 5' promoter region, at the exon-intron junctions, and in the 3' untranslated region (3'-UTR) at the end of the mRNA sequences (Figure 1.4).

The underlying pathophysiology of β thalassaemia relates to a quantifiable deficiency of functional β globin chains, which leads to an imbalanced globin chain production and an excess of α globin chains (Weatherall & Clegg, 2001). The latter aggregate in red cell precursors leading to premature destruction of red cells in bone marrow or in the spleen. Thus, anaemia in β thalassaemia is due to a combination of ineffective erythropoiesis, peripheral haemolysis and an overall reduction in haemoglobin synthesis. Factors which are able to reduce the degree of chain imbalance and the

magnitude of α chain excess in the red cell precursors have an ameliorating effect on the β thalassaemia phenotype (Thein S L, 2004).

There are over 200 different mutations reported (Olivieri, 1999), mainly of the non-deletional types that result in β-thalassaemia syndrome (Figure 1.5). These different mutations seem to vary with geographical locations and ethnic distributions with only five to six mutations causing 90 percent of the total β thalassaemia cases in each ethnic. The vast majority of β thalassaemia are caused by point mutations within the gene or its immediate flanking sequences. Rarely, it is caused by gene deletions and mutations that affect the *trans*-acting regulatory factors (Thein S L, 2004). The point mutations are the result of single base substitutions, minor insertions or deletions of a few bases within the gene or its immediate flanking sequences (Thein S L, 1998), and may affect any level of genetic regulation. Mutations in the conserved DNA sequences that form the β globin promoter or the stretch of 50 nucleotides in the 5'-UTR can directly affect transcription but they generally result in a mild to minimal deficit of β globin output that reflects the relatively mild phenotype of these β^+ thalassaemia.

Other mutations that affect RNA processing can involve either of the invariant dinucleotides (GT at 5' and AG at 3') in the splice junction in which case normal splicing is completely abolished resulting in β^0 thalassaemia phenotype. Mutations within the consensus sequences at the splice junctions reduce the efficiency of normal splicing to varying degrees and produce a β^+ phenotype that ranges from mild to severe. Mutations within introns or exons might also affect the splicing pattern of the pre-mRNAs. The most common example of this is HbE mutation. Other RNA processing mutants affect the polyadenylation signal (AATAAA) and the 3'-UTR. These are generally mild β^+ thalassaemia alleles.



(Klug & Cummings 1997)

Figure 1.4 Beta globin gene constitutes three exons, two introns, 5' untranslated region (UTR) and 3' UTR.

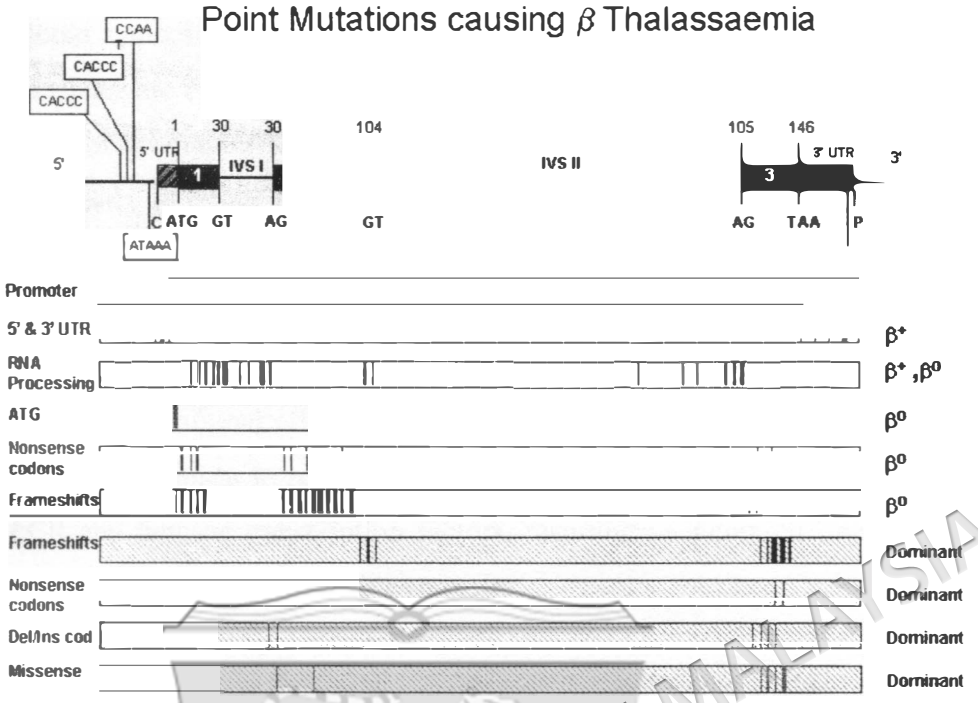


Figure 1.5 Figure of the globin gene with 3 exons and 2 introns and the different classes of point mutations causing β thalassaemia.

Note : The vast majority of βthalassaemia alleles are caused by point mutations. These result in a complete absence (β^0) or reduced production (β^+) of β globin. Mutations which affect the promoter region, the 5' and 3' UTRs and polyA site lead to minimal deficit of β chain synthesis and are usually associated with very mild β thalassaemia. A large group of mutations causes aberrant splicing of mRNA. Those which involve the invariant dinucleotide in the splice junction inactivate normal splicing and cause β^0 thalassaemia. Those which affect consensus splice site, introns or exons reduce normal splicing and cause β^+ thalassaemia. About half of the mutations cause β^0 thalassaemia from premature termination of translation due to F/S or nonsense codons. Other mutations which affect translation include those affecting ATG codons. A spectrum of underlying molecular lesions is found including missense mutations, minor insertions/deletions, leading to insertion or loss of intact codons, aberrant splicing and premature termination caused by F/S or nonsense mutations. The molecular mechanisms underlying the unusual severity of these mutations appear to be the predicted synthesis of highly unstable β^2 chain variants, so unstable that in many cases they are not detectable, and only implicated from the DNA sequence. Compared to recessive β^2 thalassaemia, the premature termination codons caused by single base substitutions and frameshift mutations are concentrated in exon 3 or 3' half of exon 2. These PTCs escape nonsense mediated decay (NMD) resulting in the synthesis of mutant β^2 mR A and highly unstable truncated β^2 chain variants.

Thein S L, 1998

Mutations that abolish mRNA translation either at the initiation or extension phases of globin synthesis are all associated with a β^0 phenotype. About half of the β thalassaemia alleles are characterized by premature termination of β chain extension due to introduction of premature termination codons, a result of frameshifts or nonsense mutations and nearly all terminate within exons 1 and 2.

Deletions causing β thalassaemia result in a complete absence of β globin product and are expected to cause a severe phenotype but in reality, they do not normally cause a β^0 phenotype. The mutational effect is compensated by unusually high Hb F and HbA2 levels. The deletions appear to remove competition by the different β-like genes for the upstream LCR and limiting transcription factors, resulting in increased interaction with the γ and δ genes in *cis*, enhancing their expression.

In Malaysia, thalassaemia and haemoglobinopathy are common similar to the other parts of the world where malaria is endemic. The carrier rate for β-thalassaemia is about 4.5% amongst the Malays and Chinese-Malaysians (George E, 2001). More interestingly, there is a large ethnic mix where various combinations of thalassaemia inheritance are seen. This lead to different types and frequencies of mutations are present in our population (E. George, 1992). Five mutations in Malays and four in Chinese comprised about 94% of the mutations seen (Table 1.2) (E. George 1992). Similar mutations and frequencies were also found in a different study (Ainoon O et al., 1994).

Table 1.2 : β-thalassaemia mutations in Malays and Chinese in Malaysia

Malays	Frequency (%)	Chinese	Frequency (%)
IVS 1-5 (G to C)	47.2	Codon 41-42 (-TCTT)	50.9
IVS 1-1 (G to T)	19.4	IVS 2-654 (C to T)	23.4
Codon 17 (A to T)	13.9	-28 (A to G)	13.3
Codon 35 (-C)	8.3	Codon 17 (A to T)	10.1
Codon 41-42 (-TCTT)	5.6	Codon 71 (+A)	2.3
IVS 2-654 (C toT)	2.8		

(E. George 1992)

1.4 Diagnosis of β thalassaemia

In 1995, it was estimated that 8000 people were affected with homozygosity for beta thalassaemia, about 40% of whom, were transfusion dependent for survival (Kaur, 1995). With gene frequencies of 3-4%, β-thalassaemia is a public health problem especially amongst Malays and Chinese (E. George, 1984). Management of thalassaemia includes transfusion support and iron chelators as well as the associated complications of the disease. These are very expensive especially where the prevalence of the disease is high. A more economical approach includes prenatal screening and genetic counseling to affected individuals. This approach has been shown to reduce the homozygous thalassaemia incidence in many studies (Mitchell et al., 1996, Lena-Russoet et al., 2002, Ghanei et al., 1997).

The diagnosis β-thalassaemia is usually made by a combination of clinical presentation, full blood count indices, haemoglobin electrophoresis and high performance liquid chromatography methods. In the British of Haematology guideline for laboratory diagnosis of haemoglobinopathies, the full blood count was proposed to be the initial screening test (BJH guideline, 1998). When there is evidence of hypochromia (MCH<27 pg) or when the patient is of ethnic origin in which thalassaemia is prevalent, further tests such as haemoglobin electrophoresis, Hb A2 quantification and other specialized tests are performed to make a diagnosis. In carrier detection, the diagnosis lies on the identification of an increased haemoglobin A_2 percentage, in which the high performance liquid chromatography (HPLC) is a good method available for classical β thalassaemia trait detection (George E, 2001). Other methods for Hb A2 quantification include microcolumn chromatography and the elution of Hb A2 portion from an electrophoretic strip. However, HPLC should be combined with haemoglobin electrophoresis at alkaline pH if variant haemoglobins and with unstable haemoglobin test if unstable haemoglobins are suspected (BJH guideline 1998). Quantification of haemoglobin F (Hb F) is not essential for the diagnosis of β thalassaemia, but it is normally performed as part of the

whole thalassaemia diagnostic work up. If HPLC is used, Hb F level is automatically available together with the other haemoglobins percentages. When Hb F is elevated, it usually comprises between 2-7% of total haemomoglobin (Bain). Elevation is usual when β thalassaemia trait is caused by deletion of the 5' part of the β globin gene. However, if a patient has red cell indices suggestive of thalassaemia, but has a normal haemoglobin A2 percentage, it is essential to exclude elevation of Hb F as δβ thalassaemia is an alternative diagnosis.

Although current available techniques are good enough to identify almost all the thalassaemia cases, there are limitations to the current algorithm. A small proportion with a very mild, silent β phenotype which has normal MCH and HbA2 levels may be mi sed. It is important to be able to identify such cases because it can cause clinically significant disease in homozygotes and compound heterozygotes, therefore it is important to identify the carriers. Another problem is making the diagnosis in the neonatal period. β thalassaemia cannot be diagnosed from the Hb A2 percentage in neonates as levels are low. Coinheritance of β thalassaemia with other haemoglobinopathy such as α or δ thalassaemia can also mask the diagnosis, as well as presence of haematinics deficiencies. Inability to accurately quantify an A2 variant as elevated may cause the diagnosis of β thalassaemia trait to be missed. Another significant area of interest in today's world is prenatal diagnosis of thalassaemia, which is not possible with the usual approach in Malaysia.

With the advent of molecular technology techniques and better resources, molecular characterization of this condition is now made possible. Molecular methods are able to overcome the limitations discussed above. They can also provide the definitive diagnosis of the condition when the need arises. However, cost and expertise are the main issues when molecular techniques are employed. Over the years, a number of studies have been performed to characterized various mutations exist using variable molecular methods. PCR-based methods have gained much popularity. The variability and specificity of these techniques come from the primers used (Clark B E. 2004). The mo t commonly used techniques used in haemoglobin diagnostics include allele-

specific oligonucleotide (ASO) hybridization or dot-blot analysis, allele-specific priming or amplification refractory mutation system (ARMS), restriction enzyme analysis and gap-PCR. These techniques are useful for identifying a known mutation. For unknown mutations, technologies that make use of altered conformation of single-stranded DNA include denaturing gradient gel electrophoresis (DGGE), single-stranded conformation polymorphism (SSCP), heteroduplex analysis and denaturing high performance liquid chromatography (DHPLC). The ultimate method of mutation identification is by direct sequence analysis of specifically amplified DNA. Southern blotting is probably the one of the few non-PCR based techniques that still has a significant role in the molecular diagnosis of haemoglobinopathy. It is particularly useful for large deletions or rearrangements and is essential in the characterization of novel deletions. Some of these methods are highly laborious and time consuming. Each laboratory should carefully select test to be employed based on cost effectiveness, population size that it caters for and the type of ethnic mix present in the population of interest. Positive selection by malaria parasites for the thalassaemia has led to an increased in frequency of some of the mutations in the malarial regions, such that each population has its own combination of a limited number of common Hb variants and thalassaemia mutations that occur in a great majority of the patients together with a larger number of rarer ones.

1.5 Denaturing high performance liquid chromatography (DHPLC)

Denaturing high performance liquid chromatography (DHPLC) is a relatively new tool for unknown mutation screening. It detects mutations on the basis of mismatches between amplified chromosomal fragments that result in the formation of heteroduplices (Xiao and Oefner, 2001) (Figure 1.6). The heteroduplices, which are thermally less stable than their corresponding homoduplices, are resolved by means of ion-pair reverse-phase liquid chromatography at elevated column temperatures typically in the range of 50 ^{0}C 70 ^{0}C depending on the GC-content of the sequences. The detection is based on fragment size and charges that result in a different retention times when D A fragments pass through the solid/liquid phases in the system. A single well-defined peak at the expected retention time for the amplicon size indicates the presence of a clean PCR product, while fragments containing mutations give rise to slicing of the peaks. DHPLC allows for an automated detection of single base DNA substitution as well as small insertions and deletion (Oefner and Huber 2002. Xiao and Oefner. 2001). When using heteroduplex analysis by DHPLC under partially denaturing conditions. heteroduplexes are retained less than their corresponding homoduplexes on a unique D A separation matrix. DHPLC uses unpurified PCR products that are subjected to a final denaturing/reannealing step to ensure adequate formation of the heteroduplex. Analysis time for such a procedure is typically within 8 mins per sample.

There are various integral components that form the whole DHPLC Helix system. These include the oven, that hosts the Helix Column, the UV light source as a means of detection system, the autosample handler unit that automates sample injection from a 96-well plate, two pump systems that regulate the flow rates of buffers, the computer unit which allow instant result visualization of the results as well as storage of data for later analysis and the valve organizer box.

All these are shown in Figure 1.8, except for the computer system which is just next to the whole integrated unit.

DHPLC was reported to have greater than 95% sensitivity in detecting single nucleotide polymorphisms (SNPS) and mutations (O'Donovan et al., 1998). A few studies have used this method for detection of β-thalassaemia gene mutations. In China, Grant W. et al have tried using multiplex primer extension combined with DHPLC for rapid and accurate genotyping of their β-thalassaemia mutations. They concluded that the technique was simple, highly accurate and cost-effective for the use of genotyping common disease-causing mutations (Grant W. et al, 2003). Similarly, an Italian study also showed that DHPLC to be sensitive and reliable for screening, with reported accuracy of 100% (Colosimo A, et al.). Taiwanese group has also studied similar aspect of DHPLC sensitivity for detection common Taiwanese β-thalassaemia gene mutation and found a similar sensitivity, of more than 99% (Yi-Ning S, 2003). Therefore, DHPLC is one of the most sensitive techniques of mutation screening, however it is costly for diseases with large number of exons.

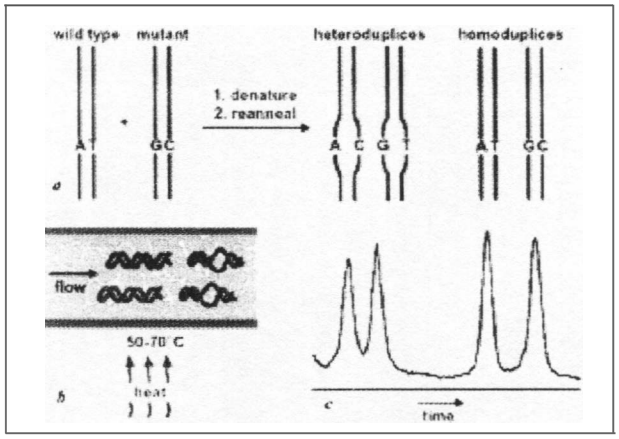
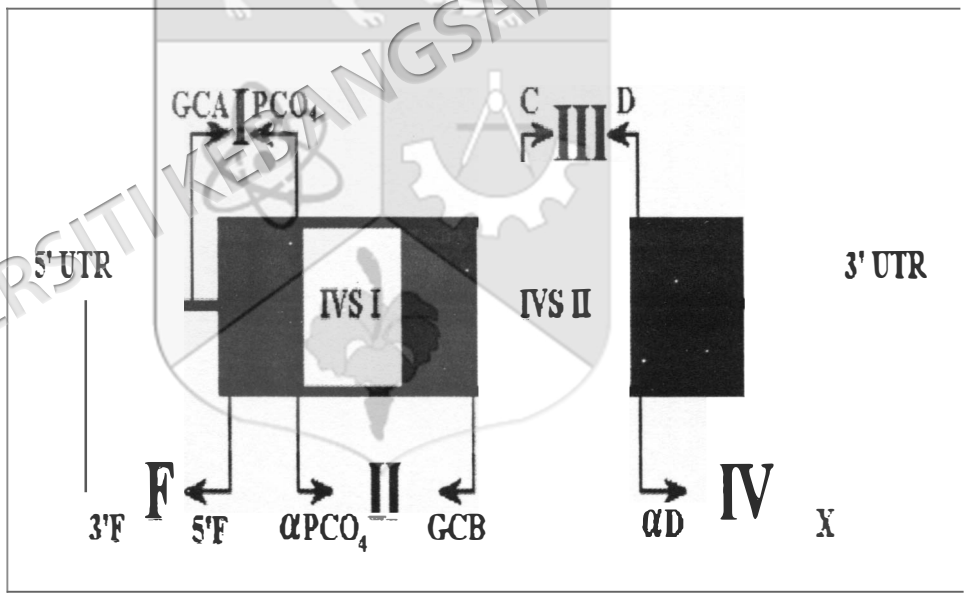


Figure 1.6 Formation of heteroduplex molecules when a mutant allele combines with a wild type allele.



(Talmaci R, 2004)

Figure 1.7 Locations of primer sequences that flank almost the entire β-globin gene where most of the mutations were found.

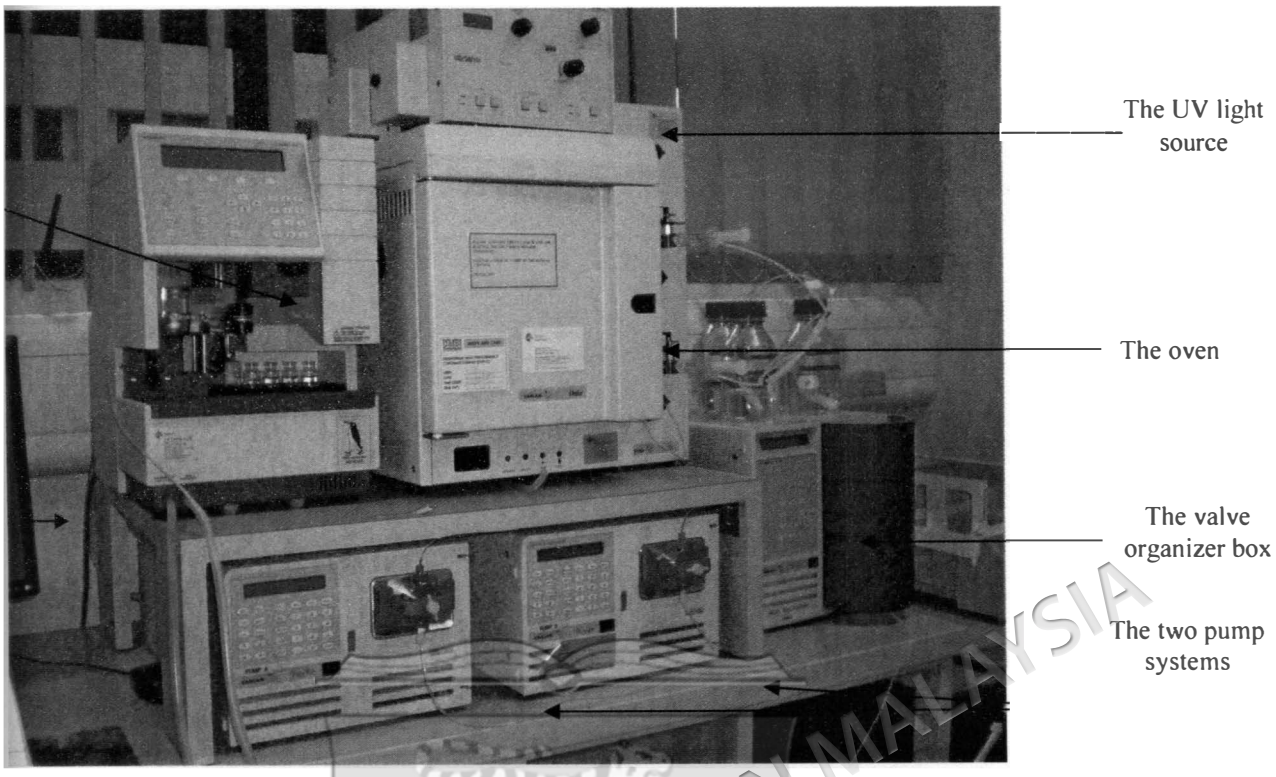


Figure 1.8 DHPLC system consists of various integral components.

1.6 Objectives of the study

1.6.1 General objective

1. To optimize the settings of denaturing high performance liquid chromatography (DHPLC) to perform mutation screening of β-thalassaemia gene.

2. To detect β-thalassaemia gene mutations by using DHPLC.

1.7 Research hypothesis

DHPLC is a sensitive method to detect the mutations in β-thalassaemia patients and carriers.

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