MOLECULAR BASIS OF ALPHA THALASSEMIA AMONG ABORIGINES IN KELANTAN

by

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"In the name of Allah, Most Gracious, Most Merciful"

"Over the knowledgeable, Allah the Most Knowledgeable"

All praises and gratitude is to Allah, the Lord to whom every single creature in the heaven and the earth belongs to. Thank Allah for giving me the strength and patient during this challenging time. May peace and blessings be on the leader of all creation, the prophet Muhammad S.A.W, his family and companion.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

%	Percent
β	Beta
α	Alpha
δ	Delta
ζ	Zeta
γ	Gamma
ε	Epsilon
ψ	Psi
θ	Theta
bp	base pair
EDTA	Ethlenediaminetetraacetic acid
FBC	Full Blood Count
Hb	Hemoglobin
Hb F	Hemoglobin F
Hb A	Hemoglobin A
Hb A_2	Hemoglobin A2
HPLC	high performance liquid chromatography
HUSM	Hospital Universiti Sains Malaysia
ddH_2O	deionized distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside trisphospate
G	gram
kb	kilo base pair

mg	milligram
$MgCl_2$	magnesium chloride
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
min	minute
mL	milliliter
mm	millimeter
PCR	polymerase chain reaction
SEA	Southeast Asian
THAI	Thailand
s	second
Taq	*Thermus aquaticus*
TAE	Tris-Acetate EDTA
Tris	tris (hydroxymethyl) aminomethane
V	volt
µl	microliter
µm	micrometer
µM	micromolar

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ABSTRAK

Talasemia alfa adalah penyakit hemoglobin (Hb) sintesis yang sering diwarisi di seluruh kawasan tropika dan subtropika di dunia. Ia biasanya hasil daripada penghapusan satu atau kedua-dua alpha gen pada kromosom 16p13.3. Pembawa gen ini mempunyai pelbagai ukuran anemia (rendah Hb), rendah (MCH/pg), rendah (MCV/fl) dan normal atau sedikit berkurang tahap HbA2. Walau bagaimanapun, analisis molekul adalah penting untuk menyokong pemerhatian hematologi. Justeru itu, tujuan kajian kami adalah untuk menyaring dan mengesan kehadiran mutasi terpadam di dalam kluster gen α-globin dalam kalangan penduduk orang asli di Gua Musang, Kelantan. Pemadaman gen alpha telah dikenal pasti melalui kaedah Multipleks-Polymerase Chain Reaction (PCR) dan 1% gel agarose elektroforesis. Mutasi pemadaman termasuk pemadaman satu gen iaitu -α3.7 dan -α4.2, dan pemadaman kedua-dua gen iaitu --SEA dan --THAI pada 52 sampel darah daripada responden yang dikaji. Keputusan mendedahkan dua daripada 52 responden mempunyai pemadaman gen alpha, satu responden dengan (1.9%) pemadaman satu gen -α3.7 dan satu responden dengan (1.9%) pemadaman kedua-dua gen --SEA. Kesimpulannya, kajian ini akan membantu dalam menentukan status talasemia alfa dalam kalangan orang asli untuk perancangan dan pengurusan kesihatan masa depan mereka.

MOLECULAR BASIS OF ALPHA THALASSEMIA AMONG ABORIGINES IN KELANTAN

ABSTRACT

Alpha thalassemia is a common inherited disorder of hemoglobin (Hb) synthesis throughout all tropical and subtropical regions of the world. It is normally results from deletion of one or both alpha genes at chromosomes 16p13.3. Carriers of these genes have a variable degree of anemia (low Hb), decreased mean corpuscular hemoglobin (MCH/pg), decreased mean corpuscular volume (MCV/fl) and a normal or slightly decreased level of HbA2. Nevertheless, molecular analysis is essential to support these hematological observations. Thus, we presented a cross-sectional study to screen for the deletional defects in the α-globin gene cluster among aborigines population in Gua Musang, Kelantan. Deletion of alpha genes was identified by single-tube multiplex-Polymerase Chain Reaction (PCR) method and 1% agarose gel electrophoresis. Deletion mutations include single gene deletions -α3.7 and –α4.2, double gene deletions --SEA and --THAI in 52 blood samples from studied respondents. The results revealed two out of 52 respondents had alpha gene deletion, one respondent with (1.9%) single gene deletion of –α3.7 and one respondent with (1.9%) double deletion of --SEA. In conclusion, this study will helps in determining the alpha thalassemia status among aborigines for their future health planning and management.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Malaysia has a multi-ethnic residents of 28.3 million, consisting of 65.1% Malays, 26% Chinese, 7.7% Indians, and 1.2% of other ethnic groups (Shaari, 2000). The "Orang Asli" or the aboriginal populations in Malaysia are descendants of the Austronesian society and they form only 0.6% from the total population (Tan *et al.*, 2010). In Peninsular Malaysia, this minority societies embraces of at least 18 different cultural-linguistic groups, officially classified into three main categories, Negrito, (Kensiu, Kintak, Jahai, Lanoh, Mendriq and Batek), Senoi (Semai, Temiar, Jah Hut, Chewong, Mah Meri and Semoq Beri) and Aboriginal Malay (Temuan, Semelai, Jakun, Orang Kanaq, Orang Kuala and Orang Seletar) (Lim *et al.*, 2009). The aboriginal groups previously lived as agricultural and fishing communities, but now many of them have integrated into urban communities and hold central administrative and academic positions (Tan *et al.*, 2010).

Thalassemia is an inherited blood disorder that results from genetic defects causing deficient synthesis of hemoglobin polypeptide chains. It occurs with substantial frequency in ethnic groups tracking their origins to countries that border the Mediterranean Sea, the Middle East, and Southeast Asia (Rosnah *et al.*, 2012). Like many other countries, thalassemia poses a crucial public health problem in Malaysia.

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4.5% of Malaysian Chinese inherit the Southeast Asian deletion that can cause Hb Bart's hydrops fetalis, a fatal condition in α-thalassaemia (Wee *et al.*, 2005).

Although thalassemia is the most widespread genetic disorder in Malaysia, there is very little data regarding the incidence of thalassemia among the indigenous population. A previous study Tan *et al*, (2010) showed that 33.6 % was confirmed of α-thalassemia in the Kadazandusuns, the largest aboriginal group in Sabah, East Malaysia. Detailed regarding molecular classification of mutations that cause thalassemia in the Malaysian aboriginal populations is restricted, as many studies have focused mainly on the major ethnic groups such Malays, Chinese and Indians (Tan *et al.*, 2010).

Previously, the standard procedure for molecular characterization of α-thalassemia was Southern blot analysis using radioactively labelled probes, a time-consuming, labour-intensive, and expensive procedure (Chong *et al.*, 2000). Thus, its appliance to any large-scale screening program was extremely difficult, even though it was very useful for diagnosis of an individual patients (Shaji *et al.*, 2000). DNA sequence analysis of each deletion breakpoint has now enabled PCR-based testing (Chong *et al.*, 2000). However, PCR-based required multiple tube amplifications for precise characterization of the two major, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, alleles. Although practicable for diagnostic purposes, multi-tube testing is burdensome for large epidemiological screening. Despite that, single-tube multiplex PCR assay is a procedure carried out in a single tube for detecting $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles (Shaji *et al.*, 2000). The aim of this molecular study was to identify the prevalence and types of α-thalassemia among aborigines population in the Gua Musang, Kelantan. Information on the prevalence

and spectrum of α-thalassemia defects will allow for control program as well as treatment strategies of thalassemia in this aborigines population.

1.2 Alpha (α)-globin Gene Cluster

1.2.1 General Structure and Development Expression of α-globin Gene Cluster

The structural gene and the other α-like genes that regulate the synthesis and structures of α-globin are found in a cluster known as the α-globin gene cluster. The α-globin cluster is located at the short arm of chromosome 16, very close approximately 150 kb to the telomere (Hua-bing *et al.*, 2002). It is an important subunit of the human hemoglobin tetramer from the sixth week of development in utero through adult life (Liebhaber *et al.*, 1986). The α-globin cluster includes the duplicated α genes (α2 and α1), an embryonic α-like gene (ζ2), three pseudogenes (ψζ1, ψα2, ψα1), and α gene of undetermined function (θ1) arranged in the order 5'-ζ2-ψζ1- ψα2-ψα1-α2-α1-θ1-3'(Higgs *et al.*, 1989).

Globin chains are differently expressed at different stages of development which give increase to various hemoglobin in embryonic, fetal and adult life. In early embryogenesis, expressions of the zeta (ζ) globin chains result predominantly in hemoglobin (Hb) Gower 1 ($\zeta_2\varepsilon_2$), whereas in the late embryo or early fetus, co-expression of the alpha (α) chains produce Hb Gower 2 ($\alpha_2\varepsilon_2$). Fetal hemoglobin, α2γ2, and adult hemoglobin, α2β2, are accumulated by combining two α-globin chains with two γ- or β-globin chains, respectively (Liebhaber *et al.*, 1986; Higgs *et al.*, 1989) (**Figure 1.1**).

Alpha-globin expression is regulated by 4 distant *cis*-acting regulatory elements (enhancers) associated with DNAase1 hypersensitive sites situated 10kb upstream of the genes (Mettananda *et al.*, 2015). A variety of trials including temporary transfections and transgenic combined with naturally occurring human mutations, suggest MCS-R2 (previously known as HS-40) is the most critical regulatory element capable of enhancing α-globin expression on its own (Higgs and Wood, 2008). Understanding the regulation of its expression is an important step toward effective gene therapy of α-thalassemia.

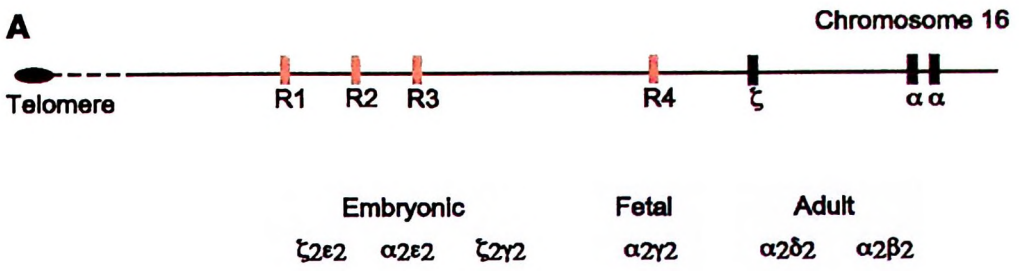

Figure 1.1: Schematic diagram of α-globin gene clusters and the types of hemoglobin produced at each developmental stage. Genes are arranged along the chromosome in the order in which they are expressed during development; (A) in the α-cluster ζ (embryonic) and α (fetal and adult). The 4 upstream regulatory elements of the α-locus are known as MCSR1 to MCSR4. The haemoglobin types expressed during different stages of development are embryonic (Hb Gower-I ($\zeta_2\varepsilon_2$), Hb Gower-II ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$), fetal; Hb F ($\alpha_2\gamma_2$), and adult; Hb A ($\alpha_2\beta_2$) and Hb A_2 ($\alpha_2\delta_2$). Figure was adapted from Mettananda *et al*, (2015) with some modifications.

1.2.2 α-globin Gene Cluster Mutation

Mutation in α-globin gene cluster can cause four clinical conditions; the silent carrier, the alpha thalassemia trait, the intermediate form of hemoglobin H disease, and the hemoglobin Bart hydrops fetalis syndrome that is lethal in utero or soon after birth. The two carrier states can be categorized into alpha^{+}-thalassemia; caused by the deletion or dysfunction of one of the four normal alpha globin genes and alpha-thalassemia; result from deletion or dysfunction of two alpha genes in *cis*. The two clinically forms are Hb H disease; only one functioning alpha gene and Hb Bart hydrops fetalis syndrome; no functioning alpha genes (Galanello and Cao, 2011).

A fetus with α-thalassemia major lacks α-globin gene expression and the Hb is γ-homotetramer, Hb Bart's (γ4). In this case, the embryonic z-globin genes remain to be active in the affected fetus and about 10% to 20% embryonic Hb Portland 1 (ζ2 γ2), and a small amount of Hb Portland 2 (ζ2β2) are produced. Hb Portland 1 is capable to transport oxygen to fetal tissues that usually allows fetuses to survive into the third trimester of gestation. However, as the fetus continues to grow, the oxygen-carrying capacity of these Hb is insufficiency to meet the fetus's need, and thus Hb Bart's hydrop fetalis acquires because of hypoxia and heart failure either in utero or shortly after birth. The diagnosis of α-thalassemia major (all four a-globin genes are mutated or deleted) and Hb H (three out of four α-globin genes are mutated or deleted) can be made based on the presence of Hb Bart's (γ4). The ζ-chain continues to be expressed in α-thalassemia-1 (two out of four α-globin genes are mutated or deleted) trait. Therefore it is positive in α-thalassemia-1 trait in cord blood. α-thalassemia-2 trait (only one out of four a-globin genes is mutated or deleted) is undifferentiated from a normal individual in electrophoresis (Pan *et al.*, 2007).

1.2.2.1 α^+ Thalassemia Deletion

The α-globin genes are implanted within two highly homologous 4 kb duplication units (Embury *et al.*, 1980; Lauer *et al.*, 1980; Harteveld and Higgs, 2010). One very common α-thalassemia deletion is the rightward deletion, a 3.7 kb deletion caused by reciprocal recombination between Z segments producing a chromosome with only one functional α-gene (α-3.7 or rightward deletion) causing α-thalassemia and an α-triplication allele without a thalassemia effect (**Figure 1.2**). Likewise a reciprocal recombination between mis-paired X-boxes results in a 4.2 kb deletion, called leftward deletion (-α4.2) (Embury *et al.*, 1980; Higgs *et al.*, 1984). An increasing number of deletions resulting in the loss of a single α-gene are reported due to non-homologous recombination events and most of it are rare, or highly region specific. More broad overviews of all deletions are reported elsewhere in: Disorders of Hemoglobin Cambridge University Press (2009) (Rugless *et al.*, 2008; Steinberg *et al.*, 2009).

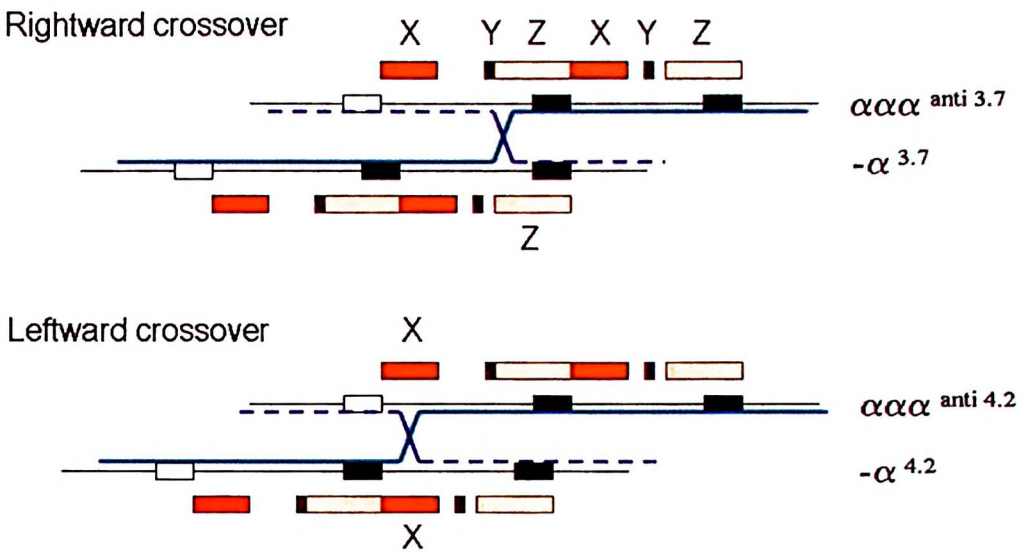

Figure 1.2: Deletions that cause α+-thalassemia. The homologous duplication units X, Y and Z in which the α-genes are embedded are indicated as colored boxes. A cross-over between the mis-paired Z boxes during meiosis gives rise to the -α3.7 and $\alpha\alpha\alpha^{anti\ 3.7}$ chromosomes. Cross-over between misaligned X-boxes gives rise to -α4.2 and $\alpha\alpha\alpha^{anti\ 4.2}$. Figure was adapted from Harteveld and Higgs (2010).

1.2.2.2 α^0 Thalassemia Deletion

The complete or partial deletion of both α-genes in *cis* results in no α-chain synthesis directed by these chromosomes *in vivo* (**Figure 1.3**). Homozygotes for such deletions will have the Hb Bart's Hydrops Fetalis Syndrome. Many deletions were described which remove the ζ- and α-genes and although heterozygotes seem to develop normally, it is unlikely that homozygotes could survive even at the early stages of gestation since neither embryonic (ζ2γ2) nor fetal (α2γ2) hemoglobin could be made. Rare deletions causing α^0-thalassemia remove the regulatory region, which lies 40-50 kb upstream of the α-globin gene cluster leaving the α-genes intact. This region comprised of four multispecies conserved sequences (MCS), called MCS-R1 to R4, correspond to the previously classified erythroid-specific DNAse1 hypersensitive sites referred to as HS-48, HS-40, HS-33 and HS-10. Of these elements, only MCS-R2 (HS-40), 40 kb upstream from the ζ globin mRNA cap-site has been shown to be crucial for α globin expression. Deletions are reviewed in detail in Higgs et al Disorders of Hemoglobin Cambridge University Press 2009 (Storz *et al.*, 2009)

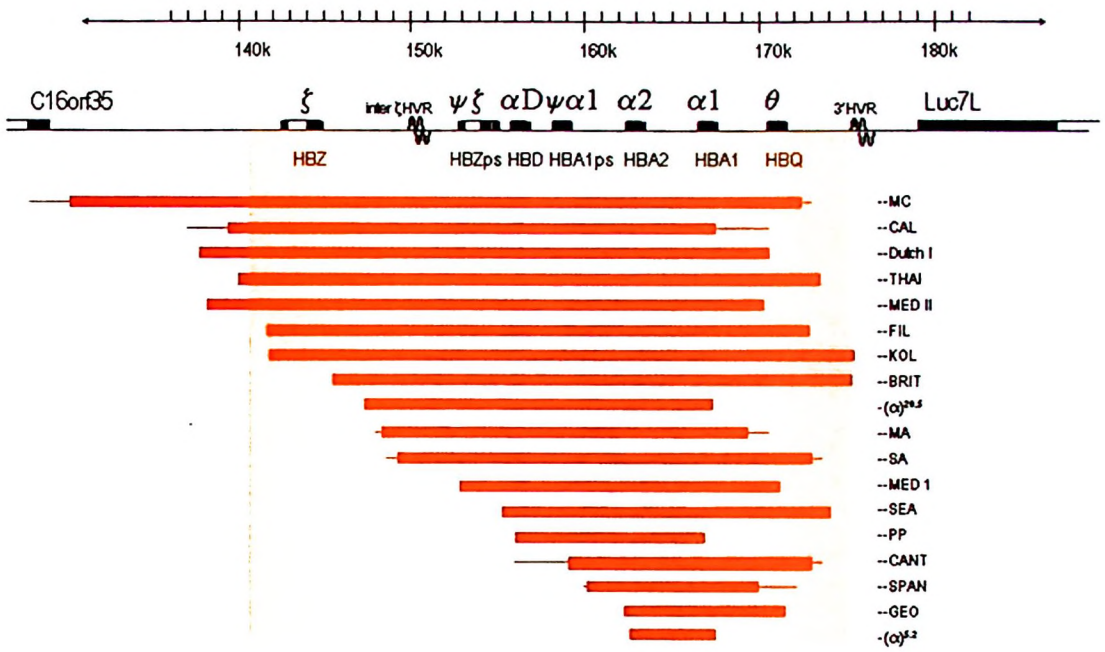

Figure 1.3 Deletions of two α-genes giving rise to α^0-thalassemia. Figure was adapted from Harteveld and Higgs (2010).

1.3 Hematological Testing of α-Thalassemia

Alpha thalassemia is often suspected primarily on the basis of a routine full blood count. Affected individuals usually have a variable degree of anemia (Hb), reduced mean corpuscular hemoglobin (MCH/pg), reduced mean corpuscular volume (MCV/fl) and normal or slightly reduced level of minor Hb A2. These parameters occur when the level of α-globin synthesis falls below ~70% of normal, in the fetal period, excess γ-globin chains will form Hb Bart's which can be detected on routine Hb analysis (Kutlar *et al.*, 1989; Lin *et al.*, 1992; Zorai *et al.*, 2002; Harteveld and Higgs, 2010). In adult life, excess of β-globin chains will form β-4 tetramers of Hb H in the cell and these can be recognized by staining the peripheral blood with 1% brilliant cresyl blue (BCB), or when present in adequate quantity by routine Hb analysis (CHUI, 2005; Pan *et al.*, 2005). Previously α-thalassemia was confirmed by globin chain biosynthesis, when the α/β-globin chain biosynthesis ratio was decreased to less than ~0.8 (Hunt *et al.*, 1980; Giordano *et al.*, 1999; Harteveld and Higgs, 2010).

Primary laboratory testing for α-thalassemia carrier identification should include MCV and MCH determination and quantitative Hb analysis which are usually performed by high-performance liquid chromatography (HPLC). However, identification of α-thalassemia carriers is difficult because they have microcytosis and hypochromia but do not have distinctive changes in Hb A2 or Hb F which characteristics of β and δ-β alpha-thalassemia carriers, respectively. Carriers with -α/-α and - -/αα genotypes have always reduced MCV and MCH, whereas -α/αα carriers may have normal red cell indices or only slightly reduced MCV and MCH. Hb A2 is normal or slightly reduced and Hb F is normal (Galanello and Cao, 2011).

After incubation of erythrocytes with 1% brilliant cresyl blue supravitally stain, some RBC with inclusion bodies; precipitated β4 tetramers, can be identified by microscope in αα-thalassemia carriers. In vitro globin chain synthesis study shows reduced α/β ratio with 0.9–0.6. Occasionally, especially in regions where thalassemia is uncommon, while the hematological parameters are quite similar, α-thalassemia trait may be mistaken with iron-deficiency anemia. Iron status assessment like serum iron and transferrin saturation or red blood cell zinc protoporphyrin determination, are usually enough to make a correct diagnosis. Newborn carriers with α-thalassemia usually have a slight to moderate (1–5%) increase in Hb Bart during hemoglobin electrophoresis or HPLC. Patients with Hb H disease have microcytic hypochromic anemia and reduced Hb A2, but the typical finding shows the presence of variable amounts of Hb H (Galanello and Cao, 2011).

Staining the peripheral blood cells with 1% Brilliant Cresyl Blue is a sensitive technique to visualize inclusion bodies in the red cells. The typical inclusion body cells have a golf-ball like appearance with stippling regularly scattered over a blue stained background (**Figure 1.4**). They appear infrequently in carriers of the --/αα genotype and in carriers of many non-deletional defects about one to two cells in approximately 10 fields under 1000x magnification. Numerous red cells containing inclusions can be seen in the BCB-stained peripheral blood smears of patients with Hb H disease (Harteveld and Higgs, 2010).

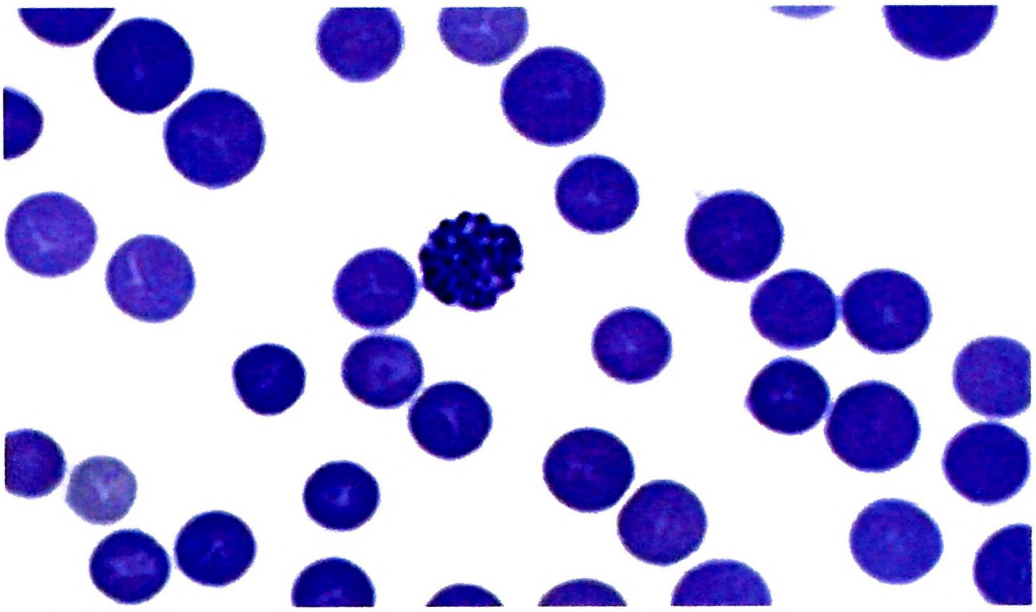

Figure 1.4: An inclusion body positive cell seen in Brilliant Cresyl Blue stained red cells of α^{o}-thalassemia carrier. Inclusion bodies are β4-tetramers precipitating on the red cell membrane, which damages the membrane and causes hemolysis. Hb H is unstable and inclusion body positive cells are harder to find in older blood samples. The number of inclusion body cells seen after staining is much lesser in α^{o}-thalassemia carriers than in patients with Hb H disease. Figure was adapted from Harteveld and Higgs (2010).

In neonates, patient with Hb H disease genotype can be identified by hemoglobin electrophoresis because they have higher levels about 25% of Hb Bart. Hematologic diagnosis of Hb Bart syndrome is characterized by the occurrence of severe macrocytic anemia and Hb Bart; 85–90%, and absence of Hb F and Hb A with hemoglobin analysis by electrophoresis or HPLC. In general, the level of microcytic (low MCV), hypochromic (low MCH) anemia (low Hb) depends on the number of α-genes mutated and correlates well with the reduction in α-chain synthesis for each mutant (Wilkie, 1991; Weatherall and Clegg, 2001; Harteveld and Higgs, 2010).

The combined use of HPLC and Capillary Electrophoresis to separate abnormal hemoglobin fractions is one of particular importance to determine Hb H in individuals with Hb H disease and Hb Bart's in newborns or any Hb variant associated with α-thalassemia phenotype. Hb Bart's is discovered in a large proportion of neonates with α-thalassemia but not detect all cases with mild α-3.7/αα interactions and cannot clearly distinguish the various α- thalassemia genotypes (Wilkie *et al.*, 1991; Zorai *et al.*, 2002). Sometimes decrease in Hb A2 level is also indicative of α-thalassemia trait. Although can distinguishes between α and β-thalassemia trait, it hardly to be relied upon as a guide to the degree or type of α-thalassemia. A reduction in the level of Hb A2 is only distinguishing in patients with Hb H disease (Van Delft *et al.*, 2009).

1.4 Molecular Diagnosis and Characterization of α-Globin Gene Cluster

Molecular analyses involve the mutation detection of α-globin gene cluster mutation by molecular scanning. Likely to diagnose α-thalassemia accurately and describe the particular defects underlying these disorders using a variety of molecular genetic approaches. Eventually, most α-globin rearrangements have been categorized by Southern blotting and DNA sequence analysis (Chong et al., 2000). However, due to high diagnostic demands, these methods are far too laborious to apply in each case, thus rapid screening assays have been developed (Liu et al., 2000).

Gap-PCR has been developed for the seven most common α-thalassemia deletions. This method is applied to discover the two most common α^+ thalassemia deletions - α3.7 and α-4.2 and the 5 α^0-thalassemia deletions -(α)20.5, --SEA, --Med I, --Thai and --Fil (Chong *et al.*, 2000; Liu *et al.*, 2000; Tan *et al.*, 2001). When a point mutation (non-deletional mutation) is suspected, re-sequencing the α-genes has become a routine method. The α genes are relatively small (~1.2 kb) which allows them to be sequenced rather easily compared to many other genes involved in human genetic disease, like for instance Duchenne Muscular Dystrophy (DMD gene; ~2.3 Mb), Cystic Fibrosis (CF-gene; ~250 kb) and Breast Cancer (BRCA1 and BRCA2 genes, ~16 and ~10 kb respectively) (Richards and Grody, 2004; Bellosillo and Tusquets, 2006; Stockley *et al.*, 2006). However, the GC-richness and the high homology between the duplicated α-genes require the use of high reliability, heat stable polymerases, specific reaction conditions using DMSO and betaine and limit the choice of particular primers for PCR. The α-genes can be conveniently sequenced in two overlapping fragments for each of the duplicated α1 and α2 genes (Molchanova *et al.*, 1994; Zorai *et al.*, 2002; Traeger-Synodinos and Harteveld,

2010). For unknown rearrangements, Southern blotting or MLPA analysis may be used. Southern blot is the classical method to discover deletions causing α-thalassemia (Higgs *et al.*, 1985; Tan *et al.*, 1991; Harteveld and Higgs, 2010). More recently Multiplex Ligation-dependent Probe Amplification (MLPA) is used, based on ligation of multiple probe-pairs hybridized across a region of interest, followed by semi-quantitative amplification using universal tag PCR primers and subsequently fragment analysis. This is a valuable alternative for Southern blot analysis and a supplementary method to gap-PCR when examining known and unknown deletions causing α-thalassemia (Schouten *et al.*, 2002; White *et al.*, 2004; Harteveld *et al.*, 2005).

1.5 Multiplex GAP Polymerase Chain Reaction Assay

Gap-PCR technique involve amplification using oligo-primers flanking deletion breakpoints, are used to detect common alpha thalassemia deletion mutations, alpha gene duplication, and other globin gene deletions, such as Hb Lepore and HPFH. Gap PCR is based upon the incapability of PCR primers complementary to DNA sequences that are far apart to direct amplification unless a deletion brings them closer together. PCR primer pairs are created to flank a known deletion, generating a unique amplicon that will be smaller in the mutant sequence compared with the rough type. The presence or absence of PCR product is detected by electrophoresis. Primers specific for seven of the most common alpha thalassemia deletions, as well as the constant spring (CS) point mutation are multiplexed to detect the mutations that most often responsible for Hb H disease. This method is also used to discover the delta or beta globin gene crossover that are responsible for Hb Lepore and the large deletions sensible for hereditary persistence of fetal hemoglobin (Chong et al., 2000).

Gap-PCR provides a quick diagnostic test for α^+-thalassaemia and α^0-thalassaemia deletion mutations but needs careful application for prenatal diagnosis. Most of the common α-thalassemia alleles that result from gene deletions can be analysed by gap-PCR. Primer sequences have now been published for the diagnosis of five α^0-thalassaemia deletions and two α^+-thalassaemia deletions (Dodé *et al.*, 1993; Chong *et al.*, 2000; Old *et al.*, 2005). The α^0-thalassaemia deletions diagnosable by PCR are: the $--^{SEA}$ allele, found in Southeast Asian individuals; the $--^{MED}$ and $-(\alpha)^{20.5}$ alleles found in Mediterranean individuals; the $--^{FIL}$ allele, found in Filipino individuals and finally the $--^{THAI}$ allele, found in Thai individuals. The two α^+-thalassaemia deletion

mutations are 3.7 kb and the 4.2 kb single α-gene deletion mutations, labelled $-\alpha^{3.7}$ and $-\alpha^{4.2}$ (Old *et al.*, 2005)

Amplification of sequences in the α-globin gene cluster is technically more difficult than the β-globin gene cluster which requires more stringent conditions for success due to the higher GC (guanine-cytosine) content of the breakpoint sequences and the considerable sequence homology within the α-globin gene cluster. Experience in many laboratories has shown that some primer pairs to be unreliable, which results occasionally in unpredictable reaction failure and the problem of allele drop out (Old *et al.*, 2005).

Currently, separate tests are needed for the different mutations due to different reagent and thermocycling requirements. Additionally, reproducibility of some PCR-based tests have been problematic, particularly those involving the $-\alpha.^{37}$ allele. These problems stem in part from the differences in GC nucleotide content of the various deletion junctions and also from the considerable sequence homology within the α-globin cluster, especially at the α2 and α1 loci. Thus, single-tube multiplex-PCR assay was developed as it is capable of detecting any combination of these six common single and double gene deletions (Chong *et al.*, 2000).

Besides, it is a simple, rapid, and robust method applicable to large population screening for epidemiological purposes. Multiplex-PCR needs to be used with caution when applied to diagnosis of individuals with compound heterozygous states for large deletions such as $--^{SEA}/-\alpha^{3.7}$ or $--^{SEA}/-\alpha^{4.2}$, which will be identified as

homozygous for the small deletion. For that case, is easily recognized on clinical and haematological features (Shaji *et al.*, 2000).

1.6 Significant of the Study

The study on hematological and molecular characterization of the α-globin gene cluster is crucial to provide knowledge for prevention and control of related disorders. Although thalassemia is the most common genetic disorder in Malaysia, there is very limited information regarding the occurrence of thalassemia in the aboriginal population. Detailed molecular characterization of α-globin gene mutations are well documented in almost main ethnic group globally, but there are still narrow studies on aborigine's population in Malaysia as studies have focused mainly on the major ethnic groups such Malays, Chinese and Indians. Moreover, carriers of α-thalassemia are asymptomatic thus the affected individuals will go through life unaware of their carrier status. Besides, screening for thalassemia is not carried out on a routine basis on the Malaysian indigenous population (Tan *et al.*, 2010). Therefore, this molecular characterization is absolutely imperative for obtaining correct diagnosis for aborigine's population as this population often marry among themselves which will increase thalassemia incidence.

To fill this gap knowledge, this study is set–up to focus on the screening of deletional defect of the α-globin gene cluster among aboriginal population in Pos Kuala Betis, Gua Musang, Kelantan by using Multiplex GAP-PCR. Knowing the prevalence of α-thalassemia and the occurrence of responsible mutations in this minority population is an imperative step in the prevention and control program as well as treatment strategies (Panyasai *et al.*, 2002; Abolghasemi *et al.*, 2007).

1.7 Objectives of the Study

1.7.1 General Objectives

The general objective of this study is to perform haematological and molecular characterization of the α-globin gene cluster deletions among aboriginal population in Kelantan.

1.7.2 Specific Objectives

The specific objectives of this study are as below:

1. To screen for α-gene deletions within this population by using Multiplex GAP-PCR.
2. To update data regarding alpha thalassemia among aborigines in Kelantan.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The materials used throughout this study were listed in **Table 2.1**. All chemicals were laboratory and molecular biology grade unless otherwise stated.

2.1.1 Peripheral Blood Samples

All peripheral blood samples of respondents were collected among aborigines population from Gua Musang, Kelantan.

2.1.2 Chemicals

All chemical used in this study were listed in **Table 2.1**.

Table 2.1: List of chemical

Chemicals	Supplier
Wright stain, Modified	Sigma-Aldrich, UK
Phosphate buffer solution	Sigma-Aldrich, UK
DPX new	Merck Millipore, Germany
Ethanol	HmbG, Malaysia
Nuclease free water	Norgen, Canada
10x Coral Load PCR buffer	Qiagen, Germany
2mM dNTP blend	Fermentas, USA
25mM MgCl2	Qiagen, Germany
Q solution	Qiagen, Germany
HotStar Taq Plus	Qiagen, Germany
TAE buffer solution, 10X, Molecular Biology Grade-Calbiochem	Merck Millipore, Germany
SeaKem LE Agarose	Cambrex, USA
Quick-Load 2-Log DNA Ladder (0.1-10.0 kb)	New England BioLabs, USA
1x Diamond Nucleic Acid Dye	Promega, USA

2.1.3 Reagents and Kits

All reagents and kit being used throughout this study were listed in **Table 2.2**:

Table 2.2: Genomic Purification Kit (QIAGEN, Germany)

Item	Specification (for each preparation)
QIAamp Midi Spin Columns	1 column
Collection Tubes (15mL)	1 tube
Buffer AL	2.4 mL
Buffer AW1	2 mL
Buffer AW2	2 mL
Buffer AE	70 µL
QIAGEN Protease	200 µL

2.1.4 Equipment

All laboratory instrument being used in this study were listed in **Table 2.3:**

Table 2.3: List of Instrument

Item	Supplier
Sysmex XE-5000 Automated Haematology System	Sysmex, Canada
VARIANT II Hemoglobin Testing System	Bio-Rad, USA
ZEISS Axiostar Plus Light Microscope	ZEISS, USA
Eppendorf Pipetting kits	Sigma-Aldrich, UK
Grant Sub 6 Water Bath	Akribis Scientific, UK
Eppendorf Centrifuge 5804 R	Eppendorf, Germany
NanoDrop ND-1000 Spectrophotometer	Thermo Fisher Scientific, USA
Veriti® 96-Well Thermal Cycler	Thermo Fisher Scientific, USA
Mupid-exU Electrophoresis System	Takara, Japan
Boeco Mini-Rocker Shaker Mr-1	Boeco, Germany
AlphaImager HP System	Alpha Innotech, Europe