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Genomic Study in β-Thalassemia

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1. Introduction

β-Thalassemia is characterized by the reduced or absent production of β-globin chains in the hemoglobin molecule leading to an excess of α-globin chains. The clinical findings of thalassemia caused by the imbalanced α/non α-globin chains synthesis include many pathological changes of various organs and a lower-than-average life expectancy. Most β-thalassemia major patients who are homozygous or compound heterozygous for $β^0$-thalassemia mutations have a severe phenotype of β-thalassemia disease and suffer from chronic anemia and requiring regular blood transfusions. In contrast, $β^0$-thalassemia/HbE disease is a very heterogeneous disorder and patients who carry identical β-thalassemia genotypes may present with a remarkable variability in disease severity. Their hemoglobin levels range from 3 to 13 g/dL with an average level of 7.7 g/dL (Fucharoen et al., 1987). Patients with mild to moderate clinical symptoms usually have normal growth development and survive without regular blood transfusions. Whereas severely affected patients have marked anemia, growth retardation, severe bone changes, hepatosplenomegaly and heavy iron overload similar to that of β-thalassemia major patients.

However, the reasons for this extraordinary clinical heterogeneity are not fully understood. It has been proposed that there are three levels of genetic control of clinical phenotypes in β-thalassemia: 1) primary modifiers due to heterogeneity of the β-thalassemia alleles, 2) secondary modifiers related to the reduced free α-globin pool or increased γ-globin production in adulthood and 3) tertiary modifiers based on candidate genes that may be involved in several pathological alterations, for example, genes that are related to iron absorption, jaundice, cardiac failure and bone defects. However, the fact that many β-thalassemia/HbE patients who have the same β- and α-globin genotypes still have variable clinical symptoms, suggests that there are still some additional factors which influence the severity of the disease. Recently, genome-wide association studies (GWAS) were performed in order to search for other genetic modifying factors. In the first GWAS, approximately 110,000 gene-based single nucleotide polymorphism (SNPs) were initially screened in pooled DNA and then validated in individual samples (Sherva et al., 2010). The second GWAS was conducted using high density SNP arrays to evaluate approximately 600,000 SNPs in individual samples (Nuinoon et al., 2010). The two SNPs association analyses identified highly significant SNPs located in 3 genes/regions (*P-value* of $1.00×10^{-7}$ to $1.00×10^{-}$

[13]): the β-globin gene cluster and olfactory receptor genes upstream of β-globin gene cluster on chromosome 11p15.5, the *HBS1L-MYB* intergenic region on chromosome 6q23 and *BCL11A* on chromosome 2p15 (Nuinoon et al., 2010; Sherva et al., 2010). The three regions have been shown to be associated with fetal hemoglobin (HbF) level by genetic linkage and association studies. Moreover, 101 SNPs on 69 genes also showed association with disease severity with *P-values* of 1.00×10^{-5} to 1.00×10^{-6}. These genes have been shown to have less effect on disease severity as compared to the three mentioned regions. However, their roles in modification of disease severity need further investigation.

2. Hemoglobinopathies

Hemoglobin (Hb) is the protein of the red blood cells that transports oxygen from the lungs to tissues, and carbon dioxide from the tissues back to the lungs. It is composed of four globin chains, two α-like and two β-like globin chains, and each globin contains iron-coordinated heme moieties. The hemoglobinopathies fall into two distinct groups, the structural hemoglobin variants and the thalassemias. Hemoglobin variants result from structural alterations in the globin chain, which are mostly due to single amino acid substitutions, thereby altering the function of the hemoglobin tetramer. The thalassemias are characterized by the absence or reduced synthesis of one of the globin chains. Some mutations also lead to both phenotypes. Hemoglobinopathies are the most common monogenic disorders and it has been estimated that approximately 5.2% of the world population are carriers. Around 1.1% of couples worldwide are at risk for having children with a hemoglobin disorder and 2.7 per 1000 conceptions are affected (Modell & Darlison, 2008).

Thalassemias can be divided according to the globin gene(s) defect into α-thalassemia, β-thalassemia, δβ-thalassemia, γ-thalassemia, δ-thalassemia and εγδβ-thalassemia. However, the major groups of this inherited disorder are α-thalassemia and β-thalassemia.

2.1 α-Thalassemia

The α-like globin locus is located on the short arm of chromosome 16 in band p13.3. It includes an embryonic gene (ζ2), two fetal/adult genes (α2 and α1), three pseudogenes (ψζ1, ψα2, ψα1) and a gene (θ) of unidentified function. α-Thalassemia is classified by a reduction or absence in synthesis of α-globin chains. Molecular defects of α-thalassemia are mainly associated with deletions or mutations of one or more of the α-globin genes, of which now more than 35 known deletional mutations have been discovered (Hardison et al., 1998). α-Thalassemia 1 (--/αα) occurs from a deletion of the duplicated α-globin genes, while deletion or mutation of one copy of the duplicated α-globin genes, which produces a reduced amount of α-globin leads to α-thalassemia 2 (-α/αα). The clinical pathology of the patients is heterogeneous depending mainly on the number of defective genes.

2.2 β-Thalassemia

The β-globin locus is located on the short arm of chromosome 11 in band p15.5. It includes an embryonic gene (ε), two fetal genes (Gγ and Aγ), two adult genes (δ and β) and a pseudogene (ψβ). β-Thalassemia occurs as a consequence of a quantitative reduction of β-globin chain production. More than 200 point mutations and, rarely deletions, have been

reported (Hardison et al., 1998). These genetic defects lead to a variable reduction in β-globin output ranging from a minimal deficit, mild β^+-thalassemia alleles, to the complete absence, β^0-thalassemia.

2.3 Hemoglobin E

HbE, the most common Hb variant among Southeast Asian populations, results from a G to A substitution at codon 26 (GAG to AAG) in exon 1 of the β-globin gene, which substitutes an amino acid residue from Glu to Lys. This abnormal gene produces a structurally abnormal hemoglobin which consists of $\alpha_2\beta^E_2$-globin chains. Moreover, the abnormal gene also activates a cryptic 5' splice site that causes abnormal pre-mRNA splicing. The aberrant splicing leads to a 16 nucleotide deletion of the 3' end in exon 1 and creates a new inframe stop codon (Orkin et al., 1982). This new cryptic splice site competes with the normal doner splice site, consequently the level of correctly spliced β^E-globin mRNA is decreased. As a result, HbE is synthesized at a reduced rate and thus the β^E-globin gene behaves like a mild form of β^+-thalassemia.

3. β-Thalassemia/HbE disease

β-Thalassemia/HbE disease is the most common form of β-thalassemia in many Asian countries. In Thailand, approximately 3,000 children are born with this condition each year, and there are some 100,000 patients in the population (Fucharoen & Winichagoon, 2000). It accounts for over 50% of cases of severe β-thalassemia in Indonesia and Bangladesh and is also very common in Vietnam, Cambodia, Laos, and Malaysia. It also occurs frequently in the eastern side of Indian subcontinent, including Sri Lanka and Maldives (Fucharoen & Winichagoon, 1997).

The major mechanism underlying the pathophysiology of β-thalassemia/HbE is due to the absence or inadequate β-globin chain production, and can be related to the deleterious effects of imbalanced globin chain synthesis. The excess α-globin chains in β-thalassemia, which are highly unstable, precipitate and lead to oxidative damage in developing (causing dyserythropoiesis) and mature red cells (causing shortened red blood cell survival). Hemolysis and ineffective erythropoiesis cause anemia in β-thalassemia (Rund & Rachmilewitz, 2005). Ineffective erythropoiesis leads to the expansion of marrow cavities and the massive medullar cell proliferation, resulting in skeletal deformities. The erythroid hyperplasia and ineffective erythropoiesis are responsible for the increased iron absorption, which together with regular blood transfusions, results in chronic iron overload and death in these patients.

3.1 Clinical Heterogeneity of β-Thalassemia/HbE disease

β-Thalassemia/HbE is generally classified as thalassemia intermedia. The patients have inherited a β-thalassemia allele and hemoglobin E, which acts as a mild β^+-thalassemia. However, despite seemingly identical genotypes, β-thalassemia/HbE patients have a remarkably wide spectrum of clinical phenotypes. Notable are variations in anemia, growth development, hepatosplenomegaly, and transfusion requirements. The clinical features range from asymptomatic or mild clinical symptoms with normal growth development and survival without transfusions, to transfusion-dependent thalassemia major who have

marked anemia, growth retardation, severe bone changes, hepatosplenomegaly and heavy iron overload. A study of 802 β-thalassemia/HbE patients showed that hemoglobin levels in the steady state range from 3 to 13 g/dL with an average level of 7.7 g/dL (Fucharoen et al., 1987). Interestingly, the discordant severity of anemia ranged from 0 to 8.6 g/dL, while the distribution of differences of hemoglobin levels in 216 sib pairs from 98 families showed a remarkable skewness toward the lower values with a mode at 0-0.5 g/dL. This suggests that multiple genetic factors must be involved in determining the clinical variability (Fucharoen et al., 1984). The reasons for this extraordinary clinical heterogeneity are not fully understood.

3.2 β-Thalassemia/HbE disease modifiers

The pathophysiologic change in homozygous β-thalassemia patients is predominantly determined by the amount of excess α-globin chains. *In vitro* globin chain synthesis techniques available in the 1960s revealed that the central mechanism underlying the pathophysiology of β-thalassemia was related to the excess α-globin chains and the degree of chain imbalance (Weatherall et al., 1965). Similar to homozygous β-thalassemia, the effect of imbalanced globin chain synthesis to disease severity of β-thalassemia/HbE patients has been demonstrated. The *in vitro* globin chain synthesis showed a significant difference between α/non α-globin chains ratio of 2.08±0.42 and 2.55±0.34 in mild and severe groups (*P-value*<0.005), respectively (Fucharoen et al., 1987). Interestingly, there were overlaps in the range of α/non α-globin chains ratio of 1.34-2.90 in the mild group and 2.23-3.30 in the severe group. This suggested that other factors also contributed to the clinical symptoms of these patients.

The well-characterized or possible genetic modifiers which influence the severity of β-thalassemia have been classified. As the amount of excess α-globin chains determined the pathophysiologic change in β-thalassaemia, the factors affecting the excess α-globin pools, which may lead to variable clinical phenotype of the patients are classified as primary and secondary modifying factors. While the tertiary modifying factors are genetic factors that do not affect globin imbalance directly but modify complications of the disease in several different ways (Thein, 2008).

Recently in order to explore disease modifying factors we recruited about 1,300 Thai β0-thalassemia/HbE patients and classified them into three groups, mild, moderate and severe using a scoring system based on criteria of 6 representative parameters; hemoglobin level, age onset, age at the first blood transfusion, requirement of blood transfusion, spleen size or splenectomy, and growth development (Sripichai et al., 2008a). The analysis of known genetic modifiers and genome-wide SNPs association studies (GWAS) were performed using MassARRAY platform (Sequenom, Inc., San Diego, CA, USA) and Illumina Human 610-Quad BeadChips array (Illumina, San Diego, CA, USA) (Nuinoon et al., 2010; Sherva et al., 2010).

3.2.1 Primary modifying factors

The primary modifying factor is described by expression of the β-globin alleles due to the nature of the underlying β-thalassemia mutation itself. β-Thalassemia mutations range from null mutations (β0-thalassaemia) that cause a complete absence of β-globin production, to those that cause a minimal deficit (β+-thalassemia). Generally, interaction of two β+-

thalassemia alleles, in which there is some β-globin production such as the mutations at -28 ATA box (A→G), codon 19 (A→G), results in a milder disease. A recent study in a Thai β-thalassemia/HbE cohort showed that all β+-thalassemia/HbE patients had mild clinical symptoms (Table 1). On the contrary, co-inheritance of a β0-thalassaemia mutation, which causes a complete absence of β-globin production from the allele, and HbE results in a wide spectrum of phenotypes.

Since mutation of the β^E-globin gene leads to the alternative splicing of β^E-globin pre-mRNA, the amount of alternative spliced β^E-globin mRNA may play a role in the variability of disease severity. Analysis of *in vitro* globin chain synthesis showed α/β^E-globin chains ratio of 2.69±0.58 and 3.18±0.36 in mild and severe groups respectively (Fucharoen et al., 1987). A study in the 1990s by the RT-PCR technique showed that aberrantly spliced β^E-globin mRNA in patients with severe clinical phenotype was higher than those of mild cases and the level of aberrantly spliced β^E-globin mRNA was correlated with the degree of anemia in the patients (Winichagoon et al., 1995). A recent study using allele specific RT-qPCR confirmed that there were differences in the alternative splicing of the β^E-globin mRNA among the patients and the correctly/aberrantly spliced β^E-globin mRNA ratio in the mild group was higher than that of the severe group (Tubsuwan et al., 2011). Excluding factors correlated to high HbF production, *Xmn*I -158 $^G\gamma$-globin, *HBS1L–MYB* intergenic region and *BCL11A*, the alternative splicing of the β^E-globin mRNA contributed to at least 7.8% of the mild group.

3.2.2 Secondary modifying factors

The severity of anemia in β-thalassemia reflects the degree of α- to non-α-globin chain imbalance and the excess of unmatched α-globin chains with all their deleterious effects on the erythroid precursor cells. Therefore secondary modifying factors are described as factors that affect the degree of globin chain imbalance and the size of the free α-globin chain pool, either via the co-inheritance of α-globin gene mutations or through genetic determinants which increase the level of HbF production.

β-Thalassemia patients who co-inherit α-thalassemia will have less excess α-globin chains and tend to have less severe symptoms (Sripichai et al., 2008b; Wainscoat et al., 1983). The different types of α-thalassemia mutations that predominate in different racial groups display a wide range of severity. The degree of amelioration depends on the number of functional α-globin genes. None of the patients carrying α-thalassemia were in the severe group. After exclusion of β+-thalassemia mutations, all patients who co-inherited with α-thalassemia 1 heterozygote or α-thalassemia 2 homozygote, who have only 2 functional α-globin genes had mild clinical symptoms. About 90% of the patients who have only one defecteive α-globin gene were in the mild group and 10% were in the moderate group (Table 2). Co-inheritance of α-thalassemia appears to be a major genetic factor underlying the mild clinical phenotype of β-thalassemia/HbE. Moreover, a considerable number of patients, especially those who have co-inheritance of α-thalassemia 1, may have not yet been diagnosed or not presented to hospitals. The prevalence of 0.5% α-thalassemia 1 and 6.9% α-thalassemia 2 in the patients was lower as compared to 4% α-thalassemia 1 and 16% α-thalassemia 2 in Thai population. We suggest that the presence of α-thalassemia genes should always be considered in apparently mild cases of β0-thalassemia/HbE in Southeast Asia and probably in other countries where α-thalassemia is also prevalent.

	Disease severity				
	Mild			Moderate	severe
β-globin genotype	β^+/β^E	β^0/β^E	β^0/β^E	β^0/β^E	β^0/β^E
α-globin genotype	αα/αα	α-thal	αα/αα	αα/αα	αα/αα
No. of patient[*]	36 (2.8)	104 (8.1)	278 (21.7)	373 (29.2)	489 (38.2)
Hemoglobin (g/dL)[**]	7.56±1.05	8.22±1.11	7.53±1.25	5.57±1.51	4.55±1.141
Hemoglobin F (g/dL)[**]	1.47±0.71	1.94±0.91	3.28±1.20	2.02±0.92	1.50±0.81
Age at first presentation[*]					
<3 yr	5 (13.9)	18 (17.3)	62 (22.3)	133 (35.7)	377 (77.1)
3-10 yr	10 (27.8)	52 (50.0)	175 (62.9)	220 (59.0)	112 (22.9)
>10 yr	21 (58.3)	34 (32.7)	41 (14.8)	20 (5.3)	0
Age at first transfusion[*]					
< 5 yr	0	2 (1.92)	11 (3.96)	121 (32.44)	403 (82.41)
5-10 yr	1 (2.78)	12 (11.54)	50 (17.99)	174 (46.65)	83 (16.97)
> 10 yr	35 (97.22)	90 (86.54)	217 (78.06)	78 (20.91)	3 (0.61)
Requirement for transfusions[*]					
regular	0	2 (1.92)	4 (1.44)	162 (43.43)	421 (86.09)
occasional	0	5 (4.81)	14 (5.04)	129 (34.58)	66 (13.50)
none	36 (100.00)	97 (93.27)	260 (93.53)	82 (21.98)	2 (0.41)
Spleen size[*]					
size <3 cm	32 (88.89)	71 (68.27)	115 (41.37)	76 (20.38)	12 (2.45)
3-10 cm	4 (11.11)	32 (30.77)	143 (51.44)	130 (34.85)	153 (31.29)
>10 cm or splenectomy	0	1 (0.96)	20 (7.19)	167 (44.77)	324 (66.26)
Height development[*]					
<3rd percentide	3 (8.33)	17 (16.35)	27 (9.71)	78 (20.91)	220 (44.99)
Weight development[*]					
<3rd percentide	2 (5.56)	5 (4.81)	19 (6.83)	73 (19.57)	156 (31.90)

[*] Number of cases (%) [**] mean±SD

Table 1. Clinical and hematologic parameters among the different severity groups of β-thalassemia/HbE disease

α-Thalassemias	α-Globin genotype	Mild	Moderate	Severe	Total
		Number (%)	Number (%)	Number (%)	Number (%)
Normal	αα/αα	321	378	501	1200 (90.2)
Triplicated α-globin	ααα$^{anti3.7}$/αα	0 (0.0)	0 (0.0)	6 (0.5)	6 (0.5)
α-thalassemias all genotypes		113 (91.1)	11 (8.9)	0 (0.0)	124 (9.3)
<u>Two α-globin genes defect</u>					
α-thalassemia 1 heterozygote	--^{SEA}/αα	7 (100.0)	0 (0.0)	0 (0.0)	7 (0.5)
α-thalassemia 2 homozygote	-α$^{3.7}$/-α$^{3.7}$	2 (100.0)	0 (0.0)	0 (0.0)	2 (0.2)
<u>One α-globin gene defect</u>					
α-thalassemia 2 (3.7 kb deletion) heterozygote	-α$^{3.7}$/αα	73 (90.1)	8 (9.9)	0 (0.0)	81 (6.1)
α-thalassemia 2 (4.2 kb deletion) heterozygote	-α$^{4.2}$/αα	6 (75.0)	2 (25.0)	0 (0.0)	8 (0.6)
HbCS heterozygote	α^{CS}α/αα	23 (95.8)	1 (4.2)	0 (0.0)	24 (1.8)
HbPS heterozygote	α^{PS}α/αα	2 (100.0)	0 (0.0)	0 (0.0)	2 (0.2)
Total		434 (32.6)	389 (29.3)	507 (38.1)	1330 (100.0)

Table 2. The effect of α-globin genotype on β-thalassemia/HbE disease phenotypes

Increased α-globin chains are also synthesized from the α-globin triplicated allele (Higgs et al., 1980). The excess α-globin chains lead to a greater globin chain imbalance in β-thalassemia, and hence, the clinical severity of patients is expected to be worsened. The effect of increased α-globin chains by two extra α-globin genes in homozygous triplicated α-globin genes (ααα/ααα) or heterozygous quadruplicated α-globin genes (αααα/αα) was clearly demonstrated in β-thalassemia heterozygotes, which resulted in thalassemia intermedia (Galanello et al., 1983; Thompson et al., 1989). However, the effect of one extra α-globin gene on heterozygous triplicated α-globin genes (ααα/αα) in heterozygous β-thalassemia is more variable and depends on the severity of the β-thalassemia allele (Camaschella et al., 1997; Traeger-Synodinos et al., 1996). Co-inheritance of triplicated α-globin genes (ααα/αα) resulting in severe anemia in β-thalassemia patients has been observed (Camaschella et al., 1995; Galanello et al., 1983; Sripichai et al., 2008a). In this β-thalassemia/HbE cohort, all patients carrying triplicated α-genes (0.5 % of patients) showed a severe clinical phenotype (Table 2). However, the triplicated and quadruplicated α-globin genes occur at a low frequency in most populations. The frequency of triplicated α-globin genes in Cambodian and Chinese cohorts was about 0.5% of patients (Carnley et al., 2006;

Ma et al., 2001). A higher frequency (1–3%) was observed in other countries such as Argentina and Mexico (Bragos et al., 2003; Nava et al., 2006). The quadruplicated α-globin genes were found at an even lower frequency than the triplicated α-globin genes. In Sri Lanka the triplicated and quadruplicated α-globin genes frequencies in the patients were 2.0% and 0.2 %, respectively (Fisher et al., 2003).

The HbF level, which is high at birth and decreases thereafter, is an extremely complex process and still poorly understood. However, the high production of HbF during the neonatal period protects the newborn from the clinical manifestations of β-thalassemia. The role of increased HbF as an ameliorating factor was shown in the group of homozygous β-thalassemia patients who are unable to produce any HbA but showed a mild disease with a reasonable level of HbF (Thein et al., 1988). Studies in β-thalassemia patients have shown that increased Hb F production can reduce the ratio of α- to non-α-globins and ameliorate the severity of the anemia (Rees et al., 1999). The absolute HbF level in mild patients who do not carry the two known modifying factors ($β^+$-thalassemia or α-thalassemia) was 3.28±1.20 g/dL. This level is significantly higher than that of severe patients, 1.50±0.81 g/dL or mild patients carrying $β^+$-thalassemia or α-thalassemia, 1.47±0.71 and 1.94±0.91 g/dL, respectively (Table 1).

The broad distribution of HbF levels in normal adults, in which HbF levels decrease steadily with age, and in which females have higher levels than males, suggests that more than one genetic factor may control HbF production. A twin study composed of 264 monozygotic and 511 dizygotic twins showed that approximately 90% of the variations in the level of HbF and F-cells (HbF containing erythrocytes) production in adult life is genetically controlled by some factor located near the γ-globin gene, while other factors are present on different chromosomes (Garner et al., 2000).

It is known that the genetic variant (C→T) at position -158 upstream of ^{G}γ-globin gene, *Xmn*I ^{G}γ-globin polymorphism, is associated with high HbF production (Labie et al., 1985a; Thein et al., 1987). In addition, more than 50% of the genetic variances in levels of F-cells are caused by factors that are not linked to the β-globin gene cluster (Garner et al., 2000). Intensive linkage studies have mapped *trans*-acting quantitative trait loci (QTLs) controlling F-cell levels to three regions of the genome: chromosome 6q23, Xp22 and 8q11 (Craig et al., 1996; Dover et al., 1992; Garner et al., 2002). The genetic factors regulating HbF production are discussed in more detail below.

3.2.3 Tertiary modifying factors

Tertiary modifying factors, while not being involved in hemoglobin synthesis, cause variation in the progression of the disease. For example, the glucuronosyltransferase 1 (*UGT1A*) gene was found to be associated with the levels of bilirubin in response to hemolysis, ineffective erythropoiesis and the incidence of gallstones (Premawardhena et al., 2001). Similarly, the hemochromatosis (Venter et al.) gene has been identified as influencing the degree of iron loading (Longo et al., 1999; Rees et al., 1997) and the apolipoprotein E (*APOE*) ε4 allele is associated with organ damage and cardiac failure (Economou-Petersen et al., 1998). Variability of at least three different loci; vitamin D receptor (*VDR*), type 1 collagen (*COLIA1*) and transforming growth factor beta 1 (*TGFB1*), has the potential to modify the severity of the bone disease, which is particularly common in patients with severe β-thalassemia (Perrotta et al., 2000).

4. General genomic study

Completion of the human genome project (Frazer et al., 2004; Lander et al., 2001; Venter et al., 2001), dramatically accelerated biomedical research. In parallel, characterization of the inherited variation in human populations, focusing mainly on DNA variants that are common in the general population and that confer increases in diseases risk, has led to exploration of disease susceptibility.

4.1 Genomic variation
Genetic and environmental factors are the two keys that cause human phenotypic variation. Although more than 99% of human DNA sequences are the same across the population there is substantial variation in the sequence at many points throughout the genome. DNA sequence variations are described as mutations and polymorphisms. A mutation is defined as any change in a DNA sequence to a rare and abnormal variant in comparison to the normal allele that is prevalent in the population. In contrast, a polymorphism is a DNA sequence variation that is common in the population. The arbitrary cut-off point between a mutation and a polymorphism is 1% (Brookes, 1999). Genetic variations are responsible for individual phenotypic characteristics and impact on how humans respond to diseases, bacterias, viruses, toxins, chemicals, drugs and other therapies.

Since the early 1980s, humans were known to carry a heterozygous site roughly every 1,300 bases. Genetic maps containing a few thousand markers, which are adequate for rudimentary linkage mapping of Mendelian diseases, were constructed in the late 1980s and early 1990s (Lander, 2011; Lander et al., 2001). A map of 1.42 million single nucleotide polymorphisms (SNPs) distributed throughout the human genome was reported in a companion to the human genome project (Sachidanandam et al., 2001). Today, the vast majority of human variants with frequency >5% have been discovered and 95% of heterozygous SNPs in an individual are represented in current databases. The combination of marker alleles on a single chromosome is called a haplotype (*Haplo*id Geno*type*). Haplotype includes markers that tightly linked with each other and these markers often display statistical dependence, called linkage disequilibrium (LD). The International Haplotype Map Project defined the SNPs patterns across the entire genome by genotyping 3 million SNPs (Frazer et al., 2005).

The presence of a specific allele variation can be implicated as a causative factor in human genetic disorders and a genetic modifying factor in disease phenotypes. Therefore, screening for such an allele in an individual might enable detection of a genetic predisposition to disease or particular phenotype. The importance of finding genetic contribution to disease, therefore, has led to collection of DNA samples from a large number of patients with particular disorders. Two major approaches have been used to map genetic variants that influence disease or phenotype: linkage analysis and association studies.

4.2 Linkage studies
Linkage analysis (family-based studies) is based on the principle that a disease locus in a family will segregate as part of an undisturbed chromosomal region. Genetic markers that tag a chromosomal region that includes the disease gene will co-segregate with the disease. In practice, highly variable markers, such as microsatellites that can be distinguished between most mating couples, have been used to tag regions of the chromosomes (Hauser et

al., 2004). Families in which sibling pairs are affected with the disorder are typed with genetic markers to find a polymorphism that is co-inherited with the disease. If the polymorphic allele is found in a large number of affected sibling pairs, the polymorphism is probably linked to a gene that confers susceptibility to that disease. The genetic markers and the disease allele must generally be inherited together within the one or two generations spanned by the family. To find polymorphisms that are linked to a specific disease requires the typing of 200–300 families with multiple affected relatives using a set of a few hundred or a few thousand markers, spaced millions of bases apart along the human genome. However, the linkages reported by one group have often not been replicated by others. Failure to replicate the linkages may result from a lack of statistical power or false positive results in the original study. Furthermore, there might also be different sets of susceptibility genes operating in different populations. Linkage analysis has been less successful for polygenic diseases and quantitative traits, perhaps in part because of a limited power to detect the effect of common alleles with modest effects on disease.

4.3 Association studies

Association studies rely on the retention of adjacent polymorphisms over several generations across the population. The prevalences of a particular genetic marker or a set of markers, in affected and unaffected individuals, are compared instead of analysis of large family pedigrees (population-based studies) (Cardon & Bell, 2001; Hirschhorn & Daly, 2005). The "common disease–common variant" hypothesis posited that common genetic variants could have a role in the etiology of common diseases (Reich & Lander, 2001). The vast majority of genetic variance in the population is due to common variants. The susceptibility alleles for a trait will include many common variants unless the alleles have had a large deleterious effect on reproductive fitness over long periods. For common diseases or traits, many susceptibility alleles may have been only mildly deleterious, neutral or even advantageous. By testing all common variants, one could pinpoint key genes and shed light on underlying mechanisms.

Genome-wide association studies (GWAS) are association studies that survey most of the genome for causal genetic variants. In GWAS, hundreds of thousand SNPs tested for association with a disease in hundreds or thousands of persons have revolutionized the search for genetic influences on complex traits (Manolio, 2010). Such conditions, in contrast with single-gene disorders, are caused by many genetic and environmental factors working together, each of which has a relatively small effect and few if any being absolutely required for the disease to occur. Rapid advances in technology and quality control now permit affordable, reliable genotyping of up to 1 million SNPs in a single scan of a person's DNA. In 2010, nearly 600 genome-wide association studies covering 150 distinct diseases and traits were published, with nearly 800 SNP–trait associations being reported as significant ($P\text{-value} < 5 \times 10^{-8}$).

5. Genomic study in thalassemias

Even after exclusion of known genetic modifying factors, the phenotypic severity in the majority of β-thalassemia/HbE patients still can not be predicted. As mention above, family studies indicated that multiple genetic factors are involved in determining the clinical variability and additional genetic modifying factors remain to be discovered. In order to

identify these variants, two GWAS were performed in Thai β-thalassemia/HbE patient cohorts, from which β- or α-globin mutations that are known to affect disease severity were excluded.

The first GWAS was carried out using a two-stage study design, which allowed cost effective identification of a targeted set of SNPs for individual genotyping. Initially pooled DNA of 197 mild and 198 severe cases was examined (Sherva et al., 2010). Then the SNPs with allele frequency differences between the pooled DNA (*P-value* < 0.02) were further genotyped in individual samples of 198 mild and 305 severe cases. The assay panel corresponded to 119,811 gene-based SNPs with a median spacing of 10.4 kilobases, covering approximately 99% of all known and predicted human genes. The pooled genotyping was conducted using the MassARRAY platform (Sequenom, Inc., San Diego, CA, USA). The results showed that 50 SNPs were significantly associated with disease severity (*P-value* < 0.05). Forty-one SNPs in a large linkage disequilibrium (LD) block within the β-globin gene cluster were associated with severe disease, of which the most significant was bthal_bg200 (odds ratio (OR) = 5.56, *P-value* = 2.6×10^{-13}).

The second GWAS was conducted using the Illumina Human 610-Quad BeadChips array (Illumina, San Diego, CA, USA) with DNAs from 235 mild and 383 severe cases of Thai $β^0$-thalassemia/HbE patients (Nuinoon et al., 2010). The result identified 27 SNPs located in three genes/regions, having *P-value* of 1.00×10^{-7} or lower to be associated with the disease severity. The quantile–quantile plot of the observed *P-value* for association to the disease severity and Manhattan plot are shown in Figure 1. The strongest SNPs associated with the disease severity were located in the β-globin gene cluster on chromosome 11p15.5 and olfactory receptor genes upstream of β-globin gene cluster. The 9 most significant disease severity associated SNPs (*P-value* < 1.00×10^{-10}) were located in a large LD spanning over 62 kb containing the locus control region (LCR) of the β-globin gene cluster, 4 functional β-globin-like genes (ε-, $^Gγ$-, $^Aγ$- and δ-globin) and 1 pseudogene (ψβ-globin). The most significantly-associated SNP, rs2071348, was in the ψβ-globin gene (*P-value* = 2.96×10^{-13}, OR = 4.33 with 95% CI; 2.74-6.84). The second most significant region was mapped to the *HBS1L-MYB* intergenic region on chromosome 6q23. Five SNPs of this region showed strong association with disease severity (*P-value* < 1.00×10^{-7}) and rs9376092 revealed the most significant association with *P-value* = 2.36×10^{-10} (OR = 3.07 with 95% CI; 2.16-4.38). The third significant gene is the *BCL11A* on chromosome 2p15. Four SNPs were significantly associated with the disease severity, with the most significant SNP rs766432 located in intron 2 (*P-value* = 5.87×10^{-10}, OR = 3.06 with 95% CI; 2.15-4.37). In addition, 101 SNPs on 69 genes also showed association with disease severity with *P-value*s of 1.00×10^{-5} to 1.00×10^{-6}. These SNPs are located on several genes/regions and can be classified into various biological functions such as cell cycle, cell growth, structural proteins, enzymes, hormones, signaling molecules, genes involved in gene expression, protein degradation, inflammatory response and hypothetical proteins with unknown functions. These genes may have less effect on disease severity compared to the three regions. However, they may play some roles in modification of disease severity and need further investigation.

No SNPs in the *BCL11A* region were typed in the first GWAS, suggested that the pooled genotyping was done on a marker panel that was less dense than the second GWAS and did not have the same level of genome coverage as the high density SNP arrays. However, the low marker density can identify two of the three SNPs highly associated with disease severity identified by the second GWAS, the β-globin gene cluster and *HBS1L-MYB* region.
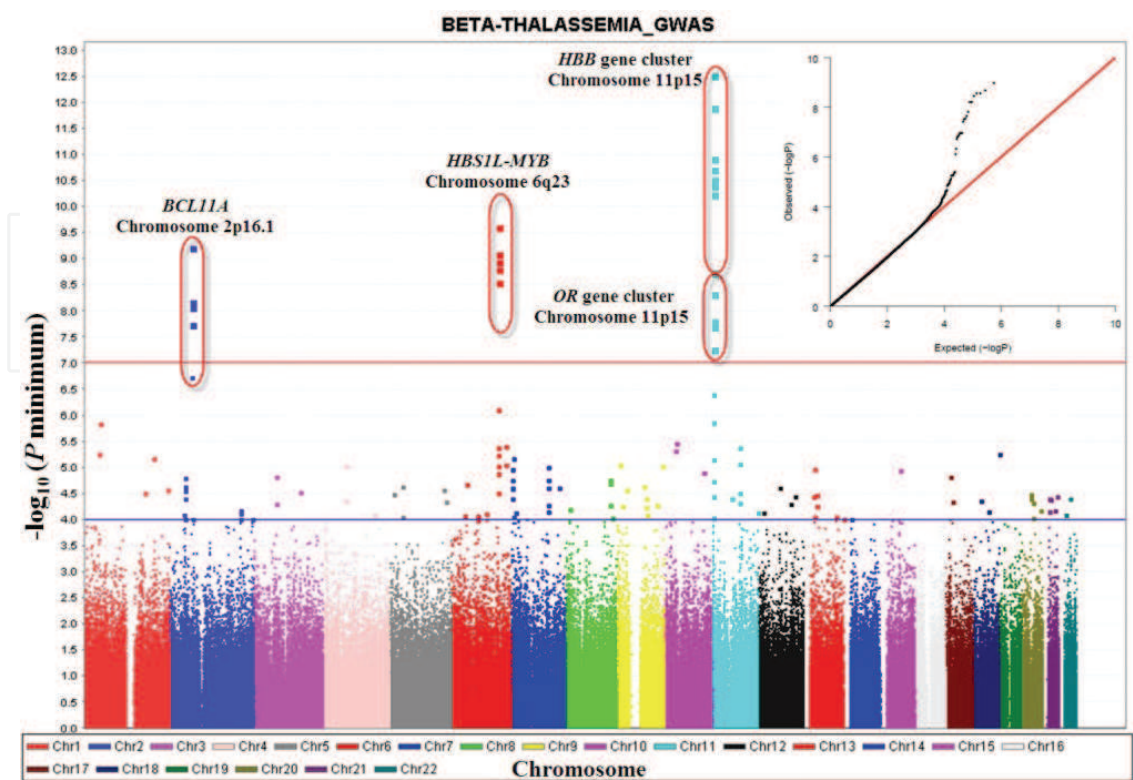
Fig. 1. Genome-wide association study scatter plots of *P-values* (analyzed with disease severity) with chromosome location. The red line denoted the significance threshold (*P-value* = 1.00 x 10^{-7}), and the blue line denoted the suggestive *P-value* (P = 1.00 x 10^{-4}). Inset shows the quantile–quantile plot of the *P-values* (allelic association) (Nuinoon et al., 2010). With kind permission from Springer Science+Business Media

Interestingly, the absolute HbF level among mild β^0-thalassemia/HbE patients without α-thalassemia co-inheritance was significantly higher than those of the mild cases who were β^+-thalassemia/HbE or having co-inheritance of α-thalassemia and the severe cases (Table 1). The three most significant regions associated with disease severity were also reported to be quantitative trait loci (QTLs) associated with HbF level in the previous genetic linkage and association studies.

6. HbF quantitative trait loci

It is clear from isolated family, linkage and GWAS studies that there are several QTLs for HbF and F-cells. Moreover, in many family studies, the high HbF phenotype segregates independently of the β-globin gene cluster, implicating the presence of *trans*-acting factors. The major QTLs for HbF and F-cells that have been extensively studied are located in three loci, one is *in-cis* with the β-globin gene cluster and two are i*n-trans* with the β-globin gene cluster, the *HBS1L-MYB* intergenic region and *BCL11A* gene.

6.1 β-globin cluster
It has been recognized since the 1980s that the C→T single base substitution at position -158 in the promoter of the $^G\gamma$-globin gene is associated with high HbF levels (Gilman &

Huisman, 1985; Labie et al., 1985a). Evidence for the influence of a genetic variant within the β-globin gene cluster came from families with β-thalassemia or sickle cell anemia which showed a tendency for co-segregation of higher or lower HbF levels with the disease mutation. The HbS mutation is found on four major β-globin cluster haplotypes (HbS-Senegal, -Benin, -Bantu, and –Arab/Indian). Carriers for the $β^S$ gene on the HbS-Senegal or HbS-Arab/Indian $β^S$ haplotype (including the T allele of *Xmn*I -158 $^Gγ$-globin) have high HbF levels, and a mild clinical course, whereas carriers of the $β^S$ gene on a HbS-Bantu haplotype (including the C allele of *Xmn*I -158 $^Gγ$-globin) have low HbF levels (Labie et al., 1985b). Despite the long time since the identification of this locus as a major QTL for HbF expression, functional studies have been inconclusive. Identification of the pinpoint causative variant is still unknown, in part because the QTL is located in a large LD block spanning over 62 kb. A plausible explanation of the genetic variant in β-globin cluster and olfactory receptor genes upstream function is the regulation of globin genes expression and hemoglobin switching. In erythroblasts, the β-globin locus forms an erythroid-specific spatial structure called the active chromatin hub (ACH). The ACH composed of the HS sites of the LCR, active β-globin genes, remote 5′ HS sites (HS-110 in human) and 3′ HS-1 (Fang et al., 2007). A chromatin hub is formed by looping 3′ HS-1, 5′ HS-110, and the 5′ part of the LCR together. The β-globin gene locus is surrounded by the genes encoding olfactory receptors that are expressed in olfactory epithelium, but not in erythroid cells (Bulger et al., 1999). The HS-110 and 3′ HS-1 site are located in the olfactory receptor genes (ORGs) cluster. An RNA FISH study in erythroid cells indicated that intergenic transcription of the β-globin locus occurs over a region of more than 250 kb including several genes in the nearby olfactory receptor gene cluster (Miles et al., 2007).

6.2 *HBS1L-MYB* intergenic region

Extensive studies over the years of Thein and colleages showed that a quantitative trait locus (QTL) for HbF expression in adults is located in the *HBS1L-MYB* intergenic region, the so called HBS1L-MYB intergenic polymorphisms (HMIP). A genome-wide linkage analysis study covered seven generations of 210 family members of an Asian-Indian kindred with heterocellular HPFH, β-thalassemia and α-thalassemia identified QTLs for HbF expression on chromosome 6q23-q24 (Lod score = 12.4) (Craig et al., 1996; Garner et al., 1998). Fine mapping of the 1.5 Mb region of human chromosome 6q23 encompassing the area showed five genes, *ALDH8A1*, *HBS1L*, *MYB*, *AHI1* and *PDE7B* (Close et al., 2004). To narrow down the candidate region, a high-resolution association study was performed in twin pairs of North European origin. Three LD blocks within the 79 kb long *HBS1L-MYB* intergenic region showed very strong associations with F-cell levels (*P-value*= 10^{-75}), with the strongest effect in the second block (24 kb) (Thein et al., 2007). The HMIP accounts for about 19% of the North European population trait variance. In Thai β-thalassemia/HbE, a GWAS study also showed that the most significantly-associated SNP with the HbF level in the *HBS1L-MYB* region, rs9399137, is also located within the HMIP second block. The HbF expression QTL at HMIP is also reported in healthy individuals of African descent (Creary et al., 2009), sickle cell patients from Tanzania, Brazil, African American (Creary et al., 2009; Lettre et al., 2008; Makani et al., 2011), β-thalassemia heterozygotes (So et al., 2008) and β-thalassemia patients (Galanello et al., 2009; Nuinoon et al., 2010).

The HMIP has been reported to contain distal regulatory elements that generate a key part of the overall control of the *MYB* expression (Wahlberg et al., 2009). In *MYB* expressing

primary human erythroid cells, the core *HBS1L-MYB* intergenic region harbors several potential cis-regulatory elements for GATA-1 signals that coincided with DNase I hypersensitive sites. HbF expression is linked to the kinetics of erythrocyte maturation and differentiation. *MYB* plays role in erythroid proliferation and differentiation, and in turn, the control of HbF levels (Jiang et al., 2006). The HMIP was associated not only with the HbF levels but also with the cell numbers of platelets and monocytes in the peripheral blood (Menzel et al., 2007). Recently, a 3-bp deletion, between 135,460,326 and 135,460,328 bp on chromosome 6q23 in HMIP was identified as the functional motif (Farrell et al., 2011).

6.3 BCL11A gene

The association of *BCL11A* and HbF expression was first identified by two GWAS studies (Menzel et al., 2007; Uda et al., 2008). The first GWAS with the F-cell trait was performed in a European twin cohort, targeting 179 individuals with contrasting extreme F-cell values. Association analysis identified not only the *Xmn*I -158 $^G\gamma$-globin and the chromosome 6 locus but also a new F-cell locus in intron 2 of the oncogene *BCL11A* on chromosome 2p15 (Menzel et al., 2007). The second GWAS based on HbF of 4,000 individuals from Sardinia also showed association to the same three loci, the β-globin locus, HMIP and *BCL11A*, which was the first replication of the *BCL11A* locus in patients with SCA and β-thalassemia (Uda et al., 2008). This locus was subsequently replicated in additional SCA patients from the USA and Brazil (Lettre et al., 2008; Sedgewick et al., 2008), and β-thalassemia heterozygotes from Hong Kong and the parents of β-thalassemia/HbE patients from Thailand (Nuinoon et al., 2010; Sedgewick et al., 2008).

The *BCL11A* gene encodes several isoforms of a zinc finger transcription factor, the shorter isoforms appeared to be restricted to primitive erythroblasts, and the full-length isoforms to adult-stage erythroblasts (Sankaran et al., 2008). Down-regulation of BCL11A expression in adult human erythroid precursors results in induction of HbF (Sankaran et al., 2008; Wilber et al., 2011). High-resolution chromatin immunoprecipitation (ChIP)–chip analysis and chromosome conformation capture (3C) assay showed that BCL11A binds the upstream locus control region (LCR), ε-globin and the intergenic regions between γ-globin and δ-globin genes cooperating with SOX6 that reconfigures the β-globin cluster by modulating chromosomal loop formation, which finally leads to transcriptional silencing of the γ-globin genes (Xu et al., 2010).

7. Conclusion

Thalassemias are the most common genetic disease in the world resulting from the defective globin chain synthesis. The main pathophysiologic feature of thalassemia is the accumulation of unpaired globin chains in erythrocyte precursors and red blood cells (β-globin in α-thalassemia and α-globin in β-thalassemia). The unmatched globin chains precipitated in the erythroid precursors in the bone marrow as well as in peripheral erythrocyte membranes contribute to ineffective erythropoiesis and shortened peripheral RBC survival resulting in chronic anemia in these patients. As a monogenic disorder, thalassemia is a very heterogeneous disorder. Patients with identical β-thalassemia genotypes show a remarkable variability in disease severity, ranging from nearly asymptomatic (mild disease) to transfusion-dependent anemia with additional complications (severe disease). Thus, thalassemias are good example of the Mendelian

genetic disease that demonstrate phenotypic variations due to the result of multigene interactions. Understanding the roles played by genetic factors in diseases will revolutionize diagnosis, treatment, and prevention, in addition to increase understanding of the environmental contributions. Three levels of genetic modifiers which influence the severity of β-thalassemia have been classified into primary, secondary and tertiary modifiers. The primary modifying factor is described by expression of the β-globin alleles due to the heterogeneity of the molecular lesions of the underlying the β-thalassemia mutation itself. Secondary modifying factors are factors that can affect the degree of globin chain imbalance, which are α-globin genotype and variation in fetal hemoglobin production. Tertiary modifiers are candidate genes which may be involved in several pathological alterations in patients. Our recent GWAS in Thai β-thalassemia/HbE patient cohorts suggests that a number of additional genetic modifier genes may account for the variability in clinical expression. The three genes/regions that are most significant in β-thalassemia/HbE disease severity are also associated with HbF expression, namely the β-globin gene cluster and olfactory receptor genes upstream of β-globin gene cluster on chromosome 11p15.5, the HBS1L-MYB intergenic region on chromosome 6q23 and the *BCL11A* gene on chromosome 2p15. It is noteworthy that the HbF expression QTL in the β-globin cluster was discovered by family and population studies in the 1980s and subsequently validated by genetic studies. The second QTL, the *HBS1L-MYB* intergenic region, was discovered by linkage analysis in extensive kindred in 1990s, and subsequently validated by genetic association studies. The third QTL, the *BCL11A*, was discovered in 2000s by GWAS. This shows the significant development of genomic technologies and knowledge of the human genome. The rapid development of next-generation sequencing technologies seems likely to accelerate further genomic studies. The many less significant variants may be identified in near future. There are several less significant genes compared to the three above mentioned regions, which may play roles in modification of disease severity. More studies are also needed to address the effect of these modifying factors.

8. Acknowledgment

9. References

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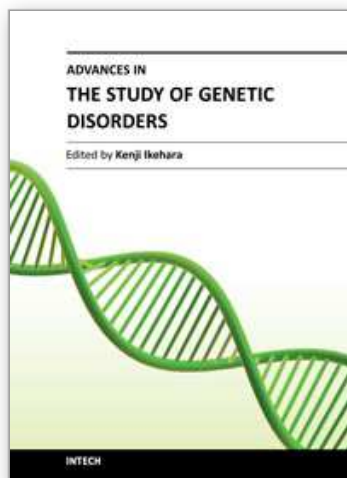
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Advances in the Study of Genetic Disorders

Edited by Dr. Kenji Ikehara

The studies on genetic disorders have been rapidly advancing in recent years as to be able to understand the reasons why genetic disorders are caused. The first Section of this volume provides readers with background and several methodologies for understanding genetic disorders. Genetic defects, diagnoses and treatments of the respective unifactorial and multifactorial genetic disorders are reviewed in the second and third Sections. Certainly, it is quite difficult or almost impossible to cure a genetic disorder fundamentally at the present time. However, our knowledge of genetic functions has rapidly accumulated since the double-stranded structure of DNA was discovered by Watson and Crick in 1956. Therefore, nowadays it is possible to understand the reasons why genetic disorders are caused. It is probable that the knowledge of genetic disorders described in this book will lead to the discovery of an epoch of new medical treatment and relieve human beings from the genetic disorders of the future.

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