QUANTIFICATION OF HUMAN GLOBIN GENE EXPRESSION IN β-THALASSEMIA

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Thesis
entitled

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QUANTIFICATION OF HUMAN GLOBIN GENE EXPRESSION IN β-THALASSEMIA

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ABSTRACT

β-thalassemia is a significant inherited disease with the occurrence of 3-9% in Thailand. Excess α- and reduced β-globin chains are the major causes of the disease pathogenesis and the compensation by HbF reproduction. We had quantitated the α, β and γ globin genes expressions in β-thalassemia by using real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and studied the relationships between mRNA expressions simultaneously with corresponding hemoglobin produced and disease severities in each β-thalassemia group and genotype. The results showed an increase of α/β globin mRNA ratios reflected more imbalance globin chains synthesis in β-thalassemia than normal controls. The differences of this ratio might indicate the severities between β^0, β^+ (severe) and β^+ genotypes of the β-thalassemia with HbE disease. For HbF compensation, the γ globin mRNA and HbF were expressed in order of homozygous β-thalassemia disease, β- thalassemia with HbE disease, and heterozygous β-thalassemia. In β-thalassemia with HbE disease, absolute HbE, but not HbF, amounts were higher in mild than moderate-severe phenotypes suggesting that increased HbE synthesis might better improved the clinical severity. The globin genes expressions in each genotype of β-thalassemia with HbE were determined and discussed.

The globin gene expressions can be used for prediction and explanation of severities in β-thalassemia. Interestingly, HbE appeared to be an additional significant factor affecting disease severity in β-thalassemia with HbE disease.

KEY WORDS: GLOBIN mRNA/ HEMOGLOBIN F/ HEMOGLOBIN E/ β-THALASSEMIA/ qRT-PCR

79 pages

การวัดปริมาณการแสดงออกของยีนโกลบินในเบต้าธาลัสซีเมีย
QUANTIFICATION OF HUMAN GLOBIN GENE EXPRESSION IN β-THALASSEMIA

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บทคัดย่อ

เบต้าธาลัสซีเมียเป็นโรคทางพันธุกรรมที่สำคัญของประเทศไทยซึ่งพบความชุกประมาณ 3-9% พยาธิสภาพของโรคเกิดจากการสร้างสายเบต้าโกลบินลดลงทำให้มีสายแอลฟ่าโกลบินส่วนเกิน ดังนั้นร่างกายจะมีการชดเชยโดยการสร้างฮีโมโกลบินเอฟสูงขึ้น ในการศึกษาครั้งนี้ทำการวัดปริมาณการแสดงออกของยีนแอลฟ่า เบต้าและแกมม่าโกลบินโดยใช้เทคนิค real-time reverse transcriptase polymerase chain reaction (qRT-PCR) และศึกษาความสัมพันธ์ระหว่างการแสดงออกของยีนและการสร้างฮีโมโกลบินกับความรุนแรงของโรคในแต่ละกลุ่มและแต่ละจีโนไทป์ของเบต้าธาลัสซีเมีย อัตราส่วนการแสดงออกของยีนแอลฟ่าต่อเบต้าโกลบินพบสูงขึ้นในกลุ่มเบต้าธาลัสซีเมียเมื่อเทียบกับกลุ่มปกติซึ่งชี้ให้เห็นถึงความไม่สมดุลของการสร้างสายโกลบิน ความแตกต่างของอัตราส่วนนี้ในกลุ่มที่มีจีโนไทป์เป็นเบต้าศูนย์ เบต้าบวก (ชนิดรุนแรง) และเบต้าบวกในโรคเบต้าธาลัสซีเมียฮีโมโกลบินอีอาจบ่งชี้ถึงความรุนแรงของโรคได้ การชดเชยโดยการสร้างฮีโมโกลบินเอฟและการแสดงออกของแกมม่าโกลบินเอ็มอาร์เอ็นเอพบจากมากไปน้อยในโฮโมไซกัสเบต้าธาลัสซีเมีย เบต้าธาลัสซีเมียฮีโมโกลบินอีและพาหะเบต้าธาลัสซีเมียตามลำดับ สำหรับความรุนแรงของโรคในกลุ่มเบต้าธาลัสซีเมียฮีโมโกลบินอีพบว่าในกลุ่มที่อาการไม่รุนแรงจะมีปริมาณฮีโมโกลบินอีสูงกว่ากลุ่มอาการรุนแรง ขณะที่ปริมาณฮีโมโกลบินเอฟของทั้งสองกลุ่มไม่แตกต่างกัน ชี้ให้เห็นว่าการสร้างฮีโมโกลบินอีที่มากขึ้นน่าจะลดความรุนแรงของโรคได้ดีกว่า ส่วนการแสดงออกของยีนโกลบินในแต่ละจีโนไทป์ของโรคเบต้าธาลัสซีเมียฮีโมโกลบินอีจะมีการอภิปรายในรายละเอียด

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LIST OF ABBREVIATIONS

Δ	delta
A	adenine
ARMS	amplification refractory mutation system
AS-PCR	allele-specific polymerase chain reaction
$^{A}\gamma$	gamma-A
BBQ	black berry quencher
bp	base pair
C	cytosine
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
Cp	crossing point
DCIP	dichlorophenol indophenol precipitation
dsDNA	double stranded deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
dl	deciliter
DNA	deoxyribonucleic acid
DW	distilled water
E	efficiency value
EDTA	ethylenediaminetetraacetic acid
EKLF	erythroidkrupple-like factor
EOF	electro osmotic flow
et al	et alii (Latin), and Other
FAM	6-carboxy-fluorescein
fL	femtoliter
FOG	friend of globin transcription factor
FRET	fluorescence resonance energy transfer
G	guanine

LIST OF ABBREVIATIONS (cont.)

GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GATA1	globin transcription factor 1
$^{G}\gamma$	gamma-G
Hb	hemoglobin
Hb CS	hemoglobin constant spring
Hct	hematocrit
HPFH	hereditary persistence of fetal hemoglobin
HPLC	high performance liquid chromatography
HS	hypersensitive site
IVS	intervening sequence
kb	kilo base pair
LC 610	light cycler-red 610
LC670	light cycler-red 670
LCR	locus control region
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
$MgCl_2$	magnesium chloride
ml	milliliter
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NF-E2	nuclear factor-erythroid derived 2
OF	osmotic fragility
ng	nanogram
NRBC	nucleated red blood cells
nt	nucleotide
PASA	polymerase chain reaction amplification of specific alleles

LIST OF ABBREVIATIONS (cont.)

PCR	polymerase chain reaction
pg	picogram
qRT-PCR	real-time reverse transcription polymerase chain reaction
RBC	red blood cells
RDW	red cell distribution width
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
T	thymine
Tm	melting temperature
U	uracil
UTR	untranslated region
WBC	white blood cells
YAK	Yakima Yellow
α	alpha
β	beta
β^E	beta-E
γ	gamma
δ	delta
ε	epsilon
ζ	zeta
^{o} C	degree celsius
µl	microlite
µM	micromolar

CHAPTER I
INTRODUCTION

Background and problems

Thalassemia is a genetic disease causing a public health problem in Southeast Asia including Thailand (1). The frequency of α-thalassemia is 30-40% whereas β-thalassemia is 3-9% in Thailand(2, 3). The most common type of β-thalassemia mutations in Thailand is codon 41/42 (-TTCT) following by codon 17 (A-T), nt -28 (A-G), IVSII-654 (C-T) and IVSI-5 (G-C), respectively(4). Hb E is found in high frequency, about 50-60%, in northeast of Thailand (3). Coinheritances of these abnormalities cause thalassemia diseases such as Hb Bart's hydropsfetalis (homozygous α-thalassemia 1), homozygous β-thalassemia and β-thalassemia with Hb E that should be prevention and control in Thai (1). Patient with β- thalassemia disease represents anemia and may require blood transfusion. Most of them get problems from iron overload resulting in cirrhosis, pancreatic failure and heart failure (5, 6).

Patients with β-thalassemia with HbE disease have a wide range of clinical severity(7). Coinheritance of α-thalassemia, types of β-thalassemia mutations, and polymorphisms (SNPs) associated with increased HbF such as XmnI and BCL11A are factors affectedthe disease severity(7).The α/β and α/non-α globin mRNA ratios had been reported to indicate the balance of Hb synthesis (8-11).Increasing of these ratios found in β-thalassemia indicating more disease severity(8). However, several studies reportedinconsistency in α/β globin mRNA ratios.In addition, the increasing of γ globin expression was associated with increasing of HbF synthesis in patients treated with hydroxyureaand improved disease severity (12). In this study, wehad quantitatedthe α, β and γ globin genes expressions and studied the relationships between globin genes expressions and disease severitiesin each group and genotype ofβ-thalassemia.

Many methods have been used to study the gene expression in thalassemia such as conventional polymerase chain reaction (13-15), northern blot(16, 17)and

reverse dot blot (18, 19) analysis. In recently, real time reverse transcription polymerase chain reaction (qRT-PCR) assay is the most effective method for gene quantitation. It has high sensitivity, high specificity, no process of agarose gel electrophoresis and high throughput (20). Thus, we were using qRT-PCR for quantitation of globin genes expressions.

The applications of globin gene expression are the uses for prediction and explanation of β-thalassemia disease severity. In addition, it may be used to describe the hemoglobin synthesis in β thalassemia.

Objectives

1.To quantitate the α, β and γ globin genes expressions in β-thalassemia by using real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

2.To study the relationships between globin genes expressions and disease severities in each β-thalassemia group and genotype

CHAPTER II
LITERATURE REVIEW

Thalassemia is an inherited disease due to defects in hemoglobin synthesis. Since it was first observed in patients near the Mediterranean Sea, the name in Greek "thalassa" means “the sea” was used. Thalassemia occurs mainly in Africa, Middle East, and Asia. In Southeast Asia, α thalassemia, β thalassemia, hemoglobin E (Hb E) andhemoglobin Constant Spring (Hb CS) are occurred in high frequencies. The prevalence of α thalassemia was 30-40% in Northern Thailand and Laos, 4.5% in Malaysia and 5% in Philippines whereas the prevalence of β thalassemia was 3-9% (2, 3). The most common type of β thalassemia mutations in Thailand is codon 41/42 (-TTCT) following by codon 17 (A-T), nt-28 (A-G), IVSII-654 (C-T) and IVSI-5 (G-C), respectively (4). Hb E is the most prevalent hemoglobin variant in Southeast Asian population especially at the area between Thailand, Laos, and Cambodia which a very high frequency (50-60%) was found (3). Hb CS is also a common hemoglobin variant in these areas with an observed frequency of 1-8% (3). Coinheritances of these abnormalities cause various thalassemia genotypes especially the clinically significant thalassemia diseases such as Hb Bart’s hydropsfetalis (homozygous α thalassemia 1), homozygous β thalassemia, β thalassemia/Hb E and Hb H diseases.

2.1 Hemoglobin

Hemoglobin is a major protein carried in all red blood cells. The hemoglobin molecule is a tetramer of two α-like and two β-like globin chains. The molecule is an oxygen transport proteinvia the binding site which is the iron in heme group. The α-globin gene locus located on chromosome 16 encodes in the order for ζ, α1, and α2 globin chains and the β-globin gene locus on chromosome 11 encodes for ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β globin chains, respectively. In embryonic yolk sac, the ε globin gene transcribes during the first 6 weeks of primitive erythropoiesis to produce

embryonic globins; hemoglobin Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), and Portland ($\zeta_2\gamma_2$). Switching to fetal definitive erythropoiesis in the liver occurs by silencing of ε globin gene and activating of $^G\gamma$ and $^A\gamma$ globin genes to produce fetal hemoglobin ($\alpha_2{}^G\gamma_2$, $\alpha_2{}^A\gamma_2$). A second switch to adult definitive erythropoiesis occurs at birth. The hematopoiesis moves from liver to bone marrow, the γ globin genes are silenced, and the δ and β globin genes are activated to produce hemoglobin A ($\alpha_2\beta_2$) and hemoglobin A_2 ($\alpha_2\delta_2$) for the remainder of life (21-23). Types of hemoglobin associated with developmental stage are shown in Table 2.1.

Table 2.1 Types of hemoglobin associated with developmental stage

Hemoglobin	Subunits	In embryo	In fetus (%)	In adult (%)
Gower 1	$\zeta_2\varepsilon_2$	Present	-	0
Gower2	$\alpha_2\varepsilon_2$	Present	-	0
Portland	$\zeta_2\gamma_2$	Few	Few	0
F	$\alpha_2\gamma_2$	Few	70-90	0-1
A	$\alpha_2\beta_2$	-	10-30	96-98
A2	$\alpha_2\delta_2$	-	1-3	1-3

2.2 Regulation of human globin genes expression

The transcription of human globin genes lead to mRNA precursor expression in the nucleus. RNA processing including RNA splicing, addition of 5’ cap and poly A tail occur to form mature globin mRNAs which translocate into the

cytoplasm. The transcriptional controls for β-globin loci include cis-acting sequences, trans-acting factors and epigenetic factors. The β-LCR containing 5 DNase 1 hypersensitive sites (HS1-5), promoter locating upstream of transcriptioninitiation site, and enhancer locating upstream of the promoter are sites for transcription factors binding. Binding ofRNA polymerase II to promoter enables the transcription process occurs. GATA-1 transcription factor binds to the (T/A)GATA(A/G) sequence, interacts with FOG (Friend of GATA),and plays role in globin gene switching and erythroidmaturation. EKLF (ErythroidKrupple-like factor) plays role in γ to β globin switching by binding to the CACCC box in β globin promoter and interact with GATA-1. NF-E2 binds to the GCTGA(G/C)TCA sequence and plays role in general initiatorfor transcription (24-27). Moreover, condensations of chromatin included heterochromatin and euchromatin play a role in activation of specific genes expression at the β globin locus. Histones acetylation in euchromatin causesloosen of histone protein and DNA binding and leads to open structure. Transcription process occursin the area which sensitive toDNase I whereas in heterochromatin loss of sensitivitytoDNase I (28-30).

2.3 Fetal hemoglobin (Hb F)

Fetal hemoglobin (Hb F) is a tetrameric protein composes of two α globin chain and two γ globin chain ($\alpha_2\gamma_2$) in which each contains a heme group. It predominantly synthesizes in fetus. The coding of the $^{G}\gamma$ or$^{A}\gamma$ is only 1 nucleotide difference in codon 136 of exon 3. Glycine (GGA) is encoded by the$^{G}\gamma$ gene while alanine (GCA) is encoded by the$^{A}\gamma$ gene (31, 32). Hb F is predominantly produced in the newborn in the amount of 70-90%, and gradually reduces to less than 8% at 6 months, less than 2% at 1 year, and less than 1% in normal adults (32). The expression ratio of $^{G}\gamma/^{A}\gamma$ in newborn is approximately 70/30 while due to hemoglobin switching the ratio is reverse to 40/60 in normal adults (33). Some pathological and non-pathological conditions affect the β-globin gene cluster and lead to the increased Hb F synthesis such as β-thalassemia, sickle cell disease, δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH). In addition, some acquired conditions also associate to an increasing of Hb F such as pregnancy, leukemia, aplastic anemia,

paroxysmal nocturnal hemoglobinuria (PNH) and pernicious anemia as shown in Table 2.2 (33).

Table2.2 Factors associated to the increasing of Hb F (33)

Hereditary disorders	Acquired conditions
-Homozygous β-thalassemia	-Pernicious anemia
-Heterozygous β-thalassemia	-Paroxysmal nocturnal hemoglobinuria
	-Refractory normoblastic anemia
-HPFH, homozygous	-Sideroblastic anemia
-HPFH, heterozygous	-Pure red cell aplasia
-δβ thalassemia, homozygous	-Aplastic anemia
-δβ thalassemia, hetorozygous	-Pregnancy
-Sickle cell anemia	-Recovery after bone marrow transplant, marrow hypoplasia, leukemia chemotherapy and transient erythroblastopenia; treatment with hydroxyurea, 5-aza-2′-deoxycytidine, butyrates, ertythropoietin
-Some other hemoglobinopathies (HbC, HbE, HbLepore, Hb Rainier and Hb Bethesda)	
-Trisomy D	
-Hereditary spherocytosis	
-Hb variants with isoelectric point identical to that of HbF*	-Hyperthyroidism
	-Juvenile chronic myeloid leukemia
-Hb variants with retention time similar to that of HbF	-Acute leukemias
	-Erythroleukemia
	-Benign monoclonal gammopathies
	-Cancer with marrow metastases, hepatoma
	-Chronic renal disease

*Pre-analytical effect on HPLC methods.

A polymorphism which changed from C to T (C-T) at the position -158 in the $^{G}\gamma$ globin gene promoter is known as XmnI.The Xmn I was found associated with Hb F synthesis in β-thalassemia (34, 35). In addition,polymorphism at theBCL11A

gene is found associated with high level of HbF inβ-thalassemia and sickle cell disease (36, 37). Up regulation of the γ-globin genes help to maintain the balance between α- and non α-globin chains thus ameliorate the red blood cell from excess α-globin precipitates. For clinical utility, Hb F inducing agents such as 5-azacytidine, hydroxyurea and butyrate analogues are used to increase Hb F level in thalassemia patients (38-40).

2.4 Hemoglobinopathies

Hemoglobinopathies are the most common genetic diseases around the world. They are divided into two groups, thalassemia and hemoglobin variants or abnormal hemoglobins. Both are caused by deletions or mutations of the α- or β-globin genes. When a quantitative defect of hemoglobin synthesis but normal structure is called thalassemia, a change in hemoglobin structure is called abnormal hemoglobin. The clinical symptoms are variable ranging from mild hypochromic microcytic anemia to severe hematological disease (41, 42).

2.5 α-thalassemia

α-thalassemia is an autosomal recessive disorder inherited on the α-globin genes. The majority of α-thalassemia are caused by deletions, and a minority of them are gene mutations(43). Various phenotypes ranging from severe anemia to asymptomatic carriers depend on the number of α globin genes remaining. Couples who both carry α^0-thalassemia allele (--/) are risk to have a child with Hb Bart's hydropsfetalis. Couples who carry α^0-thalassemia allele in one and α^+-thalassemia allele (-α/) in the other are risk to have a child with HbH disease. Therefore, couples are needed to determine whether they are α-thalassemia carriers before pregnancy and to counsel if they are risk for Hb Bart's hydropsfetalis syndrome and Hb H disease.

2.5.1α-thalassemia carriers

2.5.1.1 α^0-thalassemia (α-thal 1)

α^0-thalassemia causes by complete or partial deletion of both α-globin genes leading to absent of α-globin synthesis. The common types found in Southeast Asia are $-\,-^{SEA}$, $-\,-^{THAI}$, and $-\,-^{FIL}$ deletions (44, 45)as shown in Figure 2.1.

2.5.1.2 α^+-thalassemia (α-thal 2)

The common types of α^+-thalassemia deletions in Southeast Asia are $-\alpha^{3.7}$ (rightward deletion) and $-\alpha^{4.2}$ (leftward deletion) (46)as shown in Figure 2.1. The common α^+-thalassemia mutation are Hb Constant Spring (α^{CS}) and HbPaksé (α^{PS}) which both have mutations at the termination codon and producing elongated α-globin chains. The base change from *T*AA to *C*AA is found in Hb Constant Spring while from TA*A* to the TA*T* is found in HbPaksé(47, 48). Some mutations caused highly unstable α-globin variants such as HbQuongSze, HbSuanDok(49, 50).

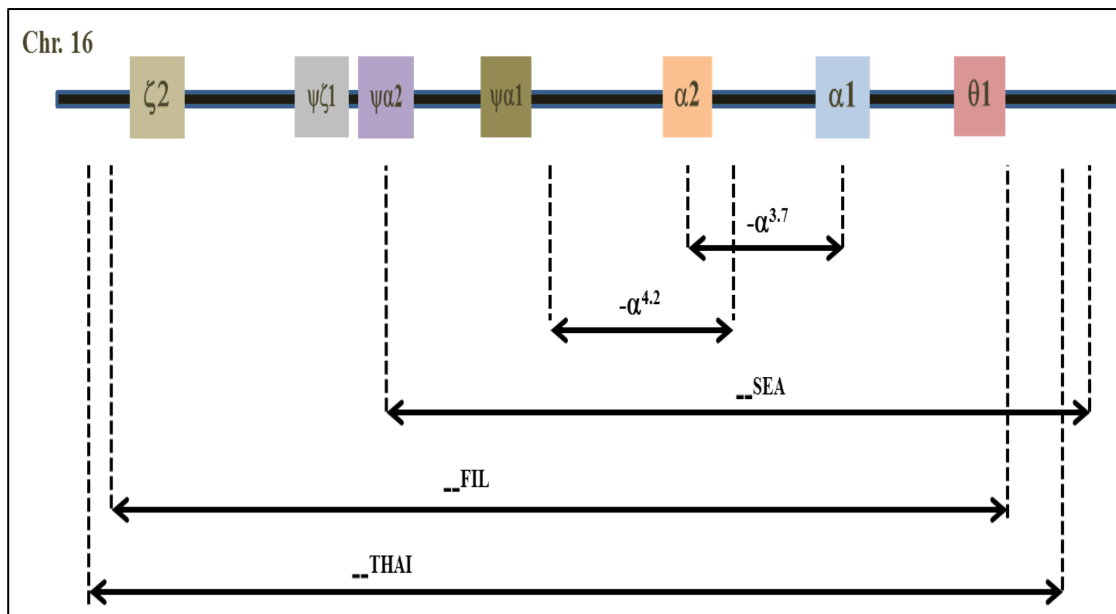


Figure 2.1 Common α-thalassemia deletions in Southeast Asia

2.5.2 α-thalassemia diseases

2.5.2.1Hb H disease

It is inherited from α^0- and α^+- thalassemia alleles and left only 1 α-globin gene expresses. Hb H (β4) is unstable hemoglobin and can precipitate in red blood cells. Staining the Hb H with Brilliant Cresyl Blue gives golf-ball morphology of red blood cells containing inclusion bodies (IB). Patients with Hb H disease have anemia with low MCV and MCH values and peak of Hb H are observed(41, 51, 52).

2.5.2.2Hb Bart's hydropfetalis syndrome

The absence of all 4 α-globin genes causes Hb Bart's hydropfetalis, the most severe thalassemia disease. Without α-globin chain, the γ-globin forms Hb Bart's (γ_4). The disease causes infants either dies in utero (23-38 weeks) or shortly after birth.Cord blood determination results in 80-90% of HbBart's, 10-20%of Hb Portland, and no Hb F and Hb A produced (41, 52). Phenotypes and genotypes of α-thalassemia are shown related to hemoglobin typing and clinical symptoms (Table 2.3) (41).

Table 2.3 Phenotypes and genotypes of α-thalassemia (41)

Phenotype	Genotype	Hemoglobin typing	Clinical Symptoms
Normal	αα/αα	Normal	-No symptoms
Heterozygous α^+-thalassemia (α-thalassemia minor)	– α/αα	Normal	-No symptoms -Slight changes to blood count
Homozygous α^+-thalassemia (α thalassemia minor)	– α/– α	Normal	-Mild anemia -Significant changes to bloodcount
Heterozygous α^0-thalassemia (α thalassemia minor)	– –/αα	Normal	-Mild anemia -Significant changes to bloodcount
Mixed heterozygosity, α^+/ α^0- thalassemia (HbH disease)	– –/– α or – –/$\alpha\alpha^{cs}$	A_2ABart's H or CS A_2AH Bart's	-Variable chronic hemolyticanemia
Homozygous α^0- thalassemia (Hb Bart's hydropsfetalis)	– –/– –	Portland, Bart's	-Life-threatening fetal anemia -Generalized hydrops

2.6 β-thalassemia

β-thalassemia is an autosomal recessive disorder inherits by defective of β globin chain synthesis. The majority of β-thalassemias are caused by point mutations or frameshift mutations, minority of them are gene deletions (53).

2.6.1 Classification of β-thalassemia alleles

2.6.1.1 β^+-thalassemia

Point mutations at the promotor region or the polyadenylation signal of the β-globin gene and some types of mutations affected mRNA processing which cause the reduction of β-globin chain synthesis lead to β^+-thalassemia. Hb A is produced in β^+-thalassemia such as heterozygous β^+-thalassemia ($\beta^+/\ \beta^A$), β^+-thalassemia with HbE ($\beta^+/\ \beta^E$) and homozygous β^+-thalassemia ($\beta^0/\ \beta^+$, $\beta^+/\ \beta^+$).

2.6.1.2 β^0-thalassemia

Mutations affected the β-globin gene resulting in absence of β-globin chain synthesis lead to β^0-thalassemia. No Hb A is produced in β^0-thalassemia with HbE ($\beta^0/\ \beta^E$) and homozygous β^0-thalassemia ($\beta^0/\ \beta^0$).

2.6.2 Mutations causing β-thalassemia

2.6.2.1 Transcriptional mutation

Common mutations affected transcriptional control of β-globin gene are changed form C to G at position -86 (-86, C-G) and changed from A to G at position -28 (-28, A-G) as shown in Table2.4. They cause β^+-thalassemia. In addition, the mutation at position -101 (-101, C-T) results in a silent β-thalassemia that represent normal hematological values but shows decreased β-globin synthesis (54-56).

2.6.2.2 Cap site and 5'-UTR mutations

The cap site and 5'-UTR mutations cause β^{+}-thalassemia. The mutationsmay affect the efficiency of 7-methylguanosine adding and decreasing β-globin gene transcription. The types such as CAP+1 (A-C), CAP+8 (C-T) and CAP+40 to +43 (-AAAC) were found in Southeast Asia. Coinheritance of CAPsite mutationwith β^{0}- and β^{+}(severe)-thalassemia lead to moderate to severe phenotypes (56-58).

2.6.2.3 Nonsense mutation

The mutation of DNA sequences that leads to premature stop codon in mRNA results in shorten protein translated. This type of mutation causes β^{0} thalassemia such as codon 17 (A-T), codon 35 (C-A) and codon 26 (G-T) mutations as shown in Table 2.4 (59-61).

2.6.2.4 Frameshift mutation

The frameshift mutation caused by deletion or insertion that is not evenly divisible by three nucleotides resulting in shift of the sequence.In Thailand, the most common β^{0}-thalassemia is frameshift mutation at codon 41/42 (-CTTT) due to four bases deletions. In addition, one base insertion at codon 71/72 (+ A) and codon 14/15 (+ G) cause frameshift mutations and represent β^{0}-thalassemia (Table 2.4) (62-65).

2.6.2.5 Mutations affected mRNA processing

The mutations that affected mRNA processing leadtoaberrant splice regions. This type of mutations causes β^{0}-thalassemia or β^{+}-thalassemia depends on whether correct spliced mRNA given β-globin chain is also occurred. For example, IVS-1 nt 1(G-T) mutationcauses aberrant splicingand no correct spliced mRNA resulting in β^{0}-thalassemia whereas IVS-1 nt 5 (G-C) and IVS-2 nt 654 (C-T) mutations lead to both correct and aberrant splicing resulting in β^{+}-thalassemia. In addition, the mutations in exonsuch as codon 26 or Hb E (G-A),codon 24 (T-A) and codon 19 orHb Malay (A-G) produce both correct and aberrant spliced mRNA

resulting in β^+-thalassemia (66-70).The mutations affected mRNA processing is shown in Table2.4.

2.6.2.6Polyadenylation mutations

The AATAAAis a conserve sequence upstream the polyadenylationsite which can bind to cleavage factor.It is also a part of signal for polyA polymerase enzyme which play role inpolyadenylation. These mutationsaffect the efficiency of the cleavage and polyadenylationprocesses. The point mutations or deletions at AATAAA region cause β^+-thalassemia. The common types found in Southeast Asia arepoint mutations, AATAAA changes to AATAGA and AATAAA changes to CATAAA. Deletions of two, five and all nucleotides of the AATAAA sequence (AA--AA, A----- and ------) werealso reported (56, 71, 72).

2.6.2.7β-globin gene deletion

The uncommon of β-thalassemia are β-globin gene deletions. Large deletions of β-globin gene cause β^0-thalassemia such as the 619-bp deletion at 3' end of β-globin gene found in India and Pakistan (73, 74). The 3,485-bp deleted entire β-globin gene and 105-bp deleted 5' end of β-globin gene were found in Thailand (75, 76). The β-thalassemia deletions,δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) were found increasing γ-globin gene expression and increasing HbF production (77, 78). The β gene deletions are shown in Table 2.4.

Table 2.4 Classification of β-thalassemia mutations

No.	Mutation	Type	Mechanism
1.	-28 (TAAA-TAGA)	β^+	Transcription mutations
2.	-31 (GCAT-GCGT)		
3.	-86 (CACCC-CACCG)		
4.	-87 (CACCC-CACAG)		
5.	+1 (A-C)	β^0	cap site mutation
6.	Codon 15 (TGG-TAG)	β^0	Nonsence mutations
7.	Codon 17 (AAG-TAG)		
8.	Codon 26 (GAG-TAG)		
9.	Codon 35 (TAC-TAA)		
10.	Codon 43 (GAG-TAG)		
11.	Codon 8/9 (+G)	β^0	Frameshift mutations
12.	Codon 14/15 (+G)		
13.	Codon 15 (-T)		
14.	Codon 27/28 (+C)		
15.	Codon 41 (-C)		
16.	Codon 41/42 (-TTCT)		
17.	Codon 71/72 (+T)		
18.	Codon 71/72 (+A)		
19.	Codon 95 (+A)		
20.	Codon 123-125 (-ACCCCACC)		
21.	IVS1-1 (G-T)	β^0	mRNA processing mutations
22.	IVS1-1 (G-A)	β^0	
23.	IVS1-5 (G-C)	β^+ (severe)	
24.	IVSII-654 (C-T)	β^+ (severe)	
25.	Codon 19 (AAC-AGC)	β^+ (Hb Malay)	
26.	Codon 26 (GAG-AAG)	β^+ (Hb E)	
27.	Codon 126 (GTG-GGG)	β^0	
28.	Poly A (AATAAA- AATAGA)	β^+	polyadenylation mutation
29.	105 bp deletion	β^0	Gene deletions
30.	619 bp deletion		
31.	3485 bp deletion		
32.	12.5 kb deletion		
33.	45 kb deletion		
34.	Asian india inversion		

2.6.3 Clinical classification of β-thalassemia

2.6.3.1 β-thalassemia major

Homozygous β-thalassemia may present as β-thalassemia major or β-thalassemia intermedia. Clinicalpresentation of β-thalassemia majoroccurs in 6 to 24 months after birth. Children have jaundice, feeding problems, diarrhea, irritability, and enlargement of the abdomen due to enlargement of spleen and liver.Patients have skeletal changes include typical craniofacial changes from extramedullaryhematopoiesis. Individual with β-thalassemia major needs medical attention in the first two years and requires regular blood transfusion.Complication after blood transfusion is iron overload leading to growth retardation and failure or delay of sexual maturation (79, 80). Phenotypes and genotypes of β-thalassemia are shown related to hemoglobin typing and clinical symptoms (Table 2.5) (41).

2.6.3.2 β-thalassemia intermedia

β-thalassemiaintermedia has less anemia than β- thalassemia major.Clinical presentation of β-thalassemia intermedia can be found in 2 to 6 years and occasionally requires blood transfusion. Patients have bone marrow hypertrophy due to compensation for anemia, skeletal changes and enlargement of the abdomen(79, 80).

2.6.3.3 β-thalassemia minor

Most of β thalassemia minor is found in carriers. No clinical symptom is presented but sometimes has mild anemia. Couples who both β thalassemia carriers are risked 25% for having a child with homozygous β-thalassemia (80).

2.6.4 Pathophysiology of β-thalassemia

The β-thalassemia causes by defect of β-globin gene that reduces β-globin chain synthesis. The excess of α-globin chains occur which is unstable and precipitated in peripheral red blood cells and red blood cells precursors in the bone marrow. These causes shorten red blood cells life span leading to extravascular

hemolysis and ineffective erythopoiesis that resulting in anemia. The excess α-globin chains can increase binding to the δ-globin chain ($\alpha_2\delta_2$) and γ-globin chain ($\alpha_2\gamma_2$) to produce high HbA_2 and HbF, respectively. Moreover, high Hb F was found in β-thalassemia due to increasing γ-globin gene expression (12, 81). The extensive destruction of red blood cellsin β-thalassemia leads tohypersplenism. Anemia induceserythropoietin production to accelerate erythropoiesis resulting in erythroid hyperplasia and skeletal change. Patients with anemia require blood transfusion. Most of them get problems from iron overload resulting in cirrhosis, pancreatic failure and heart failure. Pathophysiologyofβ-thalassemiais shown in Figure 2.3(5, 82, 83)**.**

Table 2.5 Phenotypes and genotypes of β-thalassemia (41)

Phenotype	Genotype	Hemoglobin typing	Clinical Symptoms
Normal	β^A/β^A	Normal	-No symptoms
Heterozygous β-thalassemia	β^0/β^A β^+/β^A	A_2A (Hb A_2> 3.5 %)	-Mild anemia
Homozygous β-thalassemia	β^0/β^0 β^+/β^0 β^+/β^+	A_2F or A_2FA	-Moderate to severe anemia -Occasional to regular blood transfusion
β-thalassemia with HbE	β^0/β^E β^+/β^E	EF or EFA	-Moderate to severe anemia -Rare to regular blood transfusion

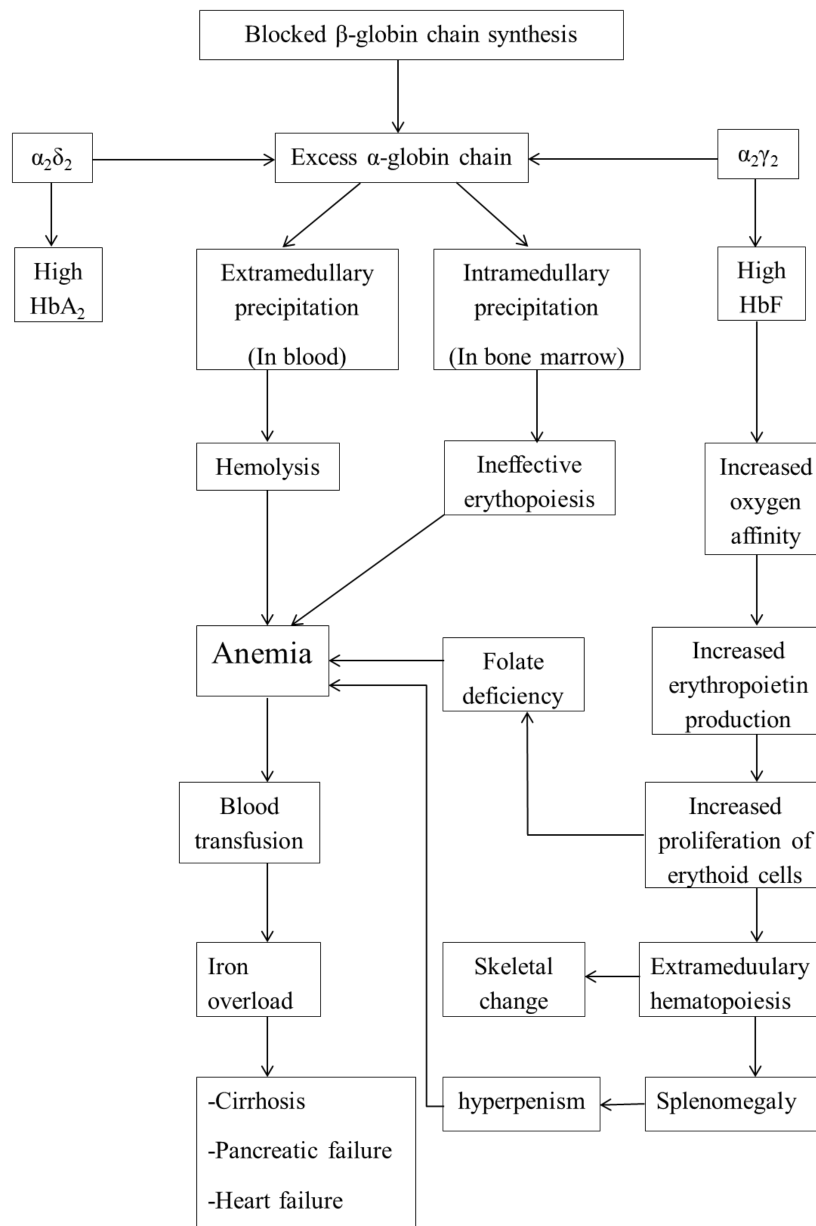


Figure 2.2 Pathophysiology of β-thalassemia

2.7 Laboratory diagnosis

2.7.1 Red blood cell morphology

Determination of blood cells morphology gives supplement information to automated blood cells count. A blood smear is prepared from whole blood and stains with Wright's stain as described elsewhere (84, 85). In thalassemia, abnormal red blood cell morphology can be observed in various grades due to severity. Mild microcytic hypochromic red blood cells and few poikilocytes are present in thalassemia carriers but are prominently found in thalassemia diseases (86, 87). Basophilic stippling can be found due to disturbance of hemoglobin synthesis. A high number of target cells is found in Hb E. Present of polychromasia indicates erythropoiesisacceleration. Nucleated red blood cells and Howell-Jolly bodies can be found especially in splenectomized patients.

2.7.2 Red blood cell indices

One of screening method for detection of thalassemia is red blood cell indices. These parameters include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Red blood cell indices are used to indicate anemia. The MCV, MCH and MCHC are calculated from hemoglobin, hematocrit and red blood cell count values. Moreover, the automated machines can directly measure MCV and calculate forRBC distribution width (RDW). The RDW value indicates variable in size of red blood cells (anisocytosis)(88).Elevation of RDW values were found in the anemic patients including sickle cell anemia, β-thalassemia disease, β-thalassemia trait, and iron deficiency (89). MCV is a mean value indicating size of red blood cells. The normal value is more than 80 fL (90) whereas low value of MCV indicates microcytic red blood cells. MCH indicates the amount of hemoglobin in red blood cells,the normal value is more than 27 pg(90). MCHC indicates hemoglobin concentration per unit volume and the normal value ismore than 32 g/dl (91). Microcytic hypochromic red blood cells with low MCV and MCH are found in β-thalassemia. Moreover, The MCV and MCH are related to the severity of the mutation in β-thalassemia (92, 93).

2.7.3 Hemoglobin Typing by automated machines

2.7.3.1 High Performance Liquid Chromatography (HPLC): The Variant™ II HPLC (Bio-Rad, France)

The machine can separate hemoglobin types and provides quantitative measurement of Hb A, Hb A_2 and Hb F. It is rapid, automatic, and capable for detection of many uncommon hemoglobin variants such as Hb S, Hb E, Hb C, and HbLepore other than common hemoglobin types (94). In cation-exchange HPLC, hemolysate is injected into a column (stationary phase) containing a negatively charged of resin which bind to the positively charged of hemoglobin. Hemoglobin is eluted by buffers composed of different ionic strengths which inject with high pressure. Each fraction is eluted in a specific retention time called "window" which is the time it retains in the column until eluting out. The elutions pass the detection part and data processing system then finally report as a chromatogram. Amounts of each fraction are reported in percentage calculated from the peak areas. Common hemoglobins are eluted separately including Hb A, Hb A2 and Hb F. However, Hb Eis eluted in the same window with Hb A2 by the Variant HPLC.

2.7.3.2 Capillary Electrophoresis (CE): The Capillarys™ 2 CE (Sebia, Lisses, France)

The principle is based on electrophoresis in a capillary tube under high voltage. Hemoglobins are migrated due to their charges and sizes by eletro-osmotic flow (EOF). Quantitative data are reported as the electropherogram. Hemoglobins are separated into zone 1 to zone 15. By using the Capillarys 2 CE, Hb E and Hb A2 are migrated in different zone thus the amount of these two hemoglobins can be detected separately.

2.7.4 DNA analysis

2.7.4.1 Amplification Refractory Mutation System (ARMS)

The amplification refractory mutation system (ARMS), also known as allele-specific Polymerase Chain Reaction (AS-PCR) or PCR amplification

of specific alleles (PASA), was first described by Newton *et al*(95). The ARMS technique is a PCR-based method for detection of known point mutations. The method relies on 'end-point' analysis and gel electrophoresis. The target DNA is amplified by using two pairs of allele-specific primers, one specific for mutation and the other for normal alleles. Internal control primers should be added to control whether the reaction is work well. Homozygous for the mutation shows only the PCR product from primers specific for mutation, other than the internal control band, while heterozygous for the mutation shows PCR products from both mutation and normal primers. This technique has been used for detection of β-thalassemia mutations (4)and non-deletional α-thalassemia such as Hb Constant Spring, HbPaksé, HbQuongSze and HbSuanDok(96-98).

2.7.4.2 Gap-PCR

The Gap-PCR technique is a PCR-based method for detection of known deletions. Two primer pairs are used, one flankingthe deletion breakpoints and the otheramplified across one of the breakpoint which detecting deletion allele and normal allele, respectively. The PCR productsare detected by gel electrophoresis. Gap-PCR has been used for detectionof common α-thalassemia deletions such as SEA deletion, Thai deletion, Filipino deletion, 3.7-kb deletion and 4.2-kb deletion(99, 100).In addition, the method was used for detection of δβ-thalassemia deletions, HPFH deletions, and HbLepore which isproduced from a 7-kb deletion δβ-hybrid genes(101, 102).

2.7.5 Real time PCR

Real time PCR is a PCR technique. Amplification is begin with a linear phase, then rapidly increases in exponential phase, and goes to end point of amplification at plateau phase (Figure 2.5) (20). Amplified PCR products of PCR technique are detected at end point or plateau phase usually by agarose gel electrophoresis whereas those of real time PCR are detected at early exponential phase such as by probes. Increasing of PCR products at early exponential phase is assumed to be 100% efficiency. The crossing point(Cp) valuesare detected in this phase. The Cpvalueis the point at which fluorescence of amplified DNA is above the background

fluorescence and is used to calculate the starting mRNA (20, 103-105). Many probes can be used for measurement light intensity of amplify PCR product during reaction such as SYBR® Green I, Hybridization probes and Hydrolysis probes. SYBR® Green I is a DNA binding fluorescent dye that binds to all dsDNA so it is not specific though the cost is cheap. High intensity of fluorescence detected indicates high dsDNA concentration (20). Hybridization probe uses two specific oligonucleotide probes,one is labeled with donor fluorescent dye on the 3′ end andthe other is labeled with reporter fluorescent dye on the 5′ end (106).The gap between two specific oligonucleotide probes should be 1-5 base pair.When both probes hybridize with DNA template in annealing step, donor dye is excited and transfer energy *to*reporter dye by fluorescence resonanceenergy transfer (FRET).Hydrolysis probes such as a Taqman™ probeis a specific single probe labeled with reporter dye on the 5′ end and with quencher dye on the 3′ end (106). When the probe specific anneals to PCR product, no FRET occurs due to the close proximity between reporter dye and quencher dye. During the extension step, 5’-3´exonuclease activity of DNA polymerase cleaves and releasing the reporter dye from the probe to emit fluorescence.

The real-time PCR technique is widely used for thalassemia detections (107-115). This method is more rapid, higher throughput and more accuracy than end-point PCR that suit for using in prenatal diagnosis (107). The applications were the use for deletional and non-deletional α-thalassemias. The SEA deletion, 3.7-kb deletion, 4.2-kb deletion and non-deletion such as Hb Constant Spring had been detected by using SYBR Green 1 combined with dissociation curve analysis (108, 109). In addition, the quantitative real time PCR using Taqman™ probe was used for detection of Hb Bart’s hydropfetalis syndrome from maternal plasma carrying fetus DNA (110, 111). Moreover, the melting curve analysis after real-time PCR was used for screening of many β-globin gene mutations such as codon 41/42, codon 37, IVSI-I, IVSI-5, IVSI-116, IVSI-110 (112-114). The detection of β-globin gene deletions were used SYBR Green 1 and relative quantitative real time PCR(115).

One-step real-time RT-PCR combines the cDNA synthesis and cDNA amplification in one reaction whereas two-step real-time RT-PCR performs in two separate reactions. Quantitation of gene expression can use either absolute or relative quantitation. The absolute quantitation directly measures the amount of target mRNA

and expresses as copy number or ng/μl (104). It requires an external standard which is a known concentration mRNA that uses for unknown sample to compare with, based on Cp values. An external standard can be either purified PCR products or plasmid DNA. A standard curve should be made by serial dilutions of standard DNA to cover the range of unknown sample concentration. The relative quantitation needs an endogenous reference gene which target gene expression should be normalized with (105). Endogenous reference gene is a gene that persistently expressesin cells and will not change in any pathological conditions (116). The data is expressed as a fold-change or a fold-difference of expression (levels target/reference ratio). The sample values should be normalized with a calibrator sample within run and between run (105). The real time PCR technique is effective method use for study gene expression. The advantages of real time PCR are a large dynamic range, high throughputand no process of agarose gel electrophoresis. However, this method is expensive, very high sensitive and difficult for experimental design(20, 117).

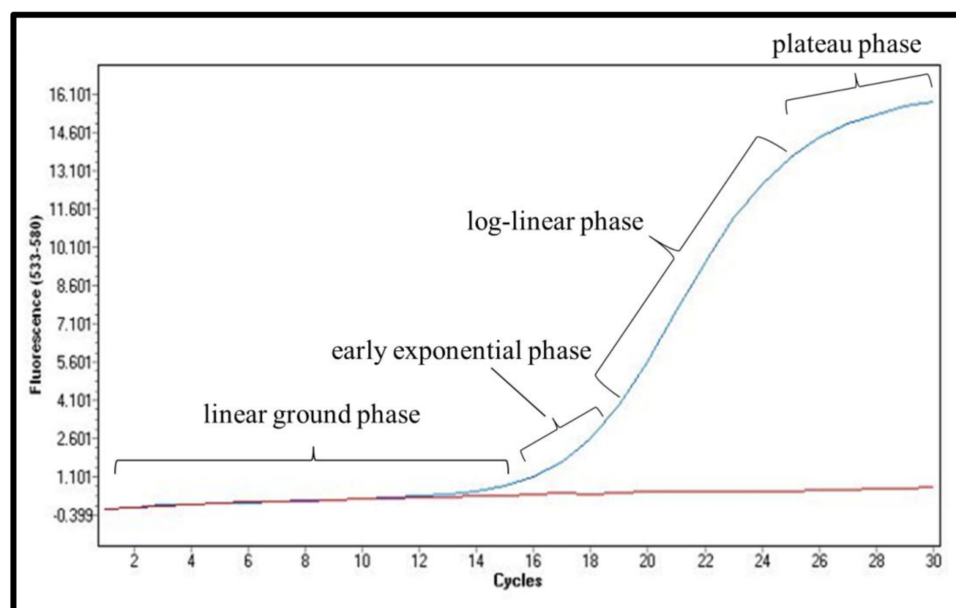


Figure 2.3 Phases of the PCR amplification curve.

CHAPTER III
MATERIALS AND METHODS

3.1 Biological samples

A 3-ml EDTA-whole blood sample was collected from each subject after given an informed consent. The sample size was calculated using the formula:

$$N = \frac{Z^2_{\alpha/2} P(1-P)}{d^2}$$

When $Z_{\alpha/2}$ = Confidence interval, P = Incident and d= Precision.

From the calculation, the numbers for each sample group used in this study are as follows.

Normal	10	cases
Heterozygous β-thalassemia	20	cases
Homozygous β-thalassemia disease	1	cases
β-thalassemia with Hb E disease	29	cases
Total	60	cases

Each sample group was discriminated by using hematological parameters and Hb typing criteria as shown in the Table 3.1.

Table 3.1 Hematological parameters and Hb typing criteria for discrimination of β-thalassemia samples

Sample group	Hematological parameters		Hb typing		
	MCV (fL)	MCH (pg)	Hb type	%HbF	%Hb A_2/E
Normal	>80	>27	A_2A	0-1.5	2 to ≤3.5
Heterozygous β- thalassemia	<80	<27	A_2A (high A_2)	1-5	3.5 to <10
Homozygous β-thalassemia disease	<80	<27	A_2F A_2FA	20-90	2-9
β-thalassemia with Hb E disease	<80	<27	EF EFA	15-40 10-30	40-60 20-50

In addition, the severities of 29 samples of β-thalassemia with Hb E disease were classified by the following criteria including Hb at steady state (g/dL), age at onset (years), age at 1st transfusion (year), transfusion requirement, spleen size(cm) and splenectomy. Sum of scores was used with some modifications from the previous report for dividing into severe to moderate (scores = 4-10) and mild (scores = 0-3.5) groups (118).

Hematological parameters were determined using automated analyzer, Sysmex XS-1000i (Kobe, Japan). Hb typing analysis was performed by the Variant™ II HPLC (Bio-Rad, France) and/or the Capillarys™ 2 CE (Sebia, Lisses, France). DNA analysis for α- and β-thalassemia genotyping was analyzed by conventional multiplex PCR.

All samples were collected from patients visited at Ramathibodi Hospital. Samples were processed and analyzed at Blood Disease Diagnostic Center, Division of Hematology, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital. The study protocol was approved by the Committee on Human Rights Related to Research Involving Human subjects of Ramathibodi Hospital (MURA2011/381).

3.2 Materials

3.2.1 Equipments

- Automated DNA Purification, Maxwell® 16 MDx Instrument (Cat.# AS3000-SC Promega, USA)
- Absorption spectrophotometer, WPA Biowave (Biochrom Ltd., UK)
- Veriti 96 well Thermocycler (ABI Applied Biosystems, USA)
- EppendorfMastercycler EP (Eppendorf AG, Germany)
- LightCycler® 480 Real-Time PCR System (Roche,Germany)
- ChemiDocTM XRS System (BIO-RAD ,USA)
- Quantity One software (BIO-RAD ,USA)
- Microwave (Sharp,Japan)
- Automated pipette : P20/100/1000 µl (Eppendorf, Germany)
- Pipette tips (Corning Incorporated)
- Biomedical Freezer, -20 ^{o}C (Sanyo, Japan)
- 4 ^{o}CRefrigerater (Sanyo, Japan)
- Microcentrifuge, Eppendorf Centrifuge 5410 (Eppendorf, Germany)
- Centrifuge, Kubota b5100 (KUBOTA, Japan)
- 12×75 mm. test tubes
- Microcentrifuge tube, Treff 1.5 ml Lab Microtube(Anachem Ltd., UK)
- Microcentrifuge, Eppendorf Centrifuge 5410 (Eppendorf, Germany)

3.2.2Reagent for DNA preparation

- Maxwell® 16 Blood DNA Purification Kits (Promega, USA)

3.2.3Reagent for Conventional PCR

- Deionized/Distilled water
- Taq DNA Polymerase (Promega, USA)
- Magnesium chloride ($MgCl_2$)(Promega, USA)
- TE buffer pH 8.0 (Promega, USA)
- Deoxynucleotide triphosphate, 10 mM each of dATP, dTTP, dCTP, dGTP (Promega, USA)

- 10 µM primers for α and β globin genes (Biodesign, Thailand)
- Agarose gel (Promega, USA)

3.2.4 Reagent for Reticulocyte separation

- Dextran, MW.150,000 (Sigma-Aldrich, USA)
- 0.85% NaCl (Sigma-Aldrich, USA)

3.2.5 Reagent for mRNA extraction

- Trizol®Reagent (Invitrogen, U.S.A.)
- Chloroform (Sigma-Aldrich, USA)
- Isopropyl alcohol (Sigma-Aldrich, USA)
- Ethanol (Sigma-Aldrich, USA)
- DEPC-treated water, (Amresco, USA)
- RNase-free DNase enzyme kit (Promega, USA)

3.2.6 Reagent for qRT-PCR

- 10 µM TaqMan™ probes for α, β, γ, E globin and GAPDH genes (Roche,Germany)
- 10 µM primers for α, β, γ, E globin and GAPDH genes (Roche,Germany)

3.3 Methods

All blood samples were extracted for DNA in order to identify α- and β-thalassemia genotypes by conventional PCR. Reticulocytes were isolated from the packed red cells using 3% dextran solution and total RNA was extracted by using Trizol® Reagent. The RNA samples were used for gene expression study. The expression of α, β, γ and β^E globin genes were determined by quantitative real-time PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene.

3.3.1 DNA preparation

EDTA whole blood sample (300μl) was used for DNA extraction using Maxwell® 16 Blood DNA Purification Kits (Promega, USA). The principle of extraction is based on cell lysis and binding of magnetized silica particles to nucleic acid which are finally eluted and resolved in buffer. The quantity of DNA was evaluated using spectrophotometer (WPA Biowave DNA-Isogen life science) by measuring at 260 and 280 nm. The OD 260/280 ratio should be in the range of 1.8 to 2 to yield the good quality DNA.

3.3.2 PCR for α- and β-thalassemia genotyping

For detection of thalassemia deletions, the Gap-PCR technique was used. PCR primers specific for each deletion and for each normal allele were used. Common α-thalassemia deletions were determined including $--^{SEA}$, $--^{THAI}$, $--^{FIL}$, $--^{MED}$ and $-\alpha^{20.5}$ deletional α-thal 1, and $-\alpha^{3.7}$ kb and $-\alpha^{4.2}$ kb deletional α-thal 2 as previously described (99, 100). Common non-deletional α-thalassemias, Hb Constant Spring and HbPaksé, were detected by ARMS-PCR technique followed previous reports with some modifications (96, 97). After amplification, PCR products were detected by agarose gel electrophoresis.

The ARMS-PCR technique was used for detection of common β-thalassemia mutations. The target DNA is amplified concurrently by sequence-specific primers for mutation and for normal alleles. PCR primers specific for twenty one of β-thalassemia mutations including codon 41/42, codon 17, nt-28, IVSI-5, Hb E, IVSII-654, codon 19, codon 71/72, IVSI-1(G-T), IVSI-1(G-A), codon 35, codon 27/28, codon 43, nt-86, codon 95, codon 126, codon 26, codon 41, nt-31, codon 8/9, codon 123-125 were followed the routine DNA analysis at Blood Disease Diagnostic Center, Ramathibodi hospital (4).

3.3.3 Reticulocyte separation

Reticulocytes were separated from EDTA whole blood samples by using 3% dextran (MW.150,000) in 0.85% NaCl solution. Each 1.5 ml of blood was mixed with 1.5 ml of dextran solution in the ratio 1:1. The mixture was incubated for 30 minutes at room temperature and packed red blood cells fraction was washed 3 times

with 0.85% NaCl solutionby centrifugation at 1,800 rpm for 5 minutes.The pellet containing reticulocytes was collected for RNA preparation.

3.3.4 Total RNA extraction

Total RNA was extracted from reticulocytes by using TRIzol® Reagent, developed by Chomczynski and Sacchi, according to the manufacturer's recommendations.In details, 1 ml of TRIzol® Reagent and 200 µl of chloroform were added into a 500-µl sample. After mixing and 12,000-g centrifugation for 15 minutes at 4°C, a clear upper aqueous layer containing RNA were transfered to a new tube. A 500 µl of isopropanol was added and incubated at room temperature for 10 minutes and then centrifuged at 12,000 g, 4°C for 10 minutes to precipitate the RNA.About 1 ml of 75% ethanol was added to the RNA pellet, mixed by vortex, and centrifuged at 7,500 g, 4°C for 5 minutes.After discarded the wash,the RNA pellet was air dried for 10 minutes and resuspended in 30 µl of DEPC treated water by 60°C incubation for 10 minutes. The prepared RNA was stored at -30°C until used. The quantity of total RNA was measured at 260 and 280 nm using spectrophotometer (WPA Biowave DNA-Isogen life science). The RNA samples were used in the experiment if having an OD 260/280 ratio in between 1.8 to 2. The contaminating DNA was removed by treating the RNA samples with RNase-free DNase enzyme prior to use in qRT-PCR.

3.3.5 Real-time reverse transcription PCR (qRT-PCR)

Globin genes expression was studied by qRT–PCR technique using the LightCycler® 480 Real-Time PCR System. The α, β, γ and β^{E} globin mRNAs were quantitated in relative to the GAPDH reference mRNA. One step qRT–PCR with Taqman™ probe was done by reverse transcribed mRNA to cDNA using reverse transcriptase and amplified cDNA using Tth DNA polymerase in the same reaction. Taqman™ probe is one of hydrolysis probe labeled the 5′ end with reporter dye and 3′ end with quencher dye. The reporter dye was quenched by the quencher dye when the probe was intact. In the extension step, DNA polymerase with 5´ to 3´exonuclease activity cleaved the probe and released the reporter dye to emit fluorescence. The fluorescence signals were detected based on the Cp value (20). The fluorescence signals

detected were directly proportional to the amount of DNA template thus relative to the amount of mRNA expressed.

The sequences of primers and probes for α, β, γ, β^E globin and GAPDH were referred to GenBank databases as shown in Table 3.2.Taqman™ probes and primers were synthesized from Roche Diagnostics, Mannheim, Germany. Each probe contained a quencher dye, the BBQ(Black Berry Quencher), at the 3' end. Different reporter dyes were labeled at the 5' end of each probe, FAM (6-carboxy-fluorescein) for the α-globin, YAK(Yakima Yellow) for the β-and β^E-globin, LC610 (LightCycler-Red 610) for the γ-globin, and LC670 (LightCycler-Red 670) for the GAPDH.

One step qRT–PCR reactions were optimized using the LightCycler® 480 RNA Master Hydrolysis Probes reagent (Roche, Germany) as shown in Table 3.3. Each reaction was prepared in a 20-µl volume including 40 ng of total RNA, master mix reagent, primers and probes for each gene. The one step qRT–PCR condition used was as follows, 3 min at 63°C for reverse transcription, 30 sec at 95°C for pre-denature cDNA, then 45 cycles of PCR with 15 sec at 95°C, 1 min at 60 °C and 1 sec at 72°C, and finally 10 sec at 40°C for cool down. Each RNA sample was analyzed in triplicate.

The result from qRT–PCR was calculated using relative quantification based on the $2^{-\Delta\Delta Cp}$ method (LightCycler® 480 Software). The calculation for relative quantification was based on the formula:

$$2^{-\Delta\Delta Cp} = \frac{2^{-(Cp\ target - Cp\ reference)\ sample}}{2^{-(Cp\ target - Cp\ reference)\ calibrator}}$$

Three of randomized normal samples were used as calibrator or reference samples. The expression data of α, β, γ and β^E globin genes were normalized according to GAPDH, the endogenous reference gene. The expression data was computed as a fold-change or a fold-difference of target to reference mRNA ratio (105).

Table 3.2 Primers and Taqman™ probes for one-step qRT-PCR assay

Gene	Primer Name	Sequence (5'-3')	Accession Number	Position (5'-3')	Length (bp)	Tm (^{O}C)
α	HBA1:F	GGAGGCCCTGGAGAGGAT	NM_000517	118-135	18	57.6
	HBA1:R	CGTGGCTCAGGTCGAAGTG		191-173	19	59.6
	HBA1:P	FAM-TGTCCTTCCCCACCACCAAGACCT-BBQ		141-164	24	67.5
β	HBB:F	CTGACACAACTGTGTTCACTAGC	NM_000518	11-33	23	54.4
	HBB:R	GGTAGACCACCAGCAGCCT		158-141	18	54.0
	HBB:P	YAK-CCCACAGGGCAGTAACGGCAGACT-BBQ		99-76	24	68.8
γ	HBG1:F	GACTTCCTTGGGAGATGCCAC	NM_000559	260-280	21	59.1
	HBG1:R	ATTTCCCAGGAGCTTGAAGTTCT		380-358	23	58.5
	HBG1:P	LC610-AGCTTGTCACAGTGCAGTTCACTCAGC-BBQ		343-317	27	64.3
β^{E}	HBB:F	CTGACACAACTGTGTTCACTAGC	NM_000518	11-33	23	54.4
	HBE:R	AGCCTGCCCAGGGCCTT		145-129	17	58.7
	HBB:P	YAK-CCCACAGGGCAGTAACGGCAGACT-BBQ		99-76	24	68.8
GAPDH	GAPDH:F	GAAGGTGAAGGTCGGAGTC	J02642	7-29	19	53.6
	GAPDH:R	GAAGATGGTGATGGGATTTC		232-213	20	51.4
	GAPDH:P	LC670-CAAGCTTCCCGTTCTCAGCCT-BBQ		204-184	21	62.9

*F: forward primer, R: reverse primer, P: probe

BBQ: Black Berry Quencher, FAM: 6-Carboxy-Fluorescein, YAK: Yakima Yellow, LC610: Light Cycler-Red 610 and LC670: Light Cycler-Red 670

Table 3.3 Optimal conditions for one-step qRT–PCR reactions

α		**β**		**γ**		**$β^E$**		**GAPDH**	
	1 rx (µl)		1 rx (µl)		1 rx (µl)		1 rx (µl)		1 rx (µl)
HBA1:F (10 µM)	0.33	HBB:F (10 µM)	0.35	HBG1:F (10 µM)	0.30	HBB:F (10 µM)	0.35	GAPDH:F (10 µM)	0.38
HBA1:R (10 µM)	0.33	HBB:R (10 µM)	0.35	HBG1:R (10 µM)	0.30	HBE:R (10 µM)	0.35	GAPDH:R (10 µM)	0.38
HBA1:P (10 µM)	0.20	HBB:P (10 µM)	0.20	HBG1:P (10 µM)	0.20	HBB:P (10 µM)	0.20	GAPDH:P (10 µM)	0.25
2.7X Enzyme mix	7.4	2.7X Enzyme mix	7.4	2.7X Enzyme mix	7.4	2.7X Enzyme mix	7.4	2.7X Enzyme mix	7.4
Activator ($Mn(OAc)_2$)	1.3	Activator ($Mn(OAc)_2$)	1.3	Activator ($Mn(OAc)_2$)	1.3	Activator ($Mn(OAc)_2$)	1.3	Activator ($Mn(OAc)_2$)	1.3
20X Enhancer	1.0	20X Enhancer	1.0	20X Enhancer	1.0	20X Enhancer	1.0	20X Enhancer	1.0
DW	7.44	DW	7.40	DW	7.50	DW	7.40	DW	7.29
Total RNA (20 ng/µl)	2.0	Total RNA (20 ng/µl)	2.0	Total RNA (20 ng/µl)	2.0	Total RNA (20 ng/µl)	2.0	Total RNA (20 ng/µl)	2.0
Total	**20**	**Total**	**20**	**Total**	**20**	**Total**	**20**	**Total**	**20**

Conditions: 3 min at 63°C for reverse transcription, 30 sec at 95°C for pre-denature cDNA, then 45 cycles of PCR with 15 sec at 95°C, 1 min at 60 °C and 1 sec at 72°C, and finally 10 sec at 40°C for cool down.

3.3.6 Statistical analysis

The Kolmogorov-Smirnov test was used to test for normal distribution of the samples. The Kruskal-Wallis test was used to compare the differences of the samples in more than two groups. Then, the Mann-Whitney U test was used to compare the differences among groups of the samples. The analysis of correlation among variables used Spearman's correlation analysis. The statistical significances were analyzed at p-value < 0.05.

CHAPTER IV
RESULTS

4.1 Hematological analysis for β-thalassemia

Hematological parametersand Hb typing were analyzedand used for classification of the cases studied into each group of β-thalassemia and normal controls. Hb typing results of A_2A was classified into normal group, A_2A with high HbA_2 was heterozygous β-thalassemia, EF or EFA was β-thalassemia with HbE disease and A_2F or A_2FA was homozygous β-thalassemia disease. The hematological parameters analysis were hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red blood cell distribution width (RDW). Ages of the patients studied were ranged from 2 to 75 years. The median (min-max) values of hematological parameters and Hb typing results from 60 β-thalassemia samples were summarized in Table 4.1.

Hb, Hct, M CV, MCH, MCHC and RDW values in four groups of samples were significant difference between each sample groups (p value <0.01 for each parameter). In normal control group, red blood cell parameters were in the normal values. These parameters were abnormal in β-thalassemia groups that represent the microcytic hypochromic anemia, mild degree in heterozygous β-thalassemia, and predominant in β-thalassemia with HbE disease and homozygous β-thalassemia. However, in some cases of β-thalassemia diseases, normal MCV were found but there were substantially high RDW thus anisocytosis should be occurred and could be confirmed by blood smear examination.

Table 4.1 Hematological parameters and Hb typing results of β-thalassemia samples.

Type (No.)	Hb (g/dl)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	RDW (%)	Reticulocyte (%)	Hb typing
Homozygous β-thalassemia (1)	7.7	22.1	84.4	29.4	34.8	25.5	11.0	A_2F
β-thalassemia with HbE (29)	8.2 (3.3-11.7)	25.2 (11.2-34.9)	62.1 (48.4-87.5)	19.5 (16.0-25.8)	31.7 (26.8-35.1)	27.6 (18.7-35.8)	4.8 (0.5-25.0)	EF or EFA
heterozygous β-thalassemia (20)	11.0 (7.6-13.6)	35.0 (22.0-42.0)	64.0 (56.0-77.0)	20.2 (18.8-26.1)	32.0 (30.5-33.9)	15.2 (12.9-17.6)	1.1 (0.2-5.0)	A_2A (high A2)
Normal (10)	15.0 (12.0-17.0)	45.2 (35.0-48.0)	86.6 (83.4-91.9)	30.0 (27.2-31.2)	34.1 (32.2-35.1)	13.0 (12.1-13.2)	0.4 (0.1-1.0)	A_2A

Patients with β-thalassemia with HbE disease have a variety of clinical severity. The clinical criteria for indicating severity in β-thalassemia with Hb E disease were Hb at steady state (g/dL), age at onset (years), age at first transfusion (year), transfusion requirement, spleen size (cm) and splenectomy. Sum of the scores was used for dividing the cases into moderate to severe (scores = 4-10) and mild (scores = 0-3.5) groups. Only 17 from 29 cases (59%) of β-thalassemia with Hb E disease that had enough data were classified, 6 cases were mild and 11 cases were moderate-severe (Table 4.2).

Table 4.2 Clinical severities of 17 cases of β-thalassemia with Hb E disease

No.	Hb Typing	Genotype	Hb at steady state			Age at onset			Age at 1st transfusion			Transfusion			Spleen size,cm			Splenectomy		Results
			>7.5 (0)	6.0-7.5 (1)	<6 (2)	>10 (0)	2-10 (0.5)	<2 (1)	>10 (0)	4-10 (1)	<4 (2)	rare (0)	occ (1)	reg (2)	<3 (0)	3-10 (1)	>10 (2)	NO (0)	Yes (2)	
1	EF	CD41/42/E			2	0			0					2					2	moderate-severe
2	EF	CD 41/42/E		1			0.5			1				2		1		0		moderate-severe
3	EF	CD17/E			2		0.5		0			0							2	moderate-severe
4	EF	CD17/E		1		0			0					2					2	moderate-severe
5	EF	CD17/E	0			0			0			0				1		0		mild
6	EF	CD17/E			2								1			1		0		moderate-severe
7	EF	CD17/E			2			1			2			2		1		0		moderate-severe
8	EF	CD17/E	0						0			0					2	0		mild
9	EF	CD17/E	0										1			1		0		moderate-severe
10	EF	IVSII-654/E			2							0							2	moderate-severe
11	EF	IVSII-654/E			2		0.5		0					2		1		0		moderate-severe
12	EF	IVSII-654/E	0									0				1		0		mild
13	EFA	P28/E	0									0						0		mild
14	EFA	P28/E	0									0						0		mild
15	EFA	P28/E	0									0						0		mild
16	EF	IVSI-1/E	0											2				0		moderate-severe
17	EFA	IVSI-5 /E	0					1			2			2		1		0		moderate-severe

*occ=occasional, reg=regular

4.2 PCR for α- and β-thalassemiagenotyping

DNA analysis by conventional multiplex PCRwas done for confirmatory and genotypic detection of α- and β-thalassemia. The multiplex Gap-PCR technique was used for detection of common α-thalassemia deletions included SEA, THAI, FIL, MED, 20.5 kb, 3.7 kb, and 4.2 kb deletions. The ARMS-PCR technique was used for detection ofHb Constant Spring, HbPaksé, and twenty one of β-thalassemia mutations including codon 41/42, codon 17, nt-28, IVSI-5, Hb E, IVSII-654, codon 19, codon 71/72, IVSI-1(G-T), IVSI-1(G-A), codon 35, codon 27/28, codon 43, nt-86, codon 95, codon 126, codon 26, codon 41, nt-31, codon 8/9 and codon 123-125. The PCR results for α- and β-thalassemia in 60 samples were related with Hb typing in all samples as shown in Table 4.3.In 29 samples of β-thalassemia with HbE disease, the β-globin mutations found were codon 41/42, codon 17, IVSI-1, IVSII-654, IVSI-5 and nt-28. Three cases of those with codon 17 and one with IVSI-1 mutations were found coinheritance with α-thalassemia ($-\alpha^{3.7}/\alpha\alpha$). Similar mutations were found in 20 samples of heterozygous β-thalassemia with one more additional mutation, codon 27/28.IVSI-1 and codon90 mutations were found in one case of homozygous β-thalassemia disease and had confirmed by DNA sequencing. No α- and β-thalassemia mutations were found in normal samples.

Table 4.3 PCR for α- and β-thalassemia genotyping

Sample group	Hb typing	PCR		Genotype		Number of sample
		α	β	α	β	
Homozygous β	A_2F	normal	IVSI-1/codon90	αα/αα	$\beta^{IVSI-1}/\beta^{codon90}$	1
β/HbE	EF	normal	codon 41/42/E	αα/αα	$\beta^{codon41/42}/\beta^{E}$	4
		normal	codon 17/E	αα/αα	$\beta^{codon17}/\beta^{E}$	10
		α-thal2, 3.7 kb del	codon 17/E	$-\alpha^{3.7}/\alpha\alpha$	$\beta^{codon17}/\beta^{E}$	3
		normal	IVSI-1/E	αα/αα	β^{IVSI-1}/β^{E}	1
		α-thal2, 3.7 kb del	IVSI-1/E	$-\alpha^{3.7}/\alpha\alpha$	β^{IVSI-1}/β^{E}	1
		normal	IVSII-654	αα/αα	$\beta^{IVSII-654}/\beta^{E}$	6
	EFA	normal	IVSI-5 /E	αα/αα	β^{IVSI-5}/β^{E}	1
		normal	-28/E	αα/αα	β^{-28}/β^{E}	3
β trait	A_2A high A_2	normal	codon 41/42	αα/αα	$\beta^{codon41/42}/\beta^{A}$	8
			codon 17	αα/αα	$\beta^{codon17}/\beta^{A}$	2
			codon 27/28	αα/αα	$\beta^{codon27/28}/\beta^{A}$	1
			IVSI-1	αα/αα	β^{IVSI-1}/β^{A}	1
			IVSII-654	αα/αα	$\beta^{IVSII-654}/\beta^{A}$	4
			IVSI-5	αα/αα	β^{IVSI-5}/β^{A}	1
			-28	αα/αα	β^{-28}/β^{A}	3
Normal	A_2A	normal	normal	αα/αα	β^{A}/β^{A}	10
Total						60

4.3 Optimization for reticulocyte separation

Reticulocytes were separated from EDTA whole blood samples by adding 3% dextran (MW.150,000) in 0.85% NaCl solution.Dextran (MW.150,000) was used for removing white blood cells and pack red blood cells fraction containing reticulocytes was retained in the bottom of tube. We studied the relationship of incubation time and the minimum amount of remaining white blood cells in pooled of 4 normal samples. The results are shown inTable 4.4.

Table 4.4 The relationship of incubation time and the minimum amount of remaining white blood cells in normal samples

Incubation time (min)	Before WBC separation (10^3/µl)	AfterWBC separation (10^3/µl)
30	9.56	1.6
45	9.56	2.3
60	9.56	2.1

We summarized that 30-minute incubation with dextran solution was appropriated for reticulocytes preparation and yielded less white blood cells contamination.

In normal control and heterozygous β-thalassemia samples, the median (min-max) of white blood cell counts before and after reticulocytes separation were reduced from 6.56 (3.33-18.68) x10^3/µl to 1.30 (0.21-4.50) x10^3/µl which was about 79.5% (30%-96%) decreasing of white blood cells contamination. On the other hand, those in β-thalassemia with HbE disease and homozygous β-thalassemia disease were reduced from 8.85 (3.40-98.24) x10^3/µl to 3.25 (0.80-164.75) x10^3/µl. The high white blood cell counts remaining in the reticulocytes fractions in β-thalassemia with HbE disease and homozygous β-thalassemia disease were interfered from nucleated red blood cells.

The reticulocyte fractions were stained with 0.5% new methylene blue and examined under light microscope. Reticulocyte count in normal control and heterozygous β-thalassemia were 0.1%-5% whereas in homozygous β-thalassemia disease and β-thalassemia with HbE disease were 0.5%-25.0%.

4.4 Total RNA extraction and RNase–free DNase enzyme treatment

Total RNA was extracted from reticulocytes by using TRIzol® Reagent as described in the methods. After RNA extraction process, the RNA samples should be treated with RNase-Free DNase enzyme to remove contaminating DNA. The present of contaminating DNA was tested by amplification using specific primers for β-actin

gene. The treated RNA samples showed no PCR product indicating there was no contaminating DNA compared with untreated samples that showed a specific band of 751 bp (Figure 4.1).

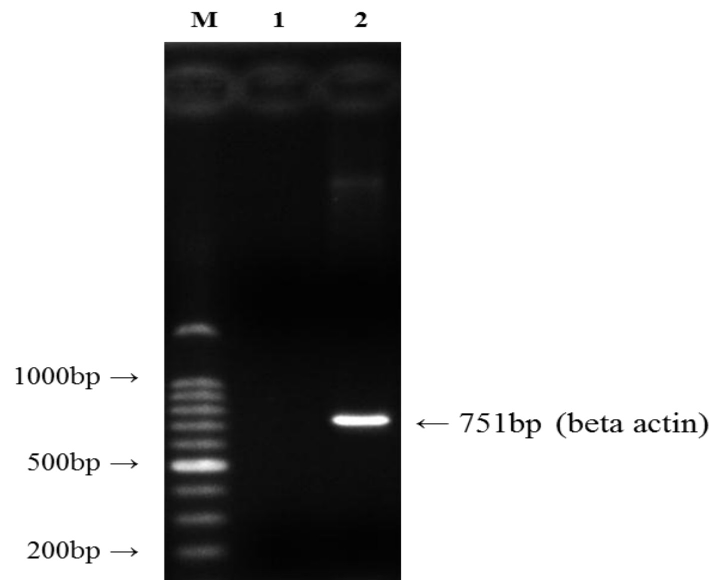


Figure 4.1 A 2% agarose gel electrophoresis of amplified PCR products for contaminating DNA testing. Lane 1 is RNA sample treated with RNase-Free DNase enzyme revealed no contaminating DNA. Lane2 is untreated RNA sample shows a specific band of β-actin gene at 751 bp.M is 1-kb ladders DNA markers (Promega, USA).

4.5 RNA analysis by real-time reverse transcription PCR (qRT-PCR)

4.5.1 Standard Curve

One step qRT–PCR for α, β, γ and β^E globin genes were developed by using GAPDH as a reference gene.The expression conditions should be appropriate for the following factors; thermal cycle condition, total RNA concentration, primers and probes concentrations, enzyme mix concentration and reaction mix concentration. The standard curve was used to determine the optimal conditions of qRT–PCR.Ten-fold serial dilutions of known concentration of specific purified PCR products were

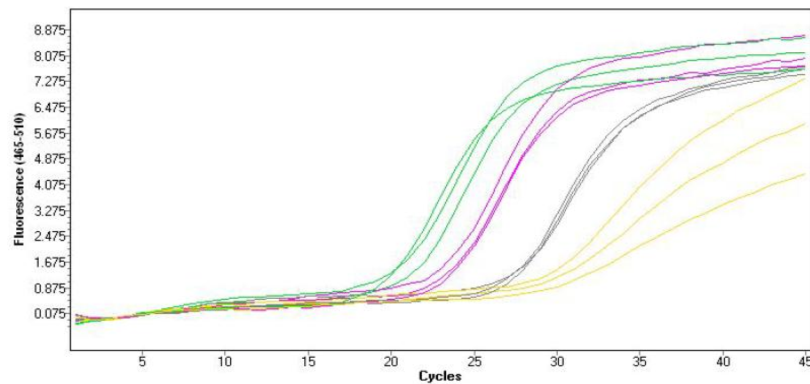
used for standard curve setting.Efficiency value of qRT–PCR was calculated from the slope of standard curve by the formula: $E = 10^{-1/slope}$ and should be in the range of 1.8-2.2 (90-110% efficiency).The best (100%) efficiency should have the value of 2 with an acceptable error of <0.2. The optimized efficiency values of each standard curve of α, β, γ, β^E and GAPDH genes were shown inTable 4.5.

Table 4.5 Optimized efficiency values of the standard curve of α, β, γ, β^E and GAPDH genes

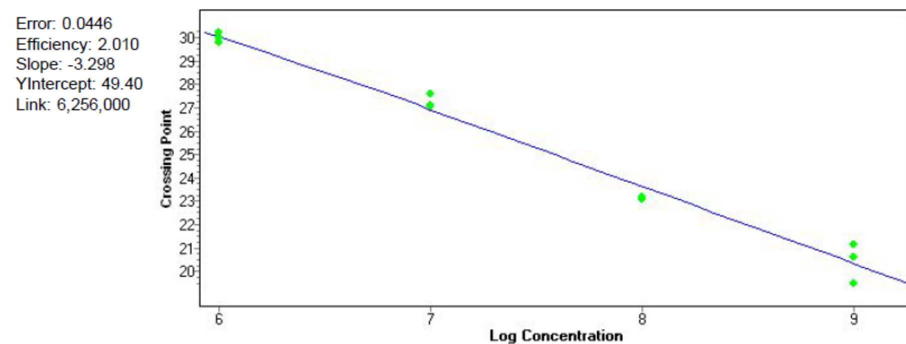
Gene	Slope	Efficiency(1.8-2.2)	Error (<0.2)
Α	-3.298	2.010	0.0446
Β	-3.736	1.852	0.0756
Γ	-3.686	1.868	0.1130
β^E	-3.541	1.916	0.0215
GAPDH	-3.919	1.800	0.0121

Since the standard curve of α, β, γ, β^E and GAPDH genes were in the acceptable ranges, these optimal conditions of qRT-PCR were used for samples analysis. The results from qRT-PCR were expressed into amplification curve and linear curve. Amplification curve was plotted between amplification cycles (X-axis) and fluorescence intensities (Y-axis), each sample was analyzed in triplicates. Linear curve was plotted between logarithm of mRNA concentrations (X-axis) and crossing point values (Y-axis). For the standard curve setting, slope, efficiency, and error values indicated whether these were optimal conditions for qRT-PCR. A 2% agarose gel electrophoresis was used for confirmation of specific amplified PCR products. The amplification curve, linear standard curve and agarose gel electrophoresis from standard curve setting for the α, β, γ, β^E and GAPDH genes were shown in Figure 4.2-4.6.

(A) Amplification curve of α globin genes.



(B) Linear standard curve of α globin genes.



(C) Specific amplified PCR products of α globin genes.

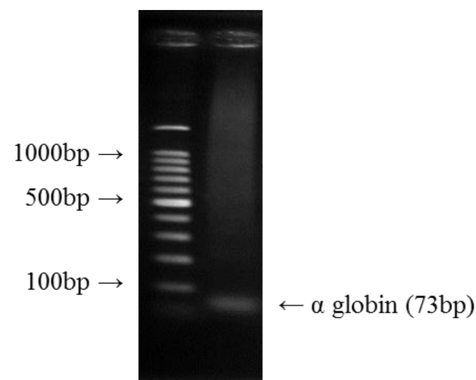
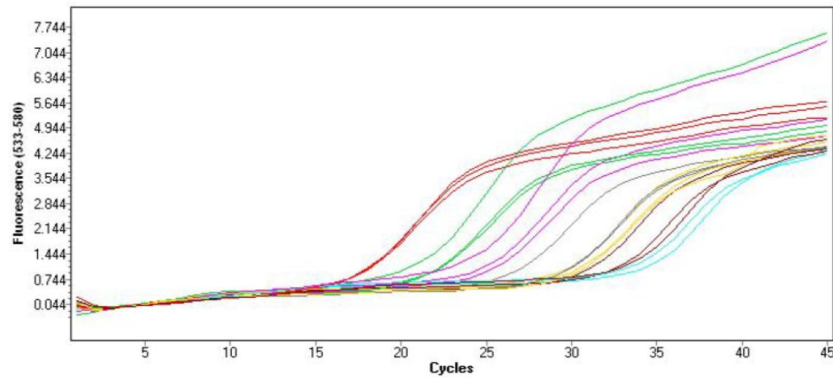
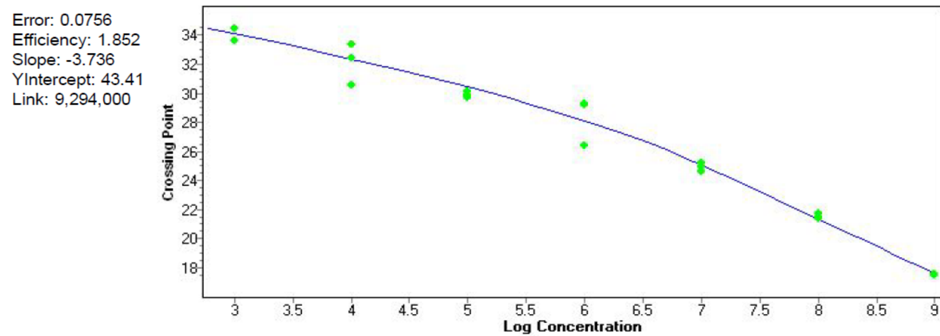


Figure 4.2 Standard curve setting for the α globin genes by qRT-PCR. (A) is amplification curve, (B) is linear standard curve and (C) is agarose gel electrophoresis of amplified PCR products.Theα globin genes were specific detected at fluorescence intensity of 465-410 nm and the standard curve was in the acceptable range. The amplification yield a specific product of 73 bp in length.

(A) Amplification curve of β globin genes.



(B) Linear standard curve of β globin genes.



(C) Specific amplified PCR products of β globin genes.

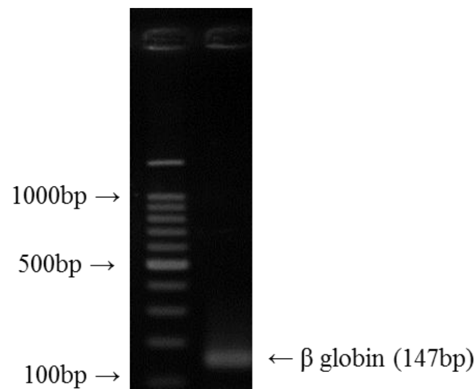
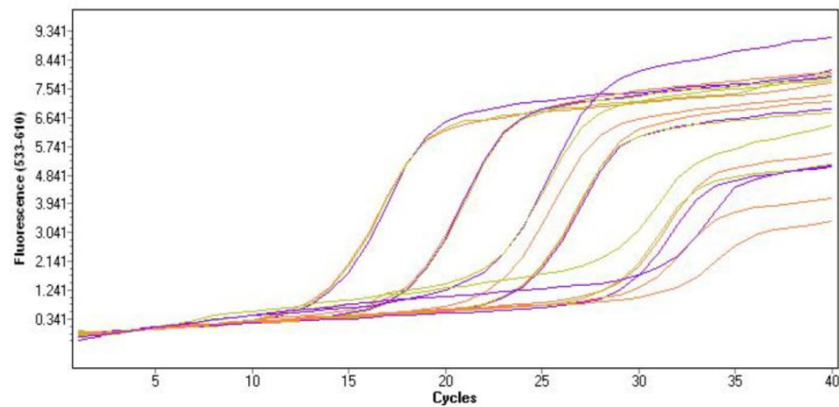
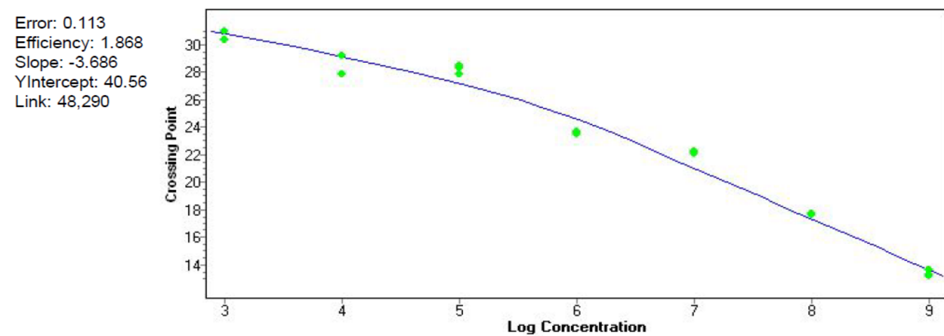


Figure 4.3 Standard curve setting for the β globin genes by qRT-PCR. (A) is amplification curve, (B) is linear standard curve and (C) is agarose gel electrophoresis of amplified PCR products.Theβ globin genes were specific detected at fluorescence intensity of 533-580 nm and the standard curve was in the acceptable range. The amplification yield a specific product of 147 bp in length.

(A) Amplification curve of γ globin genes.



(B) Linear standard curve of γ globin genes.



(C) Specific amplified PCR products of γ globin genes.

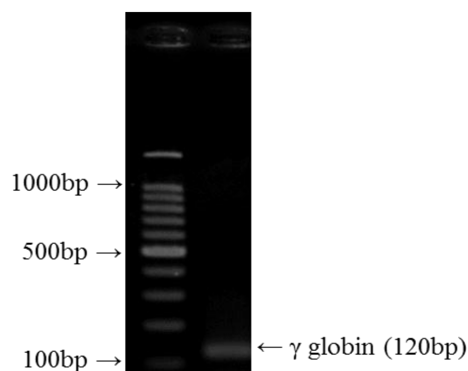
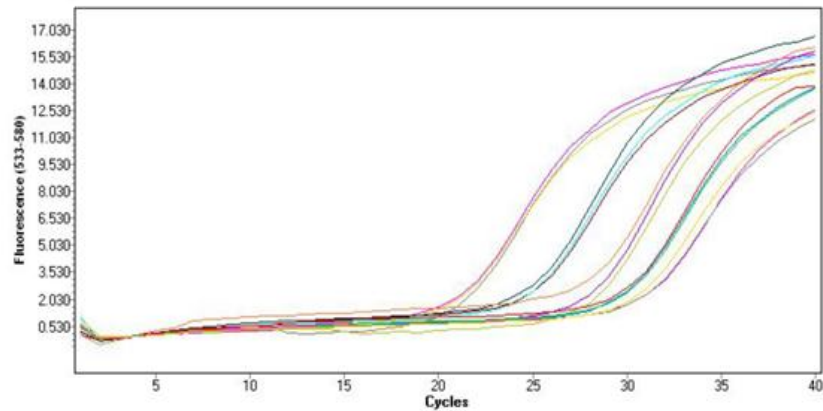
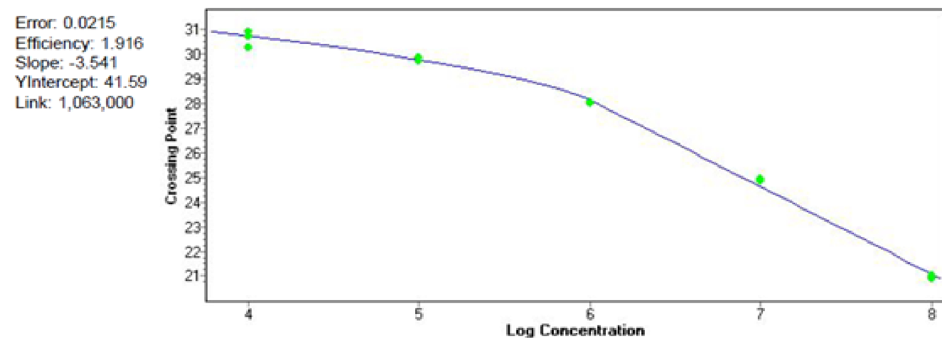


Figure 4.4 Standard curve setting for the γ globin genes by qRT-PCR. (A) is amplification curve, (B) is linear standard curve and (C) is agarose gel electrophoresis of amplified PCR products.Theγ globin genes were specific detected at fluorescence intensity of 533-610 nm and the standard curve was in the acceptable range. The amplification yield a specific product of 120 bp in length.

(A) Amplification curve of β^E globin genes.



(B) Linear standard curve of β^E globin genes.



(C) Specific amplified PCR products of β^E globin genes.

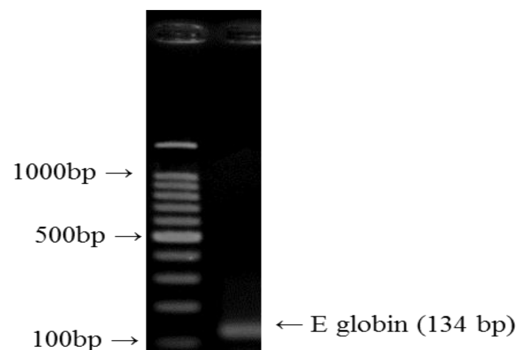
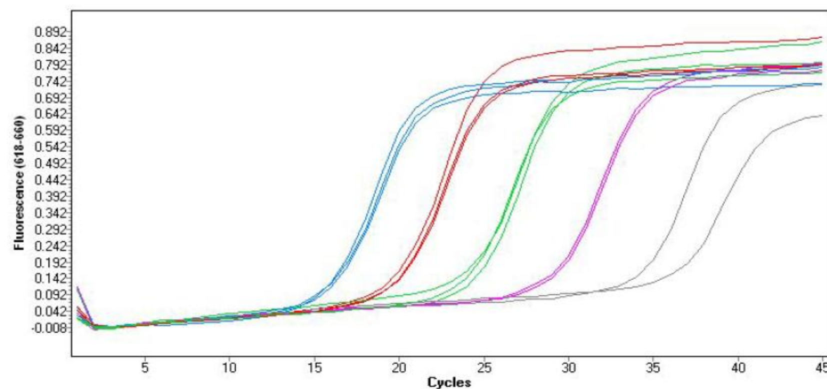
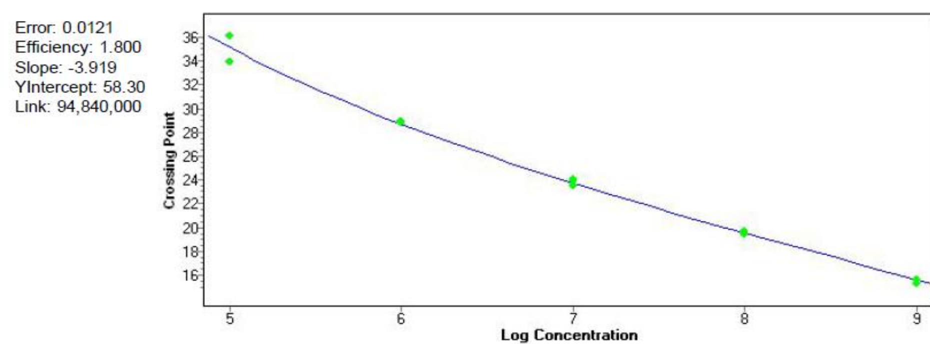


Figure 4.5 Standard curve setting for the β^E globin genes by qRT-PCR. (A) is amplification curve, (B) is linear standard curve and (C) is agarose gel electrophoresis of amplified PCR products.Theβ^E globin genes were specific detected at fluorescence intensity of 533-580 nm and the standard curve was in the acceptable range. The amplification yield a specific product of 134 bp in length.

(A) Amplification curve of GAPDH genes.



(B) Linear standard curve of GAPDH genes.



(C) Specific amplified PCR products of GAPDH genes.

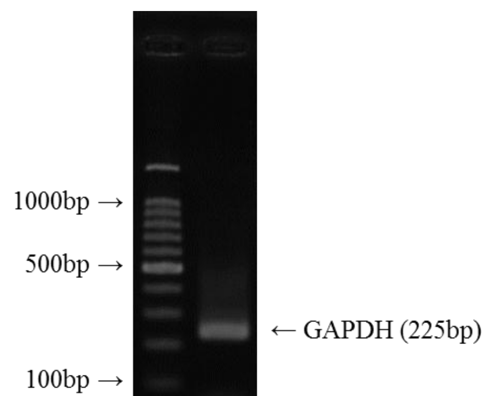


Figure 4.6 Standard curve setting for the GAPDH globin genes by qRT-PCR. (A) is amplification curve, (B) is linear standard curve and (C) is agarose gel electrophoresis of amplified PCR products.TheGAPDH globin genes were specific detected at fluorescence intensity of 618-660 nm and the standard curve was in the acceptable range. The amplification yield a specific product of 225 bp in length.

4.5.2 Globin genes expressions

The relative quantification was used for gene expression determination.The globin genes expressions were done in triplicates and the amounts were normalized with the reference (GAPDH) gene.Calibrator samples used were three normal samples. The expression levels of target/reference ratio of each sample were divided by the expression ratio of the calibrator. One sample was excluded from β-thalassemia with HbE group due to high RDW suspected for blood transfusion.

The median (min-max) of α/β, β/GAPDH, γ/GAPDH, %HbF and absolute HbF in 49 β- thalassemia and 10 normal sampleswere shown in Table 4.6**.** The β/GAPDH mRNA ratio in normal controls was 33.2 (20.6-210.0) $x10^{-2}$. No significant difference of this ratio was found between heterozygous β- thalassemia and β-thalassemia with HbE disease which were 20.0 (0.3-1110.0) $x10^{-2}$ and 20.3 (0.8-271.8) $x10^{-2}$, respectively. The β/GAPDH mRNA ratio in heterozygous β- thalassemia and β-thalassemia with HbE showed decreasing trends compared with normal controls but not statistical significance. In 3 groups of β- thalassemia with HbE disease including β^0-, β^+(severe)- and β^+-thalassemia with HbE disease, no significant difference of the β-globin gene expression was found. The lowest β/GAPDH mRNA ratio was found in homozygous β-thalassemia disease. Box plot of the β/GAPDH mRNA ratio for each group except homozygous β-thalassemia was shown in Figure 4.7.The box represents interquartile range (IQR) with the median at the central line. The upper and lower lines extended from the box are maximum and minimum values. The asterisks are the extreme values.

The median (min-max) of γ/GAPDH mRNA ratio in normal controls and heterozygous β-thalassemia were 0.3 (0.2-2.9) and 3.8 ($1.0x10^{-2}$-82.0), respectively. This ratio in heterozygous β-thalassemia showed no significant difference when compared with normal controls. The γ/GAPDH mRNA ratio in β-thalassemia with HbE disease showed significantly increased (66.3 (3.2-404.6)) when compared with heterozygous β-thalassemia ($p <0.01$) and normal controls ($p <0.01$). No significant difference was found between β^0-, β^+(severe)- and β^+-thalassemia with HbE disease. The highest γ-globin gene expression was found in homozygous β-thalassemia disease. Moreover, the increases of γ/GAPDH mRNA ratio was related to the decreases of β/GAPDH mRNA ratio in β-thalassemia with Hb E disease ($p<0.01$,

r=0.747). Box plot of the γ/GAPDH mRNA ratio for each group except homozygous β-thalassemia was shown in Figure 4.8.

Absolute HbF and %HbF were significantly different between homozygous β-thalassemia disease, β-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls ($p<0.01$). Thesignificant difference of %HbF was also found betweenβ^0-, β^+(severe) - and β^+-thalassemia with Hb E disease ($p<0.01$).However, absolute HbF was significant difference ($p<0.01$) between β^+(severe)- and β^+-thalassemia with Hb E disease but not between β^0- and β^+(severe) -thalassemia with Hb E disease.Absolute HbF was related to %HbF($p <0.01$, $r =0.984$). Box plot of absolute HbF for each group except homozygous β-thalassemia was shown in Figure 4.9. Interestingly, the γ/GAPDH mRNA ratio was related to %HbF and absolute HbF ($p <0.01$, $r =0.727$ and $p <0.01$, $r=0.682$, respectively).

The median (min-max) of α/β globin mRNA ratio in normal controls was 1.1 (0.6-1.9). Significant increasing of this ratio were found in heterozygous β-thalassemia (2.1 (1.0-4.6)) and β-thalassemia with Hb E disease (2.3 (0.8-11.2)) when compared with normal controls (p value <0.01). However, no significant difference of α/β ratio was found between heterozygous β-thalassemia and β-thalassemia with Hb E disease. The α/β globin mRNA ratio was significant difference between β^0-, β^+(severe)- and β^+-thalassemia with Hb E disease (p value = 0.025). The highest α/β ratio was found in homozygous β-thalassemia disease. Box plot of the α/β globin mRNA ratio for each group except homozygous β-thalassemia was shown in Figure 4.10.

Table 4.6The α/β, β/GAPDH, γ/GAPDH mRNA ratio, %HbF and absolute HbF in each group of β- thalassemia

Genotype (No.)	α/β ratio	β/GAPDH ($x10^{-2}$)	γ/GAPDH	%HbF	Absolute HbF (g/dl)
1.Homozygous β-thalassemia disease (1)					
$\beta^{IVSI-1}/\beta^{codon90}$ (1)	295.2	4.7 $x10^{-2}$	70.1	97.0	7.5
2.β-thalassemia with Hb E disease (28)					
β^{0}-thalassemia with Hb E disease (19)					
$\beta^{codon41/42}/\beta^{E}$ (4)	4.8 (2.6-11.2)	1.9 (0.8 -64.0)	12.8 (3.2-299.8)	48.9 (34.3-54.1)	3.8 (1.5-5.3)
$\beta^{codon17}/\beta^{E}$ (13)	1.2 (0.8-2.3)	67.9 (1.6-271.8)	99.2 (7.1-404.6)	46.6 (14.9-60.8)	2.8 (1.3-5.8)
β^{IVSI-1}/β^{E} (2)	2.8 (2.3,3.3)	7.3 (3.7,11.0)	16.3 (8.1,24.5)	35.3 (20.4,50.2)	2.7 (1.7,3.8)
Total	*1.6* *(0.8-11.2)*	*44.0* *(0.8-271.8)*	*68.6* *(3.2-404.6)*	*46.6* *(14.9-60.8)*	*2.9* *(1.3-5.8)*
β^{+}(severe)-thalassemia with Hb E disease (6)					
$\beta^{IVSII-654}/\beta^{E}$ (6)	3.6 (2.6-9.2)	10.2 (7.1-21.4)	119.2 (39.5-144.9)	55.7 (48.2-61.1)	4.9 (2.7-5.7)
β^{+}-thalassemia with Hb E disease (3)					
β^{-28}/β^{E} (3)	3.1 (2.3-3.6)	12.0 (9.1-35.1)	8.9 (7.0-27.1)	12.9 (10.8-17.8)	1.2 (1.0-1.5)
Total	***2.3*** ***(0.8-11.2)***	***20.3*** ***(0.8-271.8)***	***66.3*** ***(3.2-404.6)***	***48.7*** ***(10.8-61.1)***	***2.9*** ***(1.0-5.8)***
3.Heterozygous β-thalassemia (20)					
$\beta^{codon41/42}/\beta^{A}$ (8)	2.8 (1.4-4.6)	195.7 (6.6-1110.0)	15.7 (0.1-82.0)	0.4 (0.0-1.2)	0.1 (0.0-0.1)
$\beta^{codon17}/\beta^{A}$ (2)	1.0 (1.0,1.0)	0.3 (0.3,0.3)	1.0 $x10^{-2}$ (1.0, 1.0) $x10^{-2}$	1.1 (1.1,1.1)	0.1 (0.1,0.1)
β^{IVSI-1}/β^{A} (1)	1.2	11.2	0.6	1.0	0.1
$\beta^{codon27/28}/\beta^{A}$ (1)	2.0	145.4	12.2	0.0	0.0
$\beta^{IVSII-654}/\beta^{A}$ (4)	2.1 (1.8-3.4)	15.1 (6.0-63.0)	2.7 (0.2-5.2)	0.2 (0.0-2.1)	0.0 (0.0-0.2)
β^{IVSI-5}/β^{A} (1)	2.4	42.3	3.4	0.8	0.1
β^{-28}/β^{A} (3)	2.5 (1.0-3.9)	24.1 (1.8-57.6)	6.1 (0.2-6.3)	1.2 (0.8-1.5)	0.1 (0.1-0.2)
Total	***2.1*** ***(1.0-4.6)***	***20.0*** ***(0.3-1110.0)***	***3.8*** ***(1.0 $x10^{-2}$-82.0)***	***0.7*** ***(0.0-2.1)***	***0.1*** ***(0.0-0.2)***
4.Normal (10)					
β^{A}/β^{A} (10)	1.1 (0.6-1.9)	33.2 (20.6-210.0)	0.3 (0.2-2.9)	0.0 (0.0-0.0)	0.0 (0.0-0.0)

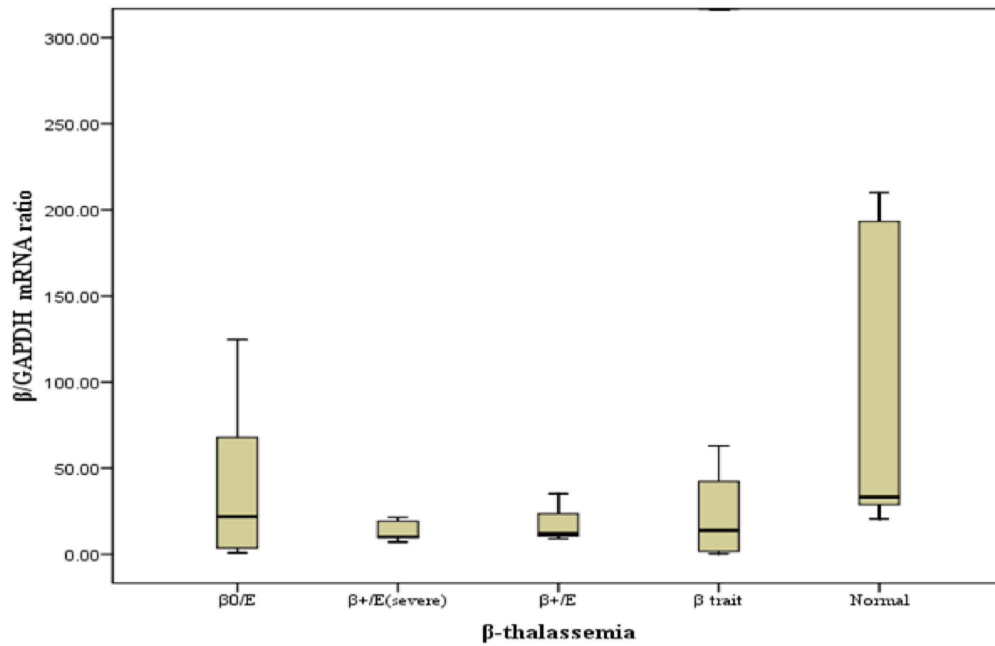


Figure 4.7 Box plot of the β/GAPDH mRNA ratio in β^0-, β^+(severe)- and β^+-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls.

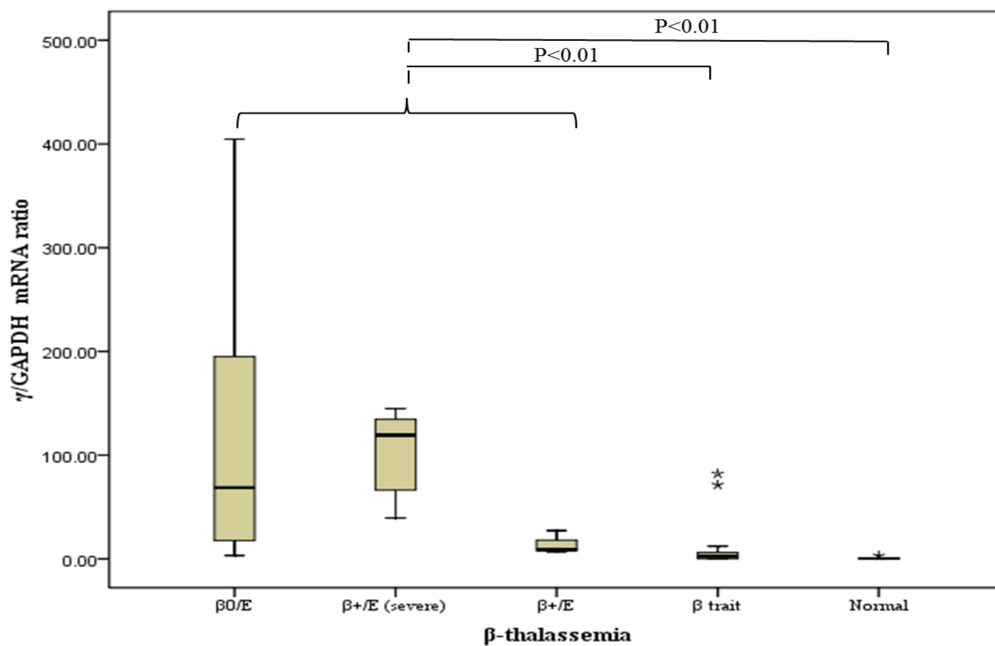


Figure 4.8 Box plot of the γ/GAPDH mRNA ratio in β^0-, β^+(severe)- and β^+-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls.

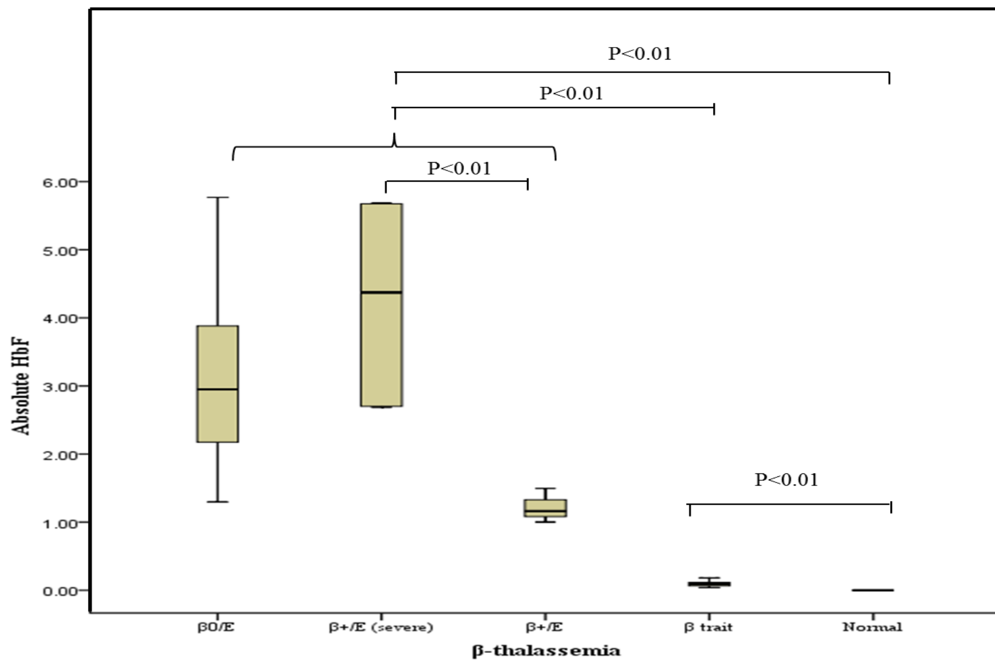


Figure 4.9 Box plot of absolute HbF in β^0-, β^+(severe)- and β^+-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls.

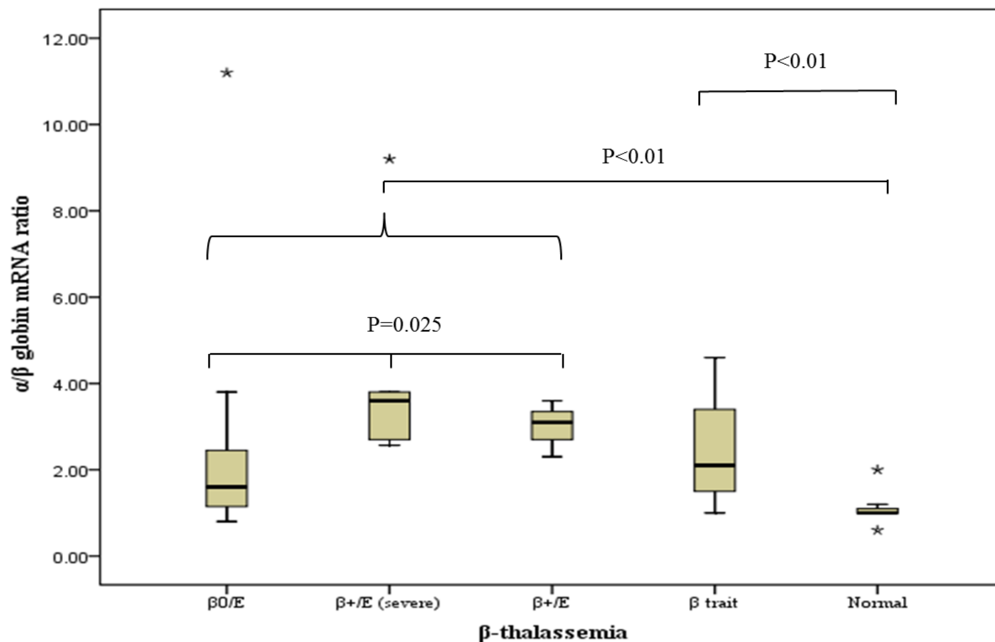


Figure 4.10 Box plot of the α/β globin mRNA ratioin β^0-, β^+(severe)- and β^+-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls.

The clinical severities were classified in 17 cases of β-thalassemia with HbE disease and revealed 6 mild and 11 moderate-severe cases. The median (min-max) of α/β, β/GAPDH, γ/GAPDH, β^E/GAPDH, absolute HbF and absolute HbEof these cases were shown in Table 4.7.The β^E/GAPDH in mild and moderate-severe were 7.3 (2.2-13.6) and 2.1 (0.1-30.8), respectively, and no significant difference was found.The absolute HbE in mild and moderate-severe were 5.1 (3.6-7.3) g/dl and 2.7 (1.7-7.5) g/dl, respectively. Significant higher of absolute HbE was found in the mild group compared with the moderate-severe group ($p<0.01$).The α/β, β/GAPDH, γ/GAPDH and absolute HbF showed no significant difference between mild and moderate-severe groups.

Table 4.7 The α/β, β/GAPDH, γ/GAPDH, β^E/GAPDH, absolute HbF and absolute HbE in mild and moderate-severe phenotypes

Severity	α/β	β/GAPDH ($x10^{-2}$)	γ/GAPDH	β^E/GAPDH ($x10^{-2}$)	Absolute HbF (g/dl)	Absolute HbE (g/dl)
Mild (6)	2.7 (1.3-3.8)	23.56 (9.1-124.7)	32.3 (7.0-119.2)	7.3 (2.2-13.6)	1.4 (1.0-5.7)	5.1 (3.6-7.3)
Moderate-severe (11)	2.3 (0.8-9.2)	20.9 (0.8-210.8)	32.0 (5.4-404.6)	2.1 (0.1-30.8)	2.7 (1.5-4.0)	2.7 (1.7-7.5)

The β^E/GAPDH, γ/GAPDH, α/β, β/GAPDH, %HbE and absolute HbE in each genotype of β-thalassemia with HbE were shown in Table 4.8. The mutations found in β^0 group were codon 41/42, codon 17, and IVSI-1, in β^+(severe) group was IVSII-654, and in β^+ group was nt-28. The highest α/β globin mRNA ratio was found in codon 41/42 while the lowest was in codon 17 (4.8 and 1.2, respectively). The increasing ratios in codon 41/42 was significant difference from that in codon 17 ($p<0.01$). The lowest β^E /GAPDH, γ/GAPDH and β/GAPDH mRNA ratios (median = 0.5, 12.8, and 1.9, respectively) were found in codon 41/42. The higher ratio of β^E /GAPDH, γ/GAPDH andβ/GAPDH (median = 6.0, 99.2, and 67.9, respectively) were found in codon 17 compared with codon 41/42 but no statistical significance. The α/β globin mRNA ratios in IVSI-1and IVSII-654 were lower than codon 41/42 but higher

than codon 17. The β^E /GAPDH, γ/GAPDH and β/GAPDH mRNA ratios in IVSI-1 were higher than codon 41/42 but lower than codon 17. The IVSII-654 mutation showed highest γ/GAPDH mRNA ratios while the β^E /GAPDH and β/GAPDH mRNA ratios were higher than codon 41/42 but lower than codon 17. For nt-28 β^+-thalassemia with HbE disease, the lowest ratio of γ/GAPDH and highest ratio of β^E/GAPDH were found compared with the other groups. Absolute HbE was related to %HbE in β-thalassemia with HbE disease ($p < 0.01$, $r = 0.631$). However, no correlation was found between β^E/GAPDH mRNA ratios and either % HbE or absolute HbE in this group.

Table 4.8 The β^E/GAPDH, γ/GAPDH, α/β,β/GAPDH, HbE and absolute HbE in each genotype of β-thalassemia with Hb E disease

Genotype (No.)	β^E/GAPDH ($x10^{-2}$)	γ/GAPDH	α/β ratio	β/GAPDH ($x10^{-2}$)	% HbE	Absolute HbE (g/dl)
β^0 - and β^+ (severe)- thalassemia with Hb E disease (25)						
$\beta^{codon41/42}/\beta^E$ (4)	0.5 (0.2-30.8)	12.8 (3.2-299.8)	4.8 (2.6-11.2)	1.9 (0.8 -64.0)	50.5 (45.9-65.2)	4.0 (1.7-5.1)
$\beta^{codon17}/\beta^E$ (13)	6.0 (0.1-25.3)	99.2 (7.1-404.6)	1.2 (0.8-2.3)	67.9 (1.6-271.8)	53.4 (38.4-84.1)	3.8 (1.9-7.5)
β^{IVSI-1}/β^E (2)	1.3 (0.6,1.9)	16.3 (8.1,24.5)	2.8 (2.3,3.3)	7.3 (3.7,11.0)	64.4 (49.3-79.6)	5.2 (3.7-6.7)
$\beta^{IVSII-654}/\beta^E$ (6)	2.0 (1.3-3.8)	119.2 (39.5-144.9)	3.6 (2.6-9.2)	10.2 (7.1-21.4)	40.6 (34.1-50.0)	3.4 (2.0-4.1)
β^+-thalassemia with Hb E disease (3)						
β^{-28}/β^E (3)	6.6 (2.2-11.0)	8.9 (7.0-27.1)	3.1 (2.3-3.6)	12.0 (9.1-35.1)	59.6 (58.7-63.2)	5.3 (5.0-5.9)

CHAPTER V
DISCUSSION

In this thesis, we had quantitated globin genes expressions in β-thalassemia by using real-time reverse transcriptase polymerase chain reaction (qRT-PCR) andstudied the relationships between globin genes expressions and disease severities in each β- thalassemia group and genotype. In this study, we had determined the α/β, β/GAPDH, γ/GAPDH and β^E/GAPDH mRNA ratios in β-thalassemia by using one-step qRT-PCR.

5.1Hematological and PCR analysis

Classification of studied cases was done by using hematological parametersand hemoglobin typing data into 4 groups of homozygous β-thalassemia disease, β-thalassemia with HbE disease, heterozygous β-thalassemia and normal controls. Ages of the patients studied were ranged from 2 to 75 years indicating complete Hb switching (23). The lower hemoglobin was suggested to occur in older age patients and affected severity. However, patients older than 40 years showed both high and low hemoglobin level and had no difference between mild and moderate-severe groups. These were replied by other factors such as thalassemia genotypes, degree of imbalance of the globin chains, and Hb F responding. All of the patients older than 40 years with nt-28 β^+-thalassemia with HbE diseasehad hemoglobin >8 g/dl which higher than β^0 thalassemia. This was the genotyping effect. However, the older patients with same age and same genotype, such as IVSII-654 β^+-thalassemia with HbE disease had different hemoglobin level. The lower hemoglobin was found in the case with higher α/β globin mRNA ratio thus representing the effect of globin chains imbalance. Red blood cell parametersfound in β-thalassemia reflected the microcytic hypochromic anemia. The normal MCV but high RDW in some cases of β-thalassemia diseases was due to anisocytosis in which both microcytic red blood cells

and a high number of reticulocytes presented. The genotypes of α- and β-thalassemia were detected by conventional multiplex PCR. The cases studied were found to be common mutations such as codon41/42, codon17, IVSI-1, IVSII-654, and nt-28. Only 4 in 49 cases of β-thalassemia were found co-inherited with α-thalassemia, the 3.7-kb deletion.

5.2 Reticulocyte separation and RNase–free DNase enzyme treatment

The procedure used for reticulocytes separation was acceptable that removed approximately 79.5% of white blood cells. The white blood cells were removed because their contamination affected the GAPDH mRNA which express in every cell types. Thus the globin gene expressions could be affected when comparison to the reference gene. Moreover, RNase-Free DNase enzyme treatment of total RNA was sufficiently cleaned out the contaminated DNA. In some cases of homozygous β-thalassemia and β-thalassemia with HbE diseases, the high numbers of white blood cells in reticulocytes fractions were from the nucleated red blood cells (NRBC) especially found in patients with splenectomy.

5.3 Standard Curve

The efficiencies of standard curve of α, β, γ, β^E and GAPDH genes were in between 1.8-2.2 indicating acceptable conditions for qRT-PCR.However, the efficiency value of GAPDH gene was at the lower acceptable value (1.8) that might be due to pipetting error or contaminating with RNA extraction solutions such as phenol, ethanol and isopropanol (119).

5.4Globin genes expressions

The β-thalassemia is an autosomal recessive disorder inherited by defective β globin chain synthesis. The excess α-globin chains occurred and caused pathology of red blood cells leading to anemia (5). The α/β globin mRNA ratio is

indicated balance of globin chains synthesis. For our study, the normal cases showed α/β globin mRNA ratios between 0.6-1.9 (mean 1.1, n=10) similar to previous studies which determined by qRT-PCR and reported the value of 0.97-1.20 (10) and 1.2-2.3 (8). The higher ratios of 4.2–4.6 were reported in normal subjects determined by conventional RT-PCR (11). The increasing of α/β globin mRNA ratio was found in β-thalassemias similar to previous studies (8, 10). Watanapokasin Y. *et al*(10) had reported the increasing of α/β ratio in Hb E trait (median =2.15, n = 8) and frameshift mutation (codon 41/42) β-thal/Hb E (median =5.70, n = 13). Similar to previous studies (8), coinheritance with α-thalassemia in β-thalassemia with HbE disease caused decreasing α/β globin mRNA ratio compared to those with normal α-globin genes. The α/β globin mRNA ratio was increased in β-thalassemia corresponded to disease severities (8). In recently, Tubsuwan A. *et al*(9) studied on the α-globin/non α-globin mRNA ratio in thalassemia disorders by one-step qRT-PCR. The γ globin mRNA was included in the non α-globin mRNA. They found that α-globin/non α-globin mRNA ratio was increased in β-thalassemia when compared with normal control. However, no significant difference of this ratio was found between mild and severe phenotypes of β-thalassemia with HbE. We found no significant difference of α/β ratio between β-thalassemia with Hb E disease and heterozygous β-thalassemia. This was due to the primers and probes for β globin genes could amplify and detect both normal β- and β^E-globin genes. Other than the mutated β-thalassemia allele, the expressed β^E mRNA was counted in β-thalassemia with Hb E disease while β^A mRNA was counted in heterozygous β-thalassemia. The total β globin mRNA thus expressed in comparable amounts in these two groups leading to similar α/β globin mRNA ratio. Nevertheless, the α/β globin mRNA ratios were significantly different between β^0-, β^+(severe)- and β^+-thalassemia with HbE disease thus the ratio might be used for severity indication in β-thalassemia with HbE diseases.

Increased HbF synthesis was known to compensate for the globin synthesis defects in β-thalassemia (33). We explored the γ globin mRNA expression in comparative to GAPDH in parallel with absolute amount and %HbF detections. The highest γ globin mRNA expression was found in homozygous β-thalassemia disease and orderly declined in β-thalassemia with Hb E disease and heterozygous β-thalassemia, respectively. The γ globin mRNA were expressed to compensate for and

in the orders of the decreasing of β globin mRNA. Heterozygous β-thalassemia showed no significant difference of γ globin mRNA expression as well as their HbF from normal controls since the remaining of one normal β globin allele was enough to function without clinically affected and no need of γ globin compensation. The β^0-, β^+(severe)- and β^+-thalassemia with HbE disease showed no significant difference of γ globin mRNA expression. Moreover, an increasing of γ/GAPDH mRNA ratio was related to decreasing of β/GAPDH mRNA ratio in β-thalassemia with Hb E disease. We suggest that β-thalassemias produced high γ globin mRNA thus lead to increased HbF synthesis for compensation of the low β globin mRNA level which lead to decreased HbA synthesis. Interestingly, the γ/GAPDH mRNA ratio was related to %HbF and absolute HbF thus the γ globin mRNA expression level directly corresponded to HbF synthesis which is a factor affected disease severity in β-thalassemias(120).

Patients with β-thalassemia with HbE disease have a variety of disease severities(7) . We expected that globin gene expression ratios, in addition of HbF, might affect the disease severity. However, the α/β, β/GAPDH, γ/GAPDH, β^E/GAPDH and absolute HbF in mild and moderate-severe phenotypes of β-thalassemia with HbE disease showed no significant difference. Our findings were similar to those of Tubsuwan A. *et al*(9) which reported no significant difference of α globin/non-α globin mRNA ratio between mild and severe phenotypes. Interestingly, we found significant higher of absolute HbE in mild than in moderate-severe phenotypes. Increased HbE synthesis might be improved clinical severity of β-thalassemia with HbE disease.

The β/GAPDH mRNA ratios in β^0-, β^+(severe)- and β^+-thalassemia with HbE disease showed no significant difference. However, in β-thalassemia/HbE, the lowest and highest β/GAPDH mRNA ratios were found in codon 41/42 and codon 17 mutations, respectively, when compared with other mutations. We suggested that the decrease of β globin mRNA depended on numbers of functional β globin genes present and β- thalassemia genotypes. Both codon 41/42 and codon 17 mutations are β^0-thalassemia, the lower β-globin mRNA detected in codon 41/42 was due to nonsense-mediated mRNA decay (NMD). The nonsense-mediated mRNA decay (NMD) is a surveillance pathway for reducing errors of gene expression by

eliminating abnormal mRNAs. mRNA containing premature termination codon (PTC) at more than 50-55 nucleotides upstream to the last exon-exon junction (50-55 nt boundary rule) within β-globin gene was known to get NMD (121, 122). Codon 41/42 causes frameshift mutation leading to premature termination at codon 59 that locates in 50-55 nucleotide boundary rule, thus nonsense-mediated mRNA decay (NMD) occurs (9, 123). On the other hand, codon 17 causes nonsense mutation leading to premature termination codon at codon 17 which locates at the 5' half of exon 1 thus escapes the NMD (121, 124).The escaped β^{cd17} mRNA were detected and resulting in the highest β-globin mRNA. Similarly, Tubsuwan A. *et al*(9) had reported higher α globin/non-α globin mRNA ratio in codon 41/42 which was affected by more decreasing β globin mRNAthan in codon 17. In addition, the β/GAPDH mRNA ratios in IVSI-1 mutation were higher than codon 41/42 but lower than codon 17. The mutation of IVSI-I activates three types of the 5' cryptic splice sites similar to the IVSI-5, but IVSI-5 mutation retains the correct splicing (125). Two cryptic splice sites at codon 18 and codon 25 cause premature termination codon at codon 55 and codon 30, respectively, leading to NMD. Another one of the cryptic splice site cause premature termination codon in exon 3 that escapes the NMD (66, 125, 126). Both NMD and escaped NMD of IVSI-I mRNA resulted in higher β globin mRNA than codon 41/42 but lower than codon 17. The β/GAPDH mRNA ratios in IVSII-654 and nt -28 mutations were higher than codon 41/42 and IVSI-1 mutations. These mutations are β^+-thalassemia that some amounts of normal β-globin mRNA were produced and normal β-globin chain were synthesized contrast to β^0-thalassemia such as codon 41/42 and IVSI-1. Nevertheless, we found the highest β/GAPDH mRNA ratio due to NMD escaped in codon 17 that causes β^0-thalassemia. We suggested that β^{cd17} globin mRNA expressed in codon 17 may be translated to short proteins which were destroyed by proteinase enzymes (122).The nt-28 β^+-thalassemia with HbE disease expressed the lowest γ/GAPDH but highest β^E/GAPDH mRNA ratios since normal β-globin chain were ably produced thus less compensation by γ globin activation was occurred. The highest β^E mRNA expression in this mutation was related to the highest HbE production as well as the highest γ mRNA expression in IVSII-654 mutation was related to the highest HbF production.

The β^E globin gene can activate the 5' cryptic splice site at codon 25 (9) leading to aberrantly spliced β^E globin mRNA in addition to the retaining correctly spliced mRNA. The correctly spliced β^E globin mRNA can be translated and produced HbE while the aberrantly spliced mRNA cannot lead to functional protein. The primers and probes for β^E globin mRNA in this study was designed for determining only the correctly spliced mRNA. No correlation was found between the β^E/GAPDH mRNA ratios and % HbE or absolute HbE suggested whether the overall β^E globin mRNA efficiently synthesized for HbE. This might be due to competing for the α globin chains binding between γ and β^E globin chains. Otherwise, if aberrantly spliced β^E globin mRNA was translated to truncated proteins, they might interfere the β^E globin chain synthesis. The truncated proteins interference of normal proteins production had been reported (127, 128).

The analysis of gene expression requires sensitive, precise, and reproducible measurement of specific mRNA sequences. Many previous studies were used conventional polymerase chain reaction (13-15), Northern blot (16, 17) and reverse dot blot analysis (18, 19) for detection of mRNA in thalassemia. However, these methods were non-quantitative and less sensitivity than real-time PCR. The qRT-PCR (Real Time Reverse Transcription Polymerase Chain Reaction) assay is the most effective method for gene quantitation. It has high sensitivity, high specificity, no process of agarose gel electrophoresis and high throughput (20).

However, globin gene expressions are only one of the factors to explain a variety of disease severities, especially in β-thalassemia with HbE disease. β-thalassemia genotypes, coinheritance of α-thalassemia, and SNPs associated with HbF such as XmnI and BCL11A had been reported to affect disease severity (7, 120). The use of globin gene expressions should be interpreted together for α/β, β/GAPDH, γ/GAPDH and β^E/GAPDH mRNA ratios. They are useful to explain the mechanism of disease severity in thalassemia.

CHAPTER VI
CONCLUSION

In this study, we had quantitated the α, β and γ globin genes expressions in β-thalassemia by using real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and studied the relationships between globin genes expressions and disease severities in each β-thalassemia group and genotype.

The α/β globin mRNA ratios indicate the balance of globin chains synthesis. In normal samples, the α/β globin mRNA ratios were 0.6-1.9 (mean 1.1, n=10).This ratio was increased in homozygous β-thalassemia disease, β-thalassemia with HbE disease and heterozygous β-thalassemia. The ratio showed no significant difference between β-thalassemia with Hb E disease and heterozygous β-thalassemia. This was due to the primers and probes for β globin genes which could amplify and detect both normal β- and β^E-globin mRNA.Interestingly, the α/β globin mRNA ratios were significant different between β^0-, β^+ (severe) - and β^+-thalassemia with HbE disease. The α/β globin mRNA ratios can be used for indicating clinical severities in β-thalassemia especially in β-thalassemia with HbE diseases.

HbF is a factor affected disease severity in β-thalassemia. We found that the increasing of γ/GAPDH mRNA expression was related toHbF production.Moreover, the increasing of γ/GAPDH ratio was found to compensatefor the decreasing of β/GAPDH ratio. The levels of γ globin mRNA expression were highest in homozygous β-thalassemia disease followed by β-thalassemia with Hb E disease and heterozygous β-thalassemia, respectively. However, no significant difference of γ globin mRNA expression as well as %HbF and absolute HbFwas found between heterozygous β-thalassemia and normal.

Patients with β-thalassemia with HbE have diversity of clinical severities. The α/β, β/GAPDH, γ/GAPDH, β^E/GAPDH and absolute HbFshowed no significant difference between mild and moderate-severe phenotypes.However, higher absolute HbE in mild than moderate-severe phenotypeswas found. Increased HbE synthesis might be improved clinical severity of β-thalassemia with HbE disease.

Both codon 41/42 and codon 17 mutations are β^0-thalassemia,the lower β-globin mRNA detected in codon 41/42 was due to nonsense-mediated mRNA decay (NMD) whereas codon 17 wasescaped the NMD.The β globin mRNA in IVSI-1 was higher than codon 41/42 but lower than codon 17 due to the occurrence of both NMD and escaped NMD.The IVSII-654 and nt -28 mutationsare β^+-thalassemia thus β globin mRNAwere higher than codon 41/42 and IVSI-1 mutations.

We found that HbE production was not correlated with β^E globin mRNA level although the primers and probes for β^E globin mRNA were designed for determining only the correctly spliced mRNA. This might be due to the competing for α globin chains binding between γ and β^E globin chains. In addition, if aberrantly spliced β^E globin mRNA were translated to truncated proteins, they might interfere the β^E globin chain synthesis.

The globin gene expressions can be used for prediction and explanation of β-thalassemia disease severities. In addition, they provideknowledge for additional explanationof hemoglobin synthesis in β thalassemia.

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APPENDIX

Stock solution for PCR and Real time PCR

1. 1X TBE buffer

10X TBE buffer 100 ml

Distill water 900 ml.

Mix, store at room temperature

2. 3% dextran in 0.85% sodium chloride

Dextran (molecular weight 150,000) 30 g

Sodium chloride 8.5 g

Distill water to 1,000 ml.

Mix, store at room temperature

3. DEPC-treated water

DEPC 10 µl

Distill water 90 µl

Mix, incubate overnight and autoclave

Store at room temperature

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