การถอดรหัสดีเอ็นเอที่อยู่ระหว่างยีน HBS1L และ MYB มีความสัมพันธ์กับระดับฮีโมโกลบินเอ๊ฟในเม็ดเลือดแดงตัวอ่อนโรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอี

นางสาวดวงกมล เลิศบรรลือชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

HBS1L-MYB INTERGENIC REGION EXPRESSION IS ASSOCIATED WITH FETAL HEMOGLOBIN LEVEL IN β-THALASSEMIA/HbE ERYTHROBLASTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Clinical Hematology Sciences
Department of Clinical Microscopy
Faculty of Allied Health Sciences
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Thesis Title	*HBS1L-MYB* INTERGENIC REGION EXPRESSION IS ASSOCIATED WITH FETAL HEMOGLOBIN LEVEL IN β-THALASSEMIA/HbE ERYTHROBLASTS
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ดวงกมล เลิศบรรลือชัย : การถอดรหัสดีเอ็นเอที่อยู่ระหว่างยีน HBS1L และ MYB มีความสัมพันธ์กับระดับฮีโมโกลบินเอ๊ฟในเม็ดเลือดแดงตัวอ่อนโรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอี (*HBS1L-MYB* INTERGENIC REGION EXPRESSION IS ASSOCIATED WITH FETAL HEMOGLOBIN LEVEL IN β-THALASSEMIA/HbE ERYTHROBLASTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.กมลลักษณ์ ลีเจริญเกียรติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.อรพรรณ ศรีพิชัย, 72 หน้า.

โรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอีเป็นโรคโลหิตจางทางพันธุกรรม มีพยาธิสภาพที่เกิดจากความไม่สมดุลในการสร้างสายโกลบินหรือโปรตีนในเม็ดเลือดแดง ความรุนแรงของโรคนี้ขึ้นอยู่กับหลายปัจจัย รวมไปถึงปริมาณฮีโมโกลบินเอ๊ฟในเม็ดเลือดแดง ซึ่งฮีโมโกลบินชนิดนี้สามารถทำหน้าที่ในการจับออกซิเจน เพื่อทดแทนฮีโมโกลบินหลักได้ จากการศึกษาข้อมูลทางพันธุกรรมของผู้ป่วยเบต้าธาลัซซีเมีย (GWAS) พบว่ากลุ่มยีนที่มีผลต่อการแสดงออกของโกลบินอยู่บริเวณใกล้เคียงกับยีนที่ผลิตทรานสคริปชั่นแฟกเตอร์ ดังเช่น บริเวณที่อยู่ระหว่างยีน *HBS1L* และ *MYB* ซึ่งพบว่าความหลากหลายทางพันธุกรรมที่บริเวณนี้ และมีความสัมพันธ์กับความรุนแรงของโรคอย่างมีนัยสำคัญ ผลการสืบค้นข้อมูลในเซลล์ K562 เกี่ยวกับการผลิตอาร์เอ็นเอ มีหลักฐานว่าบริเวณนี้สามารถผลิตอาร์เอ็นเอสายสั้นๆ ออกมาได้หรือที่เรียกว่าอาร์เอ็นเอชนิดไม่ถอดรหัส (non-coding RNA) ซึ่งอาร์เอ็นเอชนิดดังกล่าวอาจมีหน้าที่ในการควบคุมการถอดรหัสในเซลล์สิ่งมีชีวิต ผู้วิจัยจึงได้ทำการทดลองเพื่อหาปริมาณอาร์เอ็นเอชนิดไม่ถอดรหัสชนิด HMIR ที่บริเวณนี้ด้วยวิธี quantitative PCR จากเซลล์เม็ดเลือดแดงตัวอ่อนของและผู้ป่วยโรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอีเทียบกับคนปกติ พบว่าผู้ป่วยโรคเบต้าธาลัสซีเมีย/ฮีโมโกลบินอีมีการสร้างอาร์เอ็นเอชนิดไม่ถอดรหัสนี้น้อยกว่าคนปกติ จากนั้นได้ทำการทดลองเพื่อหาลำดับเบสของอาร์เอ็นเอแบบเต็มชิ้นส่วนด้วยเทคนิค rapid amplification of cDNA ends (RACE) พบว่าชิ้นของอาร์เอ็นเอที่ได้จากการทำ 5′RACE มีขนาดมากกว่า 200 เบส แต่เมื่อนำอาร์เอ็นเอนี้มาหาลำดับเบส กลับพบว่าอาร์เอ็นเอนี้ไม่อยู่บริเวณระหว่างยีน *HBS1L* และ *MYB* ผู้วิจัยจึงสรุปว่า อาร์เอ็นเอที่ได้เป็นอาร์เอ็นเอชนิดถอดรหัสสายสั้นๆ ซึ่งไม่สามารถตรวจได้ด้วยวิธีนี้ แต่อาร์เอ็นเอนี้อาจมีความสำคัญกับอาการและความรุนแรงของผู้ป่วยโรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอี

ภาควิชา จุลทรรศน์ศาสตร์คลินิก	ลายมือชื่อนิสิต .....
สาขาวิชา วิทยาศาสตร์โลหิตวิทยาคลินิก	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา 2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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DUANGKAMON LOESBANLUECHAI: *HBS1L-MYB* INTERGENIC REGION EXPRESSION IS ASSOCIATED WITH FETAL HEMOGLOBIN LEVEL IN β-THALASSEMIA/HbE ERYTHROBLASTS. ADVISOR: KAMONLAK LEECHAROENKIAT, Ph.D., CO-ADVISOR: ORAPAN SRIPICHAI, Ph.D., 72 pp.

β-thalassemia/HbE is a hematological disease caused by impairment of β-globin production. The degree of diseases severity depends on several factors including the expression of fetal globin ($\alpha_2\gamma_2$) which can compensate for an accumulation of excess α-globin chains. GWAS analysis were performed to identify loci that are linked to reduction in disease severity. A subset of key loci is located at or near the genes encoding transcription factors functionally associated with globin gene expression. The polymorphisms at the intergenic region between *HBS1L* and *MYB* gene or HMIR was shown to be one of the significant severity modifying factor. A set of short transcripts corresponding to the *HBS1L-MYB* genes intergenic region was found in RNA-seq analyses of K562 erythroid cell line, indicating that this intergenic region is actively transcribed. Recently, the non-coding RNA was shown to play roles in gene transcription control. Quantitative PCR was carried out to determine and quantify the non-coding *HBS1L-MYB* transcripts in primary human erythroblasts derived from normal individuals and β-thalassemia/HbE patients. Three clusters of HMIR transcripts were identified. Interestingly, erythroblasts from β-thalassemia/HbE patients have significantly lower levels of the HMIR transcripts than those of normal erythroblasts. In order to characterize these HMIR transcripts, the 5′ and 3′ rapid amplification of cDNA ends (RACE) were carried out to detect the full length of the transcripts. Amplifying results of RACE products show that the 5′end of each transcripts are more than 200 base pairs. The clones were selected for DNA sequencing, however, they do not contain HMIR sequences. Hence, these transcripts are the candidate for being a small non-coding RNAs that might responsible for modifying the patient's severity.

Department:	Clinical Microscopy	Student's Signature	.....
Field of Study:	Clinical Hematology Sciences	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

°C	degree celsius
µg.	microgram
µl	microliter
bp.	base pairs
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin Immunoprecipitation
CIP	calf Intestine Alkaline Phosphatase
Conc.	concentration
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EPO	erythropoietin
g	gram
GPA	glycophorinA
g/dl	gram per deciliter
Hb	hemoglobin
HbA	hemoglobin A
HbE	hemoglobin E
HbF	hemoglobin F
H3K4me2	dimethylated Histone 3 Lysine 4
H3K4me3	trimethylated Histone 3 Lysine 4
H3K36me3	trimethylated Histone 3 Lysine 36
H4K20me	monomethylated Histone 4 Lysine 20
H3K9ac	acetylated Histone 3 Lysine 9
H3K27ac	acetylated Histone 3 Lysine 27
IMDM	Iscove's modified Dulbecco's Medium
Kb	kilobase
HMIR	HBS1L- MYB intergenic region
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration

MCV	mean corpuscular volume
ng.	nanogram
ml	milliliter
min	minute (s)
PCR	polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
Pol II	RNA polymerase II
QTL	quantitative trait loci
RACE	rapid Amplification cDNA end
RNA	ribonucleic acid
RNase	ribonuclease
SD.	standard deviation
Sec.	second (s)
TAP	tobacco acid pyrophosphatase

CHAPTER I
INTRODUCTION

1. Background and significant

The inherited abnormalities of hemoglobin are classified into two categories: qualitative and quantitative disorders. The qualitative disorders are a wide range of conditions that result in abnormal globin structures, for example sickle cell anemia which is caused by a glutamic to valine mutation resulted in the generation of fiber-like structure from abnormal β-globins (1). Thalassemia is a quantitative disorder generally characterized by reduced globin chain production.

β-thalassemia/HbE disease is the most common compound heterozygous β-thalassemia in Thailand (2). It is caused by the co-inheritance of HbE and β-globin gene mutation. β-Thalassemia/ HbE patients have severe symptoms caused by accumulation of excess unmatched α globin chains within erythroid precursors. The degree of β-thalassemia/HbE severity was found to be influenced by several genetic modifying factors (3). Fetal hemoglobin (HbF) is a hemoglobin type which is predominantly expressed in fetal life (4, 5). Clear evidence showed that patients with homozygous β^0-thalassemia with a mild clinical symptom tend to have high HbF level (6). Many genomic loci have now been identified to be associated with increased HbF level and the clinical course of β-thalassemia (7, 8).

Genome wide association study (GWAS) identified SNPs at the HBS1L-MYB intergenic region (HMIR) which is the second significant polymorphism related to HbF levels (9, 10). Increased HbF level can modulate a broad spectrum of erythroid phenotypes, suggesting that this locus may have a key role in the regulation of globin gene expression and erythropoiesis (11, 12). The HMIR contains erythroid specific histone acetylation sites, GATA1 binding sites and RNA polymerase II binding sites beyond conventional promoters, indicating that this intergenic region might contain transcriptional activity.

HMIR is a region flanked between the genes encoding HBS1L and MYB. It contains putative binding sites for several erythropoiesis and hematopoiesis-related

transcription factors namely, TAL1/SCL, E47, GATA, and RUNX1/AML1 (13). HMIR contains an enhancer region controlling *HBS1L* and *MYB* genes in erythroid cells (14). The HBS1L gene encodes a GTP-binding translation elongation factor which regulates a critical cellular processes, but the function of HBS1L in hematopoiesis is not yet clear (15). A patient without functional HBS1L showed systemic abnormality without any distinct hematological defect. On the other hand, MYB is well known for its role in hematopoiesis. It is associated with proliferation survival and differentiation of hematologic progenitor cell, megakaryocyte and lymphocyte (16). MYB is also necessary for the differentiation of adult erythroid cells.

The information from the ENCODE database points toward the production of short non-coding RNA transcripts generated from HMIR. This is consistent with the enrichment of RNA polymerase II and chromatin signatures for active transcription such as H3K4me3 and H3K27Ac (17). The functional significance of the short transcripts associated with HMIR has not been validated. Considering the role of HMIR in gene regulation, these transcripts might play a role in erythropoiesis.

In this study, we report result of HMIR identifying by two techniques namely, qRT-PCR and RACE. The change in the level of short HMIR transcripts in normal individuals in comparison to those in β-thalassemia/HbE patients was studied by qRT-PCR based on available ENCODE data.

2. Objective

2.1 To characterize the HBS1L-MYB intergenic region (HMIR) transcripts in erythroblasts by qRT-PCR and RACE analysis.

2.2 To evaluate correlation of the HMIR expression to HbF production in erythroblasts.

CHAPTER II
LITERATURE REVIEW

1. Hemoglobin switching

Hemoglobin (Hb) is a tetramer of two pairs of globin chains which determines the hemoglobin types; each of which is attached to one heme molecule. The synthesis of globin chains is controlled by two different gene families (α- and β- gene clusters) that are located on different chromosomes. The α-cluster lies on chromosome 16 while the β-cluster lies on chromosome 11 (18). Sites of primitive and definitive globin production as well as the sequential waves of α, β, γ and ε-globin synthesis are shown in Figure 1. Hb is developmentally switched between globin genes, which coincides with the transition of tissue-specific erythropoiesis. The ε- and γ-genes are expressed exclusively in the yolk sac. These genes are silenced in the fetal liver where β-globin gene expression starts to occur. All the elements required for developmental regulation are included in the sequences of the globin loci and their flanking sequences (19).

The *cis* elements and transcription factors play important roles in switching from fetal to adult hemoglobins. Many transcription factors act as multiprotein complexes in γ- to β-globin switching (Figure 2). Functional genetic and biochemical studies show the roles of SOX6, TR2/TR4 erythroid definitive complex, COUPII, FOP and NF-E4 in globin gene regulation (20). ε-globin gene expression is totally restricted in the embryonic yolk sac cells. Its developmental is control autonomous with all the sequences required for silencing encoded in flanking sequences. The sequence located upstream from the promoter contains GATA site, γγ1 site and CAC motif. Evidence that this element functions as a silencer was obtained when the deletion of the element results in ε gene expression in adult transgenic mice (21). At the embryonic stage, the LCR interacts with the ε-globin gene. The downstream genes are turned off competitively. In the fetus, ε-gene is silenced, and the LCR interacts with the γ-gene.

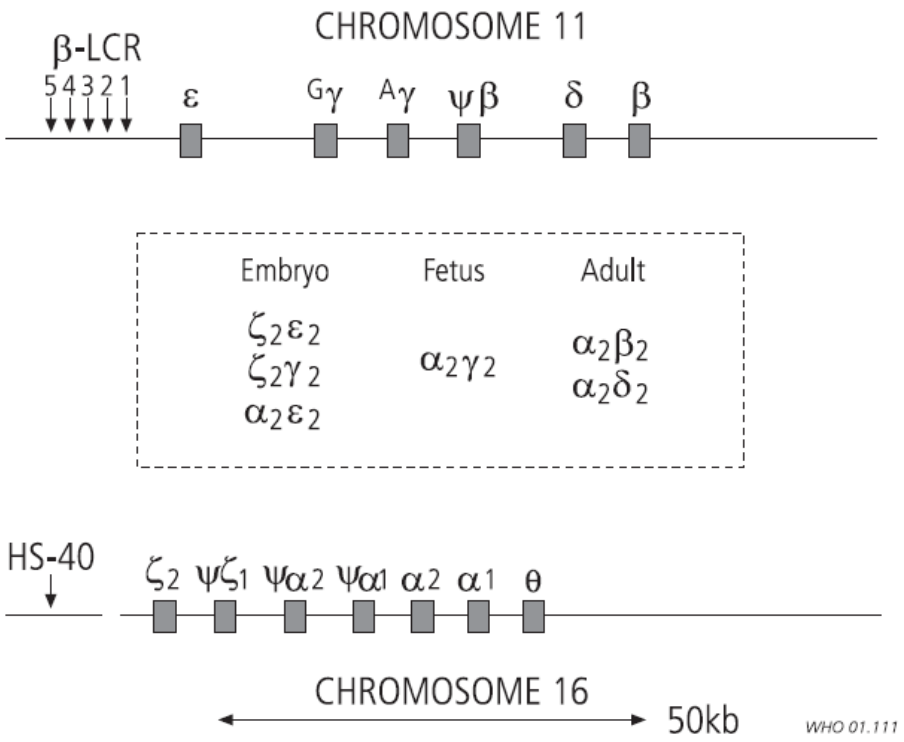

Figure 1. Normal structure of human globin gene clusters.

The α-cluster genes are located at chromosome 16 in the order 5′-ζ2-ψζ1-ψα2-ψα1-α2-α1-θ-3′, whereas the β-like genes form a cluster at chromosome 11 in the order 5′-ε-Gγ-Aγ-ψβ-δ-β-3′. The α-globin is encoded by two α-globin genes per haploid genome, either α2 or α1 gene on chromosome 16, whereas β-globin is encoded by one β-globin gene per haploid genome of chromosome 11 (18).

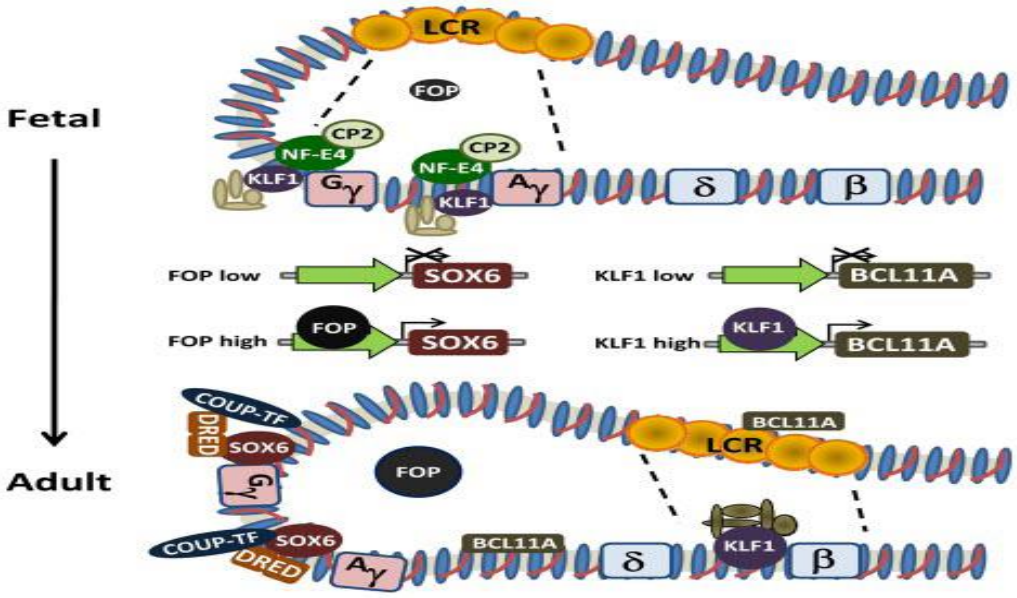

Figure 2. Schematic depiction of hemoglobin switching model, based on DNA looping and interactions of LCR and globin gene promoters (20).

2. Thalassemias

Thalassemias are a group of genetic blood diseases which characterized by deficiency or absence in globin chains production. Most of thalassemias are inherited as a recessive trait (22, 23). There are two main varieties of thalassemia, α and β-thalassemia, which are commonly found in Southeast Asia and Thailand (24, 25). The defect may affect one type of globin chain, or may affect some combination of more than one globin type in the same patient (26). The result is an imbalance of globin chain synthesis leading to abnormal morphology (hypochromic or microcytic) (27). Loss of globin production manifests as severe thalassemia types which found in Hb Bart's hydrops fetalis (homozygous α-thalassemia), homozygous β^0-thalassemia and β^0-thalassemia /HbE (Figure 3).

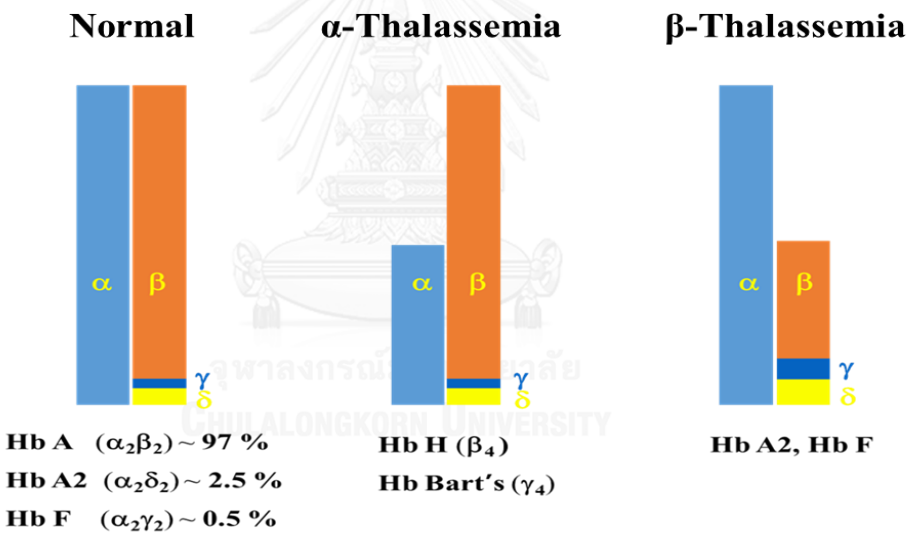

Figure 3. Diagram presenting amount of hemoglobin in normal, α-thalassemia and β-thalassemia.

3. β-Thalassemia

β-Thalassemia is resulted from defective synthesis of β-globin chains including mutations affecting transcription, processing of mRNA precursor, translation, or stability of globin chain (28). The β^0-thalassemia has no production of β-globin. Individuals with homozygous β^0-thalassemia produce only HbA_2 and HbF with accumulation of unstable α_4 tetramers that are toxic to the erythroid progenitors. β^+ Thalassemia is characterized by reduces production of β-globin chains. Patients with homozygous β^-thalassemia have problems with excess alpha globin in the erythroid progenitors inside the marrow (29, 30).

The common variant of the β-thalassemia is hemoglobin E (HbE). The HbE is caused by mutation at codon 26 of β-globin gene leading to generation of active a cryptic splice site and partial alternative splicing (31). For mRNA that is translated, β^E contains lysine instead of glutamate at codon 26. Compound heterozygosity between β^0-thalassemia and HbE leading to β^0-thalassemia/HbE disease that has a significant clinical presentation and found very common in Thailand (32).

4. Pathogenesis of β-thalassemia

β-Thalassemia/HbE patients have severe symptoms caused by accumulation within the erythroid precursors of excess unmatched α-globin chains. Hemoglobin degradation products (heme, hemin and iron) are deposited at the erythroid membrane and induce oxidative damage that interferes with erythroid cell survival (Figure 4). The free α-chains form unstable tetramers and precipitate as inclusion bodies in erythroid precursors in the bone marrow (33). They are responsible for the extensive intramedullary destruction of the erythroid precursors, a combination of ineffective erythropoiesis, peripheral hemolysis and reduction in hemoglobin synthesis (34), resulting in anemia and hypoxia in β-thalassemia. Anemia in turn stimulates the production of a relative large amount of erythropoietin (EPO) stimulates the marrow expansion and extramedullary erythropoiesis. Marrow expansion results in characteristic deformities of skull and face. Increased erythropoiesis leads to an increased rate of iron absorption. This combined with iron leaked from red cell hemolysis and iron received by blood transfusion, lead to progressive iron overload (35).

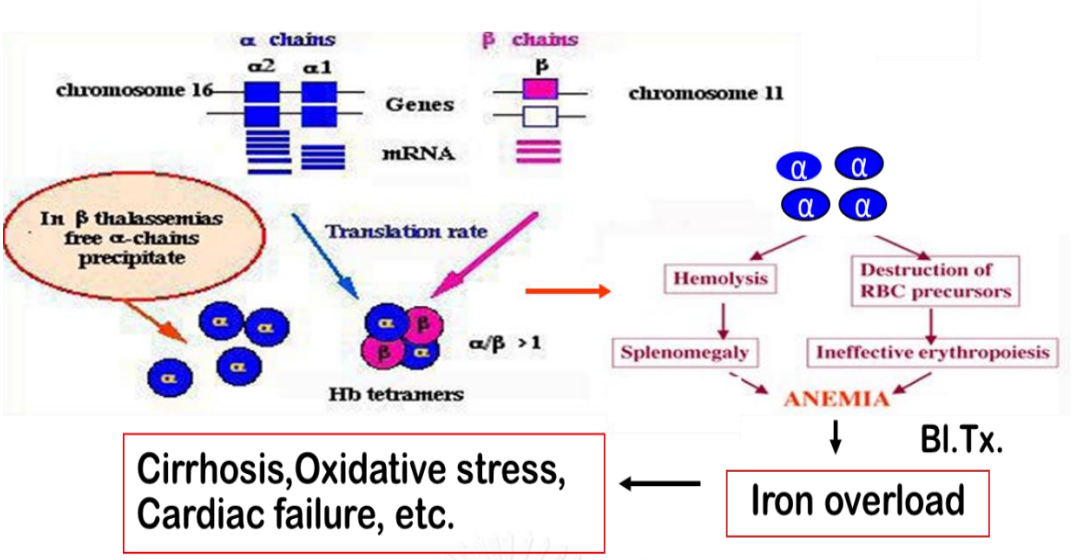

Figure 3. Pathogenesis of β-thalassemia.

Degradation products (heme or hemin) precipitate in erythroid precursors, causing ineffective erythropoiesis and hemolysis that contribute to the anemia. The picture was reprinted from http://www.slideshare.net/aabbccddeeee/beta-thalassemiappt.

5. Phenotypic diversity of β-thalassemia/HbE

One critical complication of β-thalassemia/HbE is patients with apparently similar or identical genetic defects showing different severity of presentation (36). The β-thalassemia/HbE patients have an extremely diverse clinical presentation**,** thalassemia trait, thalassemia intermediate and thalassemia major (37-39). Patients with thalassemia trait generally carry asymptomatic or the silent carrier state (40-42). Patients with thalassemia intermediate have a mild to moderate anemia and some cases require regular blood transfusion but some require few or even no transfusion. Patients with thalassemia major, referred to the most severe form of β-thalassemia, usually come to medical attention in the first year of life with a severe anemia presentation and subsequently require regular blood transfusion (39, 43). Factors affecting the phenotypic diversity of β-thalassemia have now been reported.

6. Genetic Factors reducing β-thalassemia/HbE diseases severity

β-thalassemia/HbE patients with similar genotypes could have a broad range of clinical symptoms. Understanding and perhaps mimicking these traits could pave the way toward better treatment for thalassemia patients. One of the key factors reducing diseases severity is the level of fetal hemoglobin (HbF). HbF ($\alpha_2\gamma_2$) is a major hemoglobin in human fetus. The fetal (γ) globin gene is repressed after birth, but relatively low level of HbF still remains in normal adult (19). On average, the normal physiological range of HbF is lower than 1% of hemoglobins. Increase in the HbF level is a modifier of disease severity of β-thalassemia because it is correlated with the reduction of excess α-globin (44). Induction of HbF production could be a critical and useful therapy for β-thalassemia patients (45) (Table 1). Pharmacological induction of HbF expression has been a long standing therapeutic objective for patients with severe β-thalassemia. Three different classes of fetal hemoglobin inducing drugs have been tested in patients with β-thalassemia including chemotherapeutics/hypomethylating agents (hydroxyurea, 5-azacytidine and decitabine), short chain fatty acid derivatives (sodium phenylbutyrate) and erythropoietin (46). However, all of the drugs have many side effects, and alternative strategies are needed.

Clinical and molecular genetic studies have demonstrated that modifier genes which induce γ-globin gene expression, may transform severe transfusion-dependent thalassemia into mild forms of anemia. Genetic variants that ameliorate disease severity in β-thalassemia/HbE associated with HbF modifying loci were found at three loci namely HBB gene (chr22p15), HMIR (chr6q23) and BCL11A (chr2p16) (Figure 5) (47). Twenty-three SNPs in three genes/regions were identified as being significantly associated with disease severity. These SNPs were identified in a series of genome-wide association studies (GWAS) (48). SNP in the HMIR is the second significant factor (49). Patients with the SNP have increased HbF level and improved hematological indicators including MCV, MCH and MCHC (50, 51).

Table 1. Distribution of fetal hemoglobin (HbF) in a Thai β^0-thalassemia/HbE cohort *(n = 950).* The table was modified from Sripichai O, PhD Thesis 2006.

Severity	Percentage HbF	Absolute HbF (g/dL)
Mild (n = 233)	41.0 ± 11.2	3.1 ± 1.2
Moderate (n = 310)	35.3 ± 11.1	2.0 ± 0.9
Severe (n = 407)	31.2 ± 11.2	1.4 ± 0.7

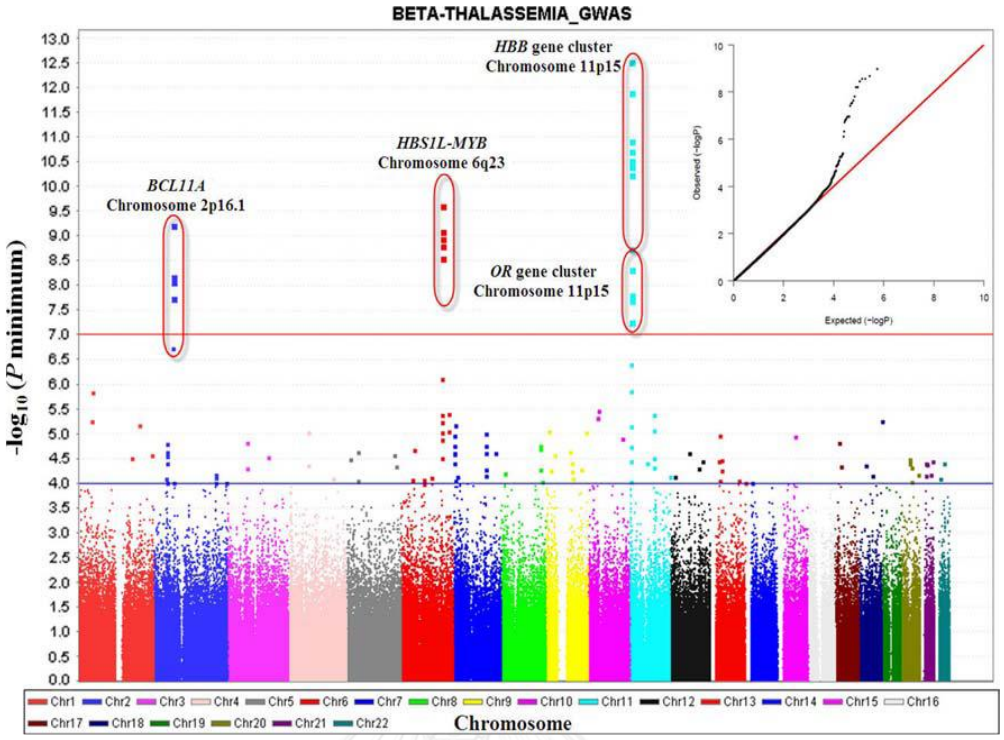

Figure 4. Genome-wide association study of 618 β^0-thalassemia/HbE patients using 548,094 SNPs (47). Scatter plots of P values (analyzed with disease severity) in log-scale with chromosome location.

7. The HBS1L-MYB intergenic region (HMIR)

HMIR is located on the chromosome 6q23 region that have nine genes encompassing approximately 1.5 Mb. These nine genes produce the total of 30 different transcripts; the majority of which are unlikely to be functional at the protein level (51). This is due to the alternate exons interrupting the open reading frame (52). HMIR is a region franking between *HBS1L* and *MYB* genes, and it contains distal regulatory elements that control HBS1L and MYB gene expression in the erythriod cell (Figure 6).

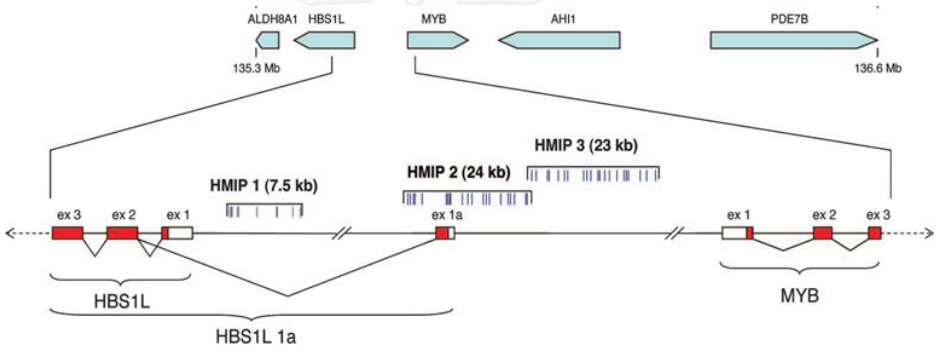

Figure 5. Diagram of HBS1L-MYB intergenic region (HMIR).
It is located between HBS1L and MYB. Exons (red bar) are spliced to generate proteins (blue arrow). The image was reprinted from Thein SL, 2007 (51).

8. Role of HBS1L and MYB in erythropoiesis

The function of HBS1L in hematopoiesis still not elucidated (11). Previous study found that patient with loss of function of HBS1L have systemic abnormality, but except hematologic (15). HBS1L (HBS 1 Like Translational GTPase) is poorly characterized gene with unknown hematological functions, whereas MYB has already been well studied for its role in hematopoiesis. MYB is associated with proliferation survival and differentiation of hematologic progenitor cell, megakaryocyte and lymphocyte (53). MYB (myeloblastosis) is necessary for the normal differentiation kinetics of adult erythroid cells and reduction in the level of this gene results in altered erythroid differentiation kinetics and the increased presence of cells from other lineages. Down-regulation of *MYB* expression by miRNA (16), resulting in delayed switching from fetal to adult hemoglobin and increase in the HbF level.

MYB activates repressors of fetal hemoglobin through BCL11A and TR2 or TR4 pathways. Both pathways repress gamma-globin gene expression (54, 55) The TR2 and TR4 genes encode the DNA binding subunits of DRED (direct repeat erythroid definitive). Disruption of the HBS1L locus reduces the level of DRED and causes HPFH (Hereditary Persistence of fetal Hemoglobin) (Figure 7) (14).

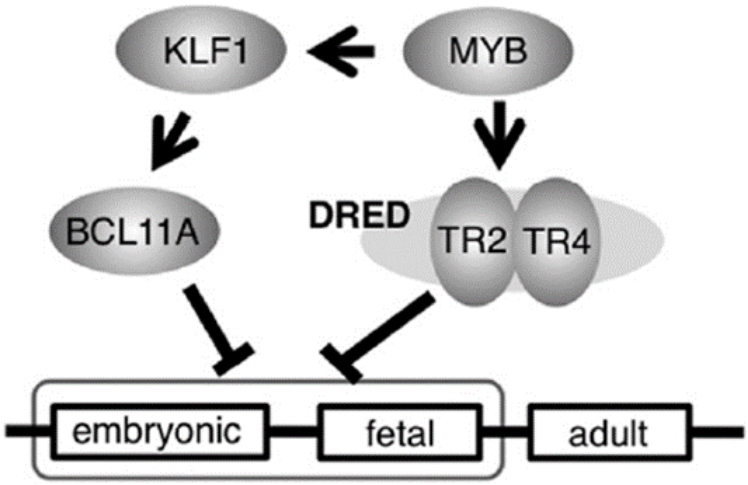

Figure 6. Repression of fetal hemoglobin via MYB, KLF1 and DRED.

MYB represses embryonic and fetal globin genes by up-regulating both TR2/TR4 and KLF1/BCL11A pathways, resulting in switching expression to adult hemoglobin. In contrast, the down-regulated MYB, both the TR2/TR4 and KLF1/BCL11A are suppressed, activates the fetal globin gene expression (14).

9. HBS1L-MYB intergenic region (HMIR) in thalassemia

HMIR was associated with controlling the fetal hemoglobin production in β-thalassemia (9). Previous study of John J. Farrell and his college revealed the HbF QTL on chromosome 6q23 in Chinese β-thalassemia heterozygotes by genome-wide association study approach. Identification of the candidate polymorphisms on chromosome 6q23 for the functional motif was 3 bp. deletion in HMIR. This polymorphism was performed by Chromatin Immunoprecipitation (ChIP) assay, many transcription factors bound to this region to play role in switching of globin gene including; TAL1/SCL, E47, RUNX1/AML1 and GATA2 (Figure 8). Moreover, the sequences flanking the 3-bp deletion polymorphism are highly conserved among 44 vertebrate species (13). They conclude that the 3-bp deletion polymorphism is probably the most significant functional variant within HMIR accounting for modulation of HbF production.

Disruption of HB1SL-MYB locus from study of Susuki M. and college, was indicate the change in globin level of mouse. Embryonic or fetal hemoglobin were increase significant, but α-globin was reduced, cause hereditary persistence of fetal hemoglobin. The reason is deletion in MYB gene enhancer in HMIR. Thus, this region might control erythropoiesis in mouse and human.

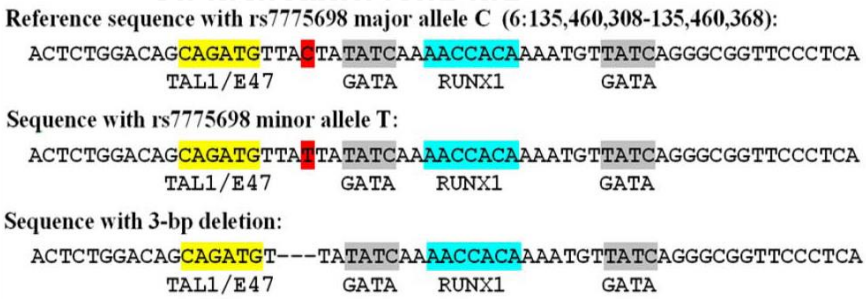

Figure 7. Binding of transcription factor on HB1SL-MYB intergenic region.

The HMIR contains binding region of many transcription factors including TAL1/SCL and E47 which are the basic helix-loop-helix transcription factors binding to E-box region. The spatial orientation of the E-box and GATA motif is required for enhancing TAL1/SCL binding affinity. Nearby is a binding motif for RUNX1/AML1 trascription factor (13).

Stadhourder R. and colleagues showed enrichment in histone modification using ChIP-seq (chromosomeimmunoprecipitation sequencing) in many tissue including K562 cell or erythroluekemic cell line, and predicted that this region might be transcribed to mRNA or non-coding RNA from results of RNA-seq (10). They also study chromosome looping in HMIR by chromosome conformation capture. The location of single nucleotide polymorphisms (SNPs) in HMIR is distance 84 bp. from MYB transcription start site which affect transcription of the *MYB* gene. They proposed that the transcription factors cannot bind to region appeared SNPs, RNA polymerase II decrease transcription activity to activate MYB, resulting in function of MYB in repression gamma globin is disrupted (Figure 9).

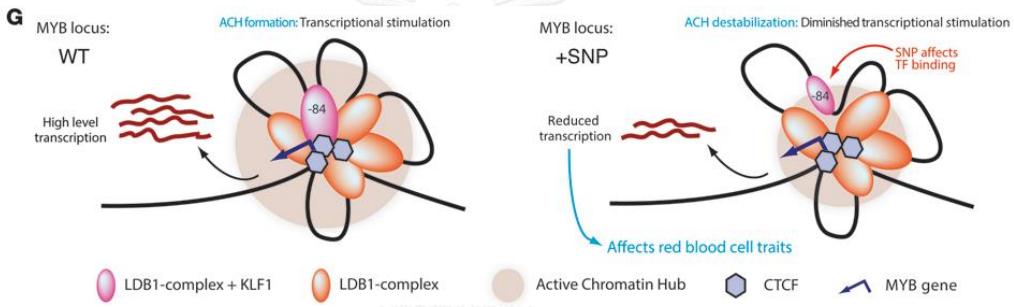

Figure 8. Model depicting the effect of SNPs on MYB regulation.

Intergenic polymorphisms, through their effect on transcription factor recruitment to the enhancers, could partially destabilize the *MYB gene.*

10. Non-coding RNA

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into a protein. This RNA species is highly abundant and has an important role in to the human cells. The ncRNAs are classified into many subtypes including transfer RNA (tRNA), ribosomal RNA(rRNAs), snoRNAs, miRNAs and siRNAs (56, 57). The ncRNAs are found to be deregulated in several human diseases (58, 59).

ncRNAs can be categorized based on their size. Long-noncoding RNAs have the size above 200 nucleotides. Shorter noncoding RNAs can be found as microRNA (miRNA), piwi-interaction RNA (piRNA) and enhancer RNA (eRNA). Based on origin, the ncRNAs can be grouped into long intergenic ncRNAs (lincRNAs), natural antisense transcripts, psuedogenes, long intronic ncRNAs, divergent transcripts, promoter associated transcripts and enhancer RNAs (eRNAs) (Figure 10). The ncRNAs are transcribed by RNA Polymerase II. They can exist in the forms with or without polyadenylation and capping (17). ncRNAs were found to have functions in the regulation of biological processes (Figure 11). Xist RNA establishes heterochromatin by repressive H3H27me3 modifications by recruiting PRC2 (60). Transcription of ncRNAs at the enhancer region is proposed to play a role in enhancer activation by mediating the deposition of H3K4 mono and dimethylation (61). ncRNAs can form a tripex with an *DHFR* promoter and bind to TFIIB to displace DHFR complex, resulting in gene silencing (62). Post-transcriptional processes including splicing, transport and translation of mRNA are possibly controlled by ncRNAs. ncRNAs can interact with mRNA to close splice junctions, causing production of alternative isoforms (63).

Long intergenic ncRNAs (lincRNA) or stand-alone ncRNA do not overlap with protein coding genes. They were identified though chromatin signatures for actively transcribed gene (H3K4me3 and H3K36me3). Natural antisense transcripts, which is generated from the template opposite to the sense DNA strand, are mostly enriched around the gene promoter and terminator (64). Pseudogenes create extra gene copies that are degraded under selective condition (65). They have lost their coding potential due to nonsense, frameshift and other mutations. Long intronic ncRNAs are encoded within the introns (66). The well-characterized RNA in noncoding RNA is Xist RNA. It functions in X-chromosome inactivation (XCI). Xist RNA is a 17-kb transcript

expressed from X-inactivation center (XCI) (67). Xist RNA coats inactivated X chromosome causing chromosomal silencing. It recruits a chromatin-modifying complex to specific sites. Polycomb repressive complex2 (PRC2) binds to a repeat at the 5′-end of RNA through EZH2 which is a subunit of PRC2 (68). Another long-noncoding RNA that plays a role in X-inactivation is Tsix (69). It controls Xist expression by modulating the chromatin structure and DNA methylation of the Xist promoter. Tsix recruits repressor and methytransferase Dnmt3a (Figure 12).

The last class of ncRNAs is promoter associated transcripts and enhancer RNA that are transcribed from the promoter and enhancer regions, respectively (70). These ncRNAs are oftenly produced by bidirectional promoters with both sense and antisense transcripts, sometimes even with overlapping RNAs (71). Their presence was originally spotted using chromatin and transcriptional signatures from whole-genome studies (72). eRNA was found to enrich the transcription factors to the promoter region, making transcriptional machineries ready for the act of transcription. eRNA transcripts have been reported in several genes, and their role appears to be general in transcription control (73).

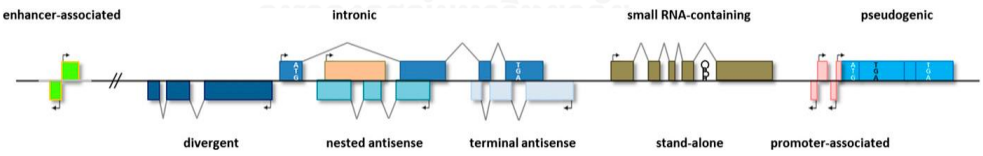

Figure 9. Classes of noncoding RNAs (74).
There are long intergenic ncRNAs (lincRNAs), natural antisense transcripts, psuedogenes, long intronic ncRNAs, divergent transcripts, promoter associated transcripts and enhancer RNAs (eRNAs).

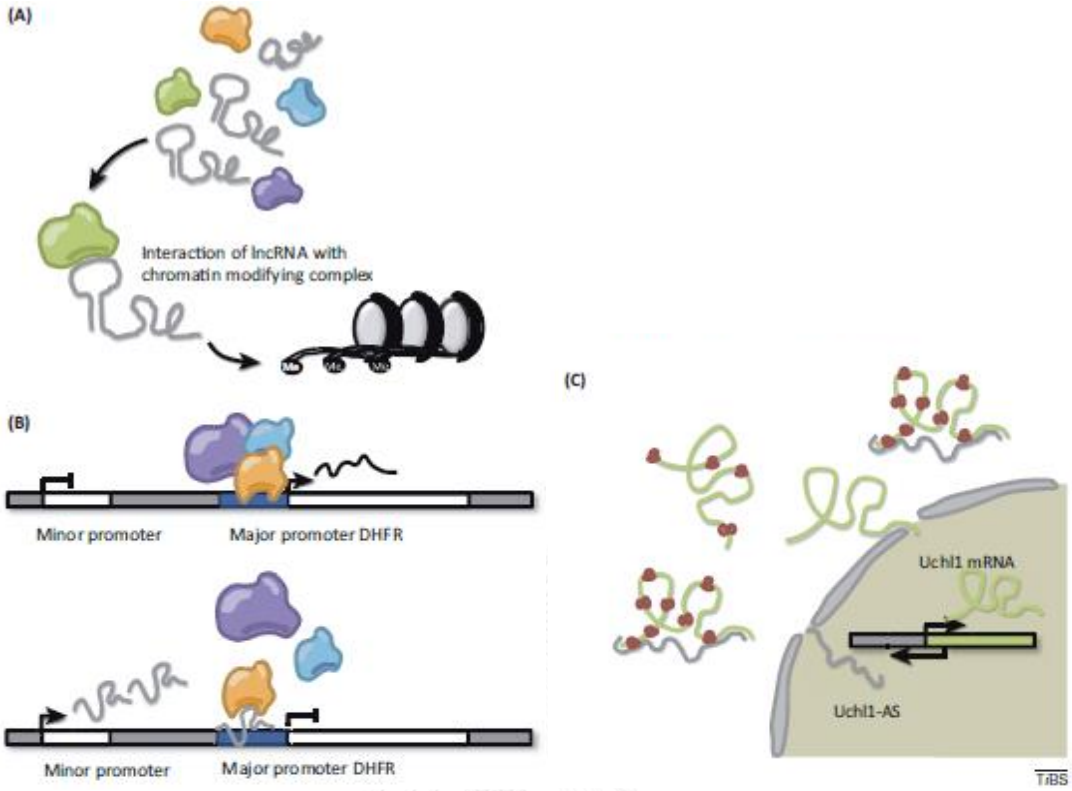

Figure 10. Function of noncoding RNA (ncRNA) (17).

(A) lncRNAs (represented in gray), Xist have been found to interact with chromatin remodeling proteins such as polycomb repressive complex 2 and G9a (represented in green) to mediate deposition of repressive chromatin marks.

(B) In case of dihydrofolate reductase (DHFR) regulation, an upstream lncRNA transcribed from the minor promoter has been shown to bind both the major DHFR promoter as well as transcription initiation factor TFIIB, leading to displacement of TFIIB from the major promoter.

(C) lncRNAs can regulate co-transcriptional processes such as RNA splicing and translation. The

ubiquitin carboxy-terminal hydrolase L1 (Uchl1)-AS RNA is transcribed in times of cellular stress and acts to speed up translation of Uchl1 mRNA.

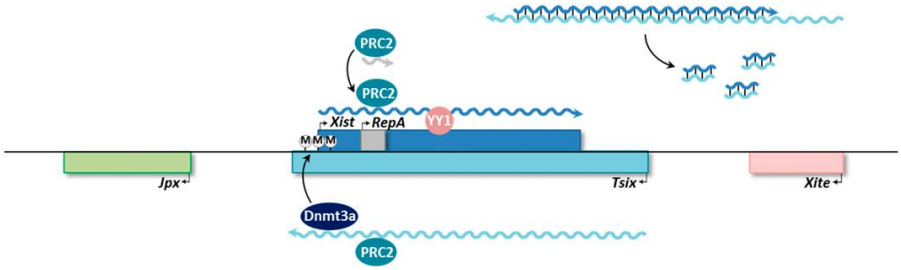

Figure 11. Diagram of X-chromosome inactivation (74).
RepA is thought to recruit PRC2 to the Xist promoter to (paradoxically) upregulate transcription. PRC2 may then be loaded onto Xist, which remains tethered to its allele of origin via YY1 interactions with RNA and DNA. Meanwhile on Xa, Tsix expression is believed to repress Xist through a combination of several mechanisms: titrating away PRC2, preventing its proper docking onto RepA/Xist; recruiting Dnmt3a.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1. Instruments and laboratory supplies

- Automated cell counter (ADVIA210, Bayer, Tarrytown, NY, USA)
- FACSCalibur flow cytometer (BD Bioscience, San Jose, CA)
- CFX realtime PCR instrument (Bio-Rad)
- High speed refrigerated centrifuge (Labnet, Toronto, Ontario, Canada)
- Biological safety cabinet (NUAIRE, Plymouth, MN, USA)
- CO_2 incubator (SHEL LAB, Cornelius, OR, USA)
- LS-Magnetic cell separator (Stem Cell Technologies Inc, Vancouver, BC, Canada)
- MS-Magnetic cell separator (Miltenyi Biotech, Auburn, CA, USA)
- Separation column (Miltenyi Biotech, Auburn, CA, USA)
- 12-well plate (Corning Incorporated, Grand Island, NY, USA)
- 15 mL centrifuge tube (Corning Incorporated, Grand Island, NY, USA)
- 24-well plate (Corning Incorporated, Grand Island, NY, USA)
- 50 mL centrifuge tube (Corning Incorporated, Grand Island, NY, USA)
- 6-well plate (Corning Incorporated, Grand Island, NY, USA)

1.2. Reagent for erythroblast culture

- Culture material including IMDM media (Invitrogen, USA)
- Fetal bovine serum (Sigma-Aldrich, USA)
- Holo-transferrin (Sigma-Aldrich, USA)
- Penicillin and streptomycin (Sigma-Aldrich, USA)
- Erythropoietin (Eprex, USA)

1.3. Reagent for RT-qPCR

- RNase-free DNase I (Fermentas, Canada)
- Random hexamer (Thermo Scientific, USA)
- SYBR™ Real-time PCR Master Mixes (Applied Biosystems, USA)

1.4. Reagent for Rapid Amplification of cDNA ends

- FirstChoice RLM-RACE kit (Ambion, USA)
- Hot Start Taq DNA polymerase (Qiagen, USA)
- Agarose low EEO Molecular Biological Grade (Research Organics, Cleveland, OH, USA)
- DNA ladder 100 bp. plus (Fermentus, Canada)
- Gel extraction kit (Geneaid, Taiwan)
- pGEM-T easy vector (Promega, USA) and reagent.

2. Methods

2.1. Bioinformatic prediction of putative transcripts from HMIR

Chromatin modification profiles at HMIR from the erythroid leukemic K562 cell line were obtained from the ENCODE project (https://www.encodeproject.org/). HMIR is located at chromosome 6:135,300,000 - 135,550,000 (Human hg19 Assembly). RNA polymerase II enrichment and histone modification marks including methylated histone (H3K4me2, H3K4me3, H3K36me3 and H4K20me1) and acetylated histone H3 (H3K9ac and H3K27ac) which are associated with active chromatin were analyzed (57).

We searched the ENCODE database for obtaining evidence of active transcripts. RNA-Polymerase II data was collected using a ChIA-PET experiment with two strands. Histone modification data was collected using a ChIP-seq method. The coverage data of each experiment in a Bigwig format was chosen to determine the number of reads for each specific regions using a bedtools program. All reads with an average of ten base pair are shown.

2.2. Identification of HMIR transcripts

2.2.1 β-Thalassemia/HbE samples

All β-thalassemia/HbE peripheral blood samples were obtained from Nakhonpathom hospital, Thailand. The study was approved by the Ethical Committee, Mahidol University Institutional Review Board. All subjects above 18 years old without blood transfusion for at least one month were enrolled in this study. Patients who have hemoglobin level less than 5 g/dL were excluded. Complete blood counts and RBC indices of β-thalassemia/HbE patients were determined using an automated cell counter (ADVIA210, Bayer, Tarrytown, NY, USA), providing parameters of red blood cell count, hemoglobin level, hematocrit MCH, MCH, MCHC and RDW. Hemoglobin typing and molecular analysis were performed.

2.2.2 Primary erythroblast culture

$CD34^+$ cells were selected from peripheral blood mononuclear cells using a positive immunomagnetic selection method according to the manufacturer's protocol. The selected $CD34^+$ cells were cultured under cell culture conditions for driving erythroid proliferation and differentiation. The $CD34^+$ cells were cultured in IMDM media (Invitrogen, USA) with 20% of fetal bovine serum (Sigma-Aldrich, USA), 300 ng/mL holo-transferrin (Sigma-Aldrich, USA), 0.1 mg/mL penicillin and streptomycin (Sigma-Aldrich, USA), and 5U/mL erythropoietin (Eprex, USA) (75). The culture was maintained in 5% CO_2 at 37 °C for 14 days.

Expression of CD71 (BD Biosciences, US) and GlycophorinA (BD Biosciences, US) in erythroblasts was followed by flow cytometer (BD FACScalibur) to observe cellular differentiation. Erythroblasts were incubated with CD71 PE and Glycophorin APC for 15 minutes. The samples were operated in duplicate reaction. The fraction were analyzed by flow cytometry analysis (BD FACSComp™ software).

2.2.3 RNA extraction and reverse transcription

Total RNA was extracted from day 8 cultured erythroblasts using Trizol reagent (Invitrogen) and sequentially treated with RNase-free DNase I (Fermentas, Canada). Concentration of RNA was determined by *NanoDrop*® instrument. cDNA was generated by reverse transcription with RevertAid First Strand cDNA Synthesis kit using Random hexamer (Thermo Scientific, USA), after comparing the efficiency between random hexamer and oligo-dT. The synthesized cDNA was used to measure the expression of HMIR transcript.

2.2.4 Quantitative real-time PCR (qPCR)

qPCR assays were performed with primers specific to the HMIR transcripts (Table 3). Primers were designed specific to 3 regions of histone modification sites after bioinformatics analysis using primer3 version 2.2.3, synthesized by idtDNA (Singapore) (Figure 13). Each of the region has 3-4 fragments for amplifying by qPCR to cover desired area. The length of PCR products are around 200 bp. PCR reaction and master mix condition was done following the manufacturer's recommended SYBR™ Real-time PCR Master Mixes (Applied Biosystems, USA) (Table 2). Real-time PCR analysis was performed with the CFX instrument (Bio-Rad). Expression of GAPDH transcript was used to normalize and the transcript level of HMIR was quantitated using ΔCt value. For each PCR, two control reactions of no reverse transcriptase and no template were included to monitor genomic DNA contamination.

Table 2. PCR profile for detection of HMIR transcript.

Step	Temperature and Time	Cycles
Initial denaturation	95 °C 10 minutes	1
Amplification	95 °C 10 seconds 58 °C 45 seconds	55

Table 3. Primers for detection of HMIR transcript by qPCR analysis.

Name	Forward primer (5´ to 3´)/ Reverse primer (5´ to 3´)	Product sizes
HMIR#A1	CCCAGTGTGATGGCATTAGAA/ GTGAAGGCTGAAGGAGAACAA	221 bp.
HMIR#A2	TCATCCTGGCTCATTCTCAAAT/ AACTAAATAGAGTCTCTGAGGCAAA	203 bp.
HMIR#A3	GCCTGCTGTTTACAGTCCATA/ GAGGCATTTCACAGAATCCAAAG	264 bp.
HMIR#B1	CCCAGGGCAACAATTAAGAAA/ CAGGGAGAGTCTATGTCATCTG	246 bp.
HMIR#B2	AAGACATGCACCAACCAAATC/ CAGCACATCAGTCTCTCTGTC	273 bp.
HMIR#B3	ACCAGTGGTGAACTGGTAAG/ ATCATGGGAGTGATGCAATAGT	258 bp.
HMIR#B4	GACCAGCCTGGACAACATAG/ CATTCCTCCTGTCGATGAAAGA	239 bp
HMIR#C1	GCTAAGGGTTCAAGGTCATACA/ ATCCTAGCCTCTTACCCTAACA	254 bp.
HMIR#C2	GATCGTGCTATCCAGAACTAACC/ GGGCCCAATGTTAGTGAATCT	249 bp.
HMIR#C3	CCTAGGTTGTCTTCTAGGGTTT/ ACAGAATAGAGGCCTCAGAAATAG	270 bp.
HMIR#D1	GCCTTTCTGAGAAGGTGACAT/ TATTGTTGGAGCCTGGATTCTT	159 bp.

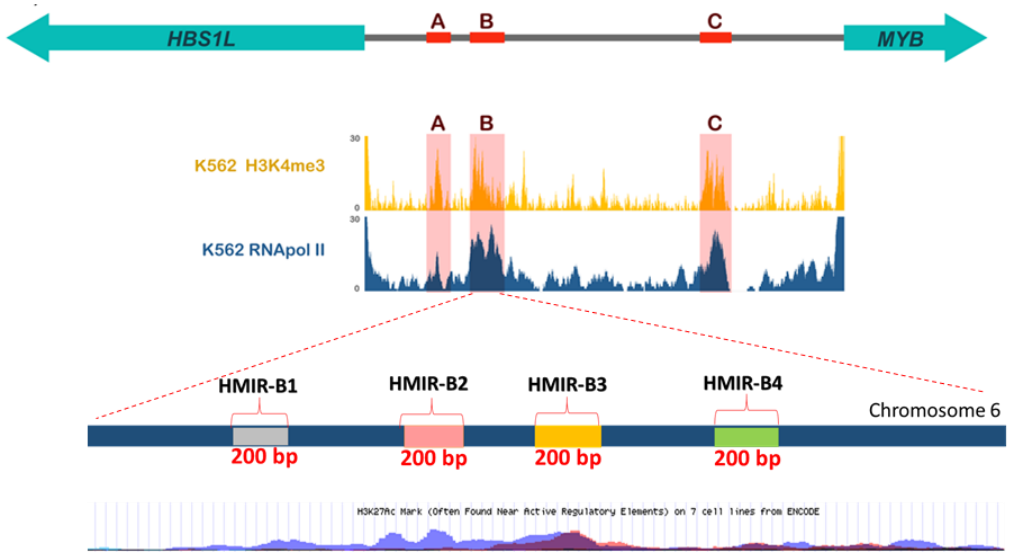

Figure 12. Primers for amplifying HMIR transcripts.
All primers were designed binding to 3 regions of histone modification site; A, B and C. and region D was used as a negative control.

2.3. Expression of the *HBS1L-MYB* intergenic region (HMIR) transcripts in β-thalassemia/HbE erythroblasts

CD34+ hematopoietic progenitor cells were collected from five healthy donors and ten β-thalassemia/HbE patients. β-thalassemia/ HbE patients were divided into two groups based on the HbF level from HPLC with the high HbF level group (HbF > 30%) and the low HbF level group (20%<HbF<30%). Erythroid cultures and RNA analysis were conducted as described above.

2.4. Determination of γ-globin expression

cDNAs of normal erythroblasts and β-thalassemia/HbE erythroblasts were used to determine the level of gamma-globin. Standard curve of gamma-globin plasmid was performed by varying plasmid amount as follows: 10^7, 10^6, 10^5, 10^4 and 10^3 copies. Amount of gamma-globin was quantified based on the standard curve.

2.5. RACE (Rapid amplification of cDNA ends)

2.5.1 5′RACE and 3′RACE analysis

RACE analysis was performed to identify the exact sequence of the transcripts using the FirstChoice RLM-RACE kit (Ambion, USA). 10 µg of RNA was extracted from day 8 cultured normal erythroblasts. 5′ RACE and 3′ RACE reactions were performed with DNase RNA template to remove DNA contamination.

To determine the 5′-end of cDNA, RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) and incubated at 37°C for 1 hour to degrade 5′-phosphate from molecules of ribosomal RNA, fragmented mRNA and the rest of contaminated DNA. RNA was then extracted using phenol:chloroform. After precipitation, RNA was treated with Tobacco acid pyrophosphatase (TAP) and incubated at 37°C for 1 hour to remove the cap structure. Ligation adapter was linked to the 5′-ends using T4 RNA ligase at 37°C for 1 hour. RNA was converted from RNA to cDNA by reverse transcriptase enzyme and random hexamer. Nested PCR was then performed to amplify the 5'-end of a specific transcript with two primers: outer and inner primers corresponding to the 5' RACE Adapter sequence (Figure 14, 16). For the 3´-RACE reaction, RNA was converted to cDNA with the 3′-adapter which contains poly-T

sequence using reverse transcriptase enzyme. cDNA was then amplified by nested PCR with inner and outer primers (Figure 15, 16).

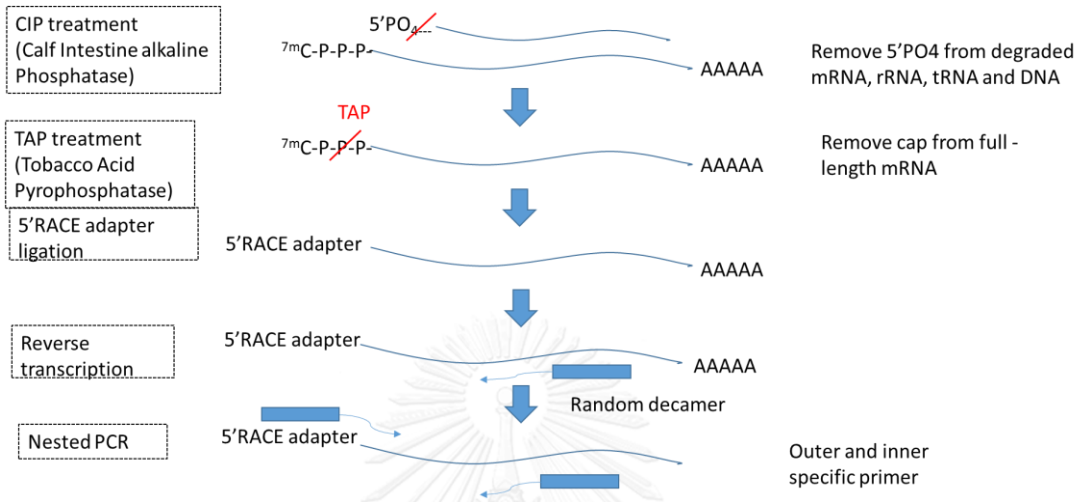

Figure 13. Schematic of 5′-RACE protocol.
Poly-A RNA (blue line) were treat with CIP and TAP (red text) to ligate with adapter. Random hexamer, outer and inner primer (arrow plus square blue) were used to amplify the product.

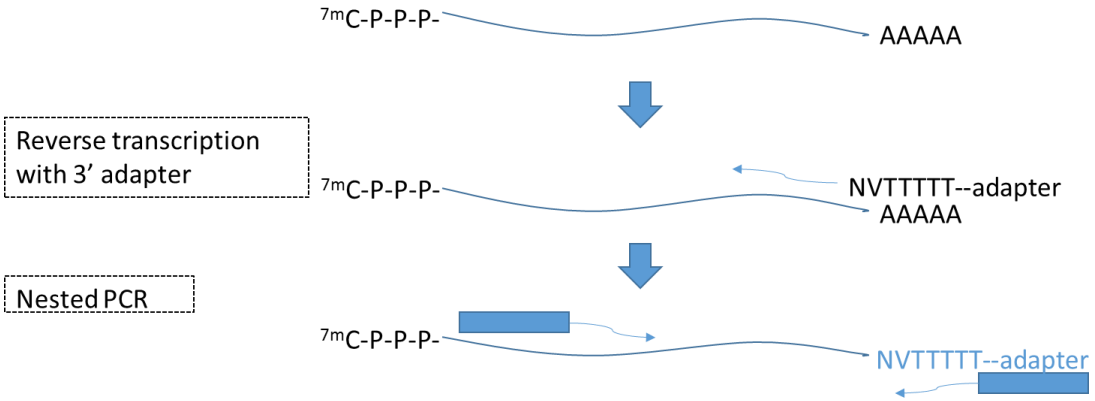

Figure 14. Schematic of 3′RACE protocol.
Poly-A RNA (blue line) were amplified with adapter and outer and inner primer (arrow plus square blue).

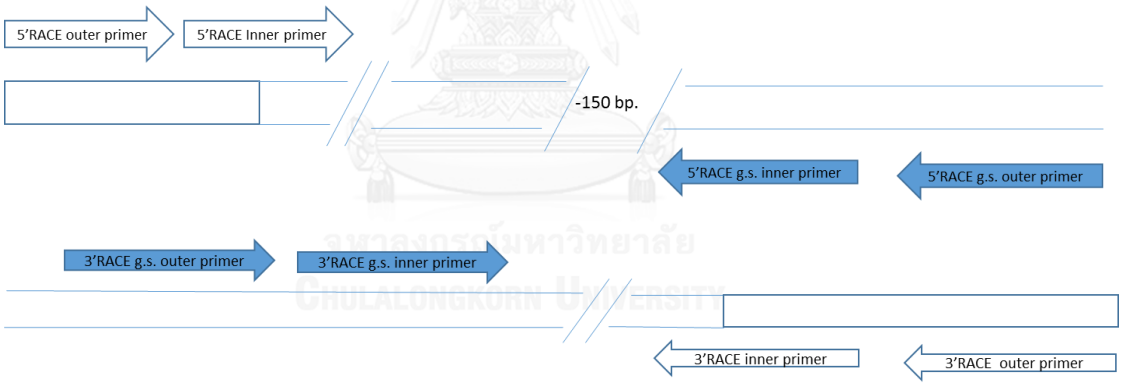

Figure 15. Diagram of Outer and inner primer RACE.
Upper picture show position of 5′ RACE and lower picture show position of 3′ RACE.

2.5.2 Cloning of RACE product

5′RACE and 3′RACE products were amplified using Hot Start Taq DNA polymerase (Qiagen, USA) in optimized condition (Table 4). All products were separated on a 1.5% agarose gel. The specific bands were cut and purified using a gel extraction kit (Geneaid, Taiwan). RACE products were then cloned into a vector. The RACE products were inserted in to pGEM-T easy vector (Promega, USA) (Figure 17). The inserts were ligated with the pGEM-T easy vector which contains T/A overhang. The reaction was incubated at 4°C overnight. The ligation reaction was transformed into competent cells, which was checked for successful insertion on X-gal plate.

Table 4. PCR condition for amplifying RACE products.

Step	Temperature and Time	Cycles
Initial denaturation	98 °C 5 minutes	1
Amplification	98 °C 30 seconds 60 °C 90 seconds 72 °C 135 seconds	35
Extension	72 °C 5 minutes	1

* Vary annealing temperature

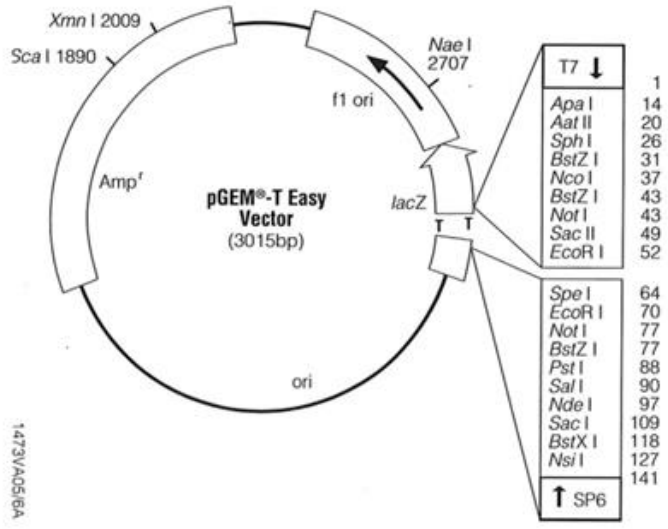

Figure 16. Structure of pGEM-T Easy Vector.

This vector is linear vector which contains extra T and many cut sites of restriction enzymes between T7 promoter and SP6. Length of vector is 3015 bp. Ampicillin resistant gene is drug marker. lacZ promoter was insert in this vectors.

2.5.3 Blue/white colony selection

pGEM-T Easy Vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase (Figure 15). Insertional inactivation of the fragments allows identification of recombinants blue/white screening on indicator plates. White colonies that contain RACE fragment was picked up. White colonies are inoculated and cultured overnight in LB media with ampicillin for antibiotics selection.

2.5.4 Sequencing analysis

Plasmid DNA was extracted using plasmid extraction kit (Geneaid, Taiwan). After adjusting concentration of plasmid, DNA are sent for sequencing with primer T7 promoter and SP6 (idtDNA).

CHAPTER IV
RESULTS

1. Active chromatin at HMIR region predicted by ENCODE database

The ENCODE data from the UCSC Genome Browser was used by retrieving the information specific to chromosome 6:135,300,000 - 135,550,000 (Human hg19 Assembly). Three regions were chosen as putative actively transcribed DNA sites including region A (135,392,581-135,398,941) size 6360 bp., region B (135,403,981-135,413,101) size 9120 bp. and region C (135,464,581-135,472,861) size 8280 bp. Transcriptional activity in all three HMIR regions (region A, B and C) of the erythroid leukemic K562 cell line were shown at RNA Polymerase II, methylated histone H3 (H3K4me2, H3K4me3 and H3K36me3) and acetylated histone H3 (H3K9ac and H3K27ac) (Figure 18). All these regions are generally associated with active chromatin. Region D is a negative control that has no major altered in these histone marks and Pol II enrichment was presented.

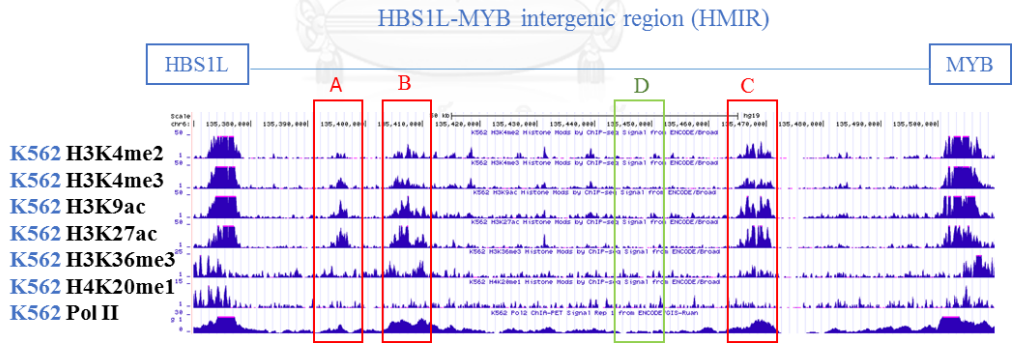

Figure 17. Evidence of active chromatin at HMIR region of K562 cell line.
The high expression peaks were showed at histone modification (blue peak) including H3K4me2, H3K4me3 H3K9ac, H3K27ac, H3K36me3 and H4K20me1 regions. RNA Polymerase II (blue peak at lower line) was generated from UCSC Genome Browser chromosome. Red Square represents region A, B and C. Green square represent region D which used as a negative control.

So far, little is known about the functional role of specific motif at HMIR. A 3-bp deletion polymorphism at the HMIR region (John J. Farrell, 2011) was shown to affect HbF expression. The 3-bp region was located outside the histone modification sites and region A, B and C (Figure 19).

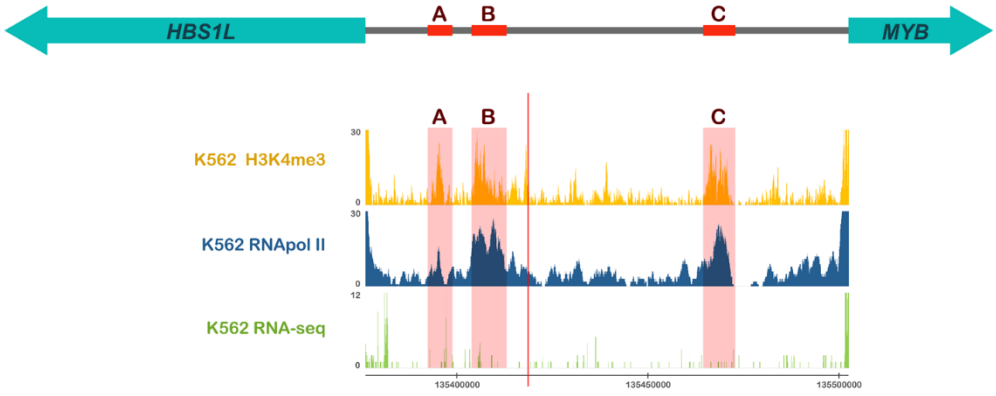

Figure 18. Position of a 3-bp deletion polymorphism in HMIR.

HMIR in A, B and C correspond to histone modification site. Red line indicates a 3-bp deletion polymorphism near region B.

2. qPCR analysis optimal for amplifying HMIR in DNA template

The optimal real-time PCR condition was optimized by using genomic DNA of normal sample as a template. All regions could be amplified with Ct values in range 23 – 32 cycles, suggesting that all designed primer sets could be used for detection HMIR transcript (Figure 20).

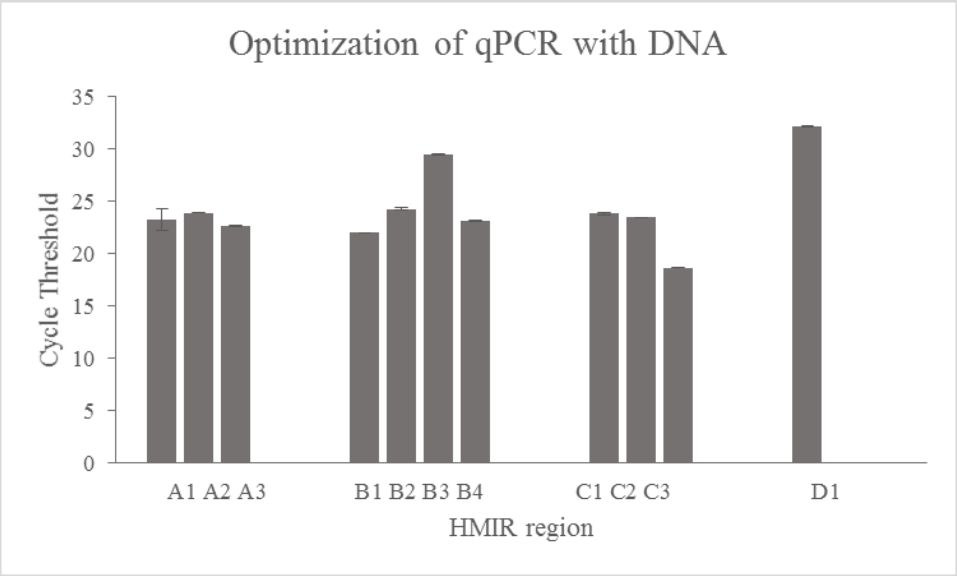

Figure 19. Optimization of qPCR with DNA to find proper primers.

Primers were tested with DNA 40 ng per well. The designed primers are specific to HMIR regions. Region A, B, and C were actively expressed, and region D as negative control. Graph was shown by cycle threshold of HMIR non-coding RNA.

3. qPCR analysis reveals expression of HMIR non-coding RNAs

The optimized condition of real-time PCR was then used to detect HMIR transcript with cDNA as template. The PCR reaction with no reverse transcriptase in combination with DNase treatment was included to exclude DNA contamination. The optimized PCR condition with specific primers was successfully detected HMIR when cDNA was used as a template. The signals of HMIR were shown at Ct values more than 30 cycles in all region (A, B and C). Highest signal was observed in region C with Ct values of 30 cycles. Melting temperature analysis showed only a single peak. Region A of HMIR showed melting temperature at 75 degree Celsius, region B and C showed melting temperature at 80 degree celsius, indicate that this optimized protocol can used to amplify HMIR transcripts and generated specific products (Figure 21).

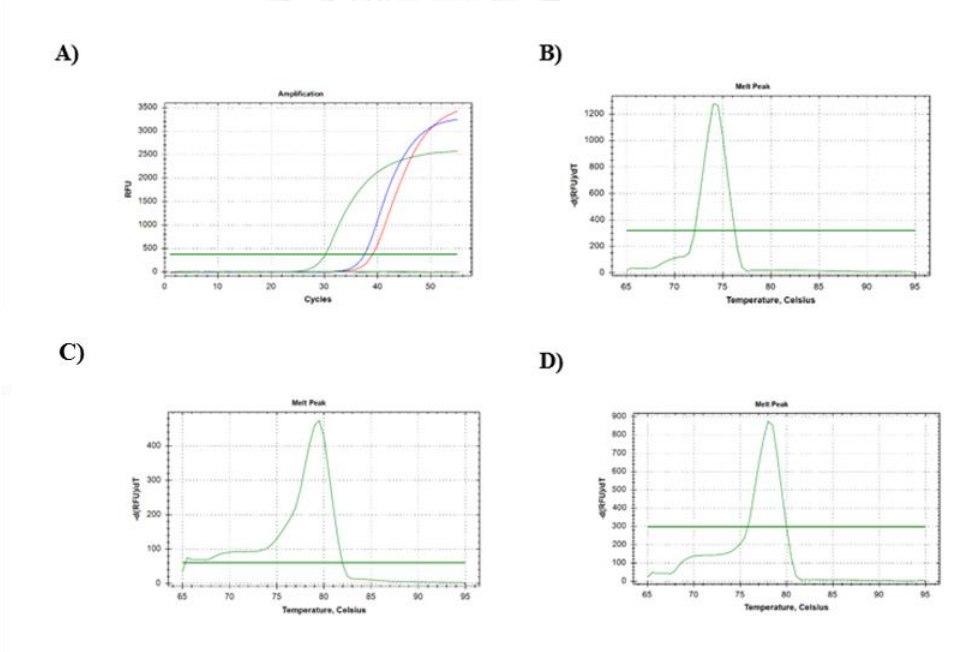

Figure 20. Cycle threshold and Melting curve in amplifying HMIR transcripts.

Amplification of HMIR transcripts from RNA of normal erythroblasts (A). Red line represent fluorescence signal of region A, Blue line represent fluorescence signal of region B and green line represent signal of region C. Melting curve of region A, B and C are shown in graph (B), (C) and (D), respectively.

The level of HMIR expression in K562 were also determined by qPCR. The expression of HMIR in K562 were shown as cycle threshold, high cycle threashold indicated low amont of HMIR transcripts. In region A and region C, HMIR expession are lower 40 cycle, region C are above 40 cycles. We cannot detected the signal at region A1 and C1, suggesting that K562 have low HMIR expression (Figure 22).

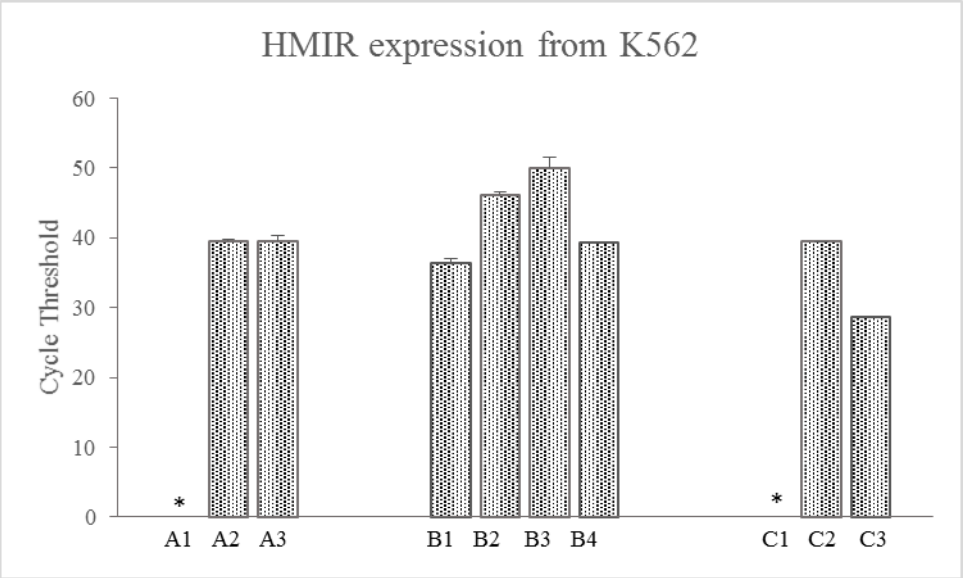

Figure 21. HMIR expression in K562 erythroluekemic cell line.

40 ng of cDNA of K562 were detect expession using qPCR in all primer. The graph were shown as cyclcle threshold.(*) are undetectable signal.

4. Efficiency of random hexamer and oligo-dT primer for the detection of HMIR transcripts

Efficiency of random hexamer and oligo-dT primer were compared for the synthesis of cDNA used for future amplifying with qPCR. Same concentration of RNA was added as a template for reverse transcription reaction. Three primer pairs showed low Ct values (A3, B4 and C1) which were chosen for the test. The A3 and C1 regions could not be detected when used oligo-dT primer for synthesis cDNA, suggesting that these HMIR transcripts are not polyadenylated. On the other hand, there is no difference between oligo-dT and random hexamer in the amplification of coding GAPDH and actin genes as well as B4 region (Figure 23). However, the signals of HMIR transcripts are much lower than those of coding mRNAs of actin and GAPDH.

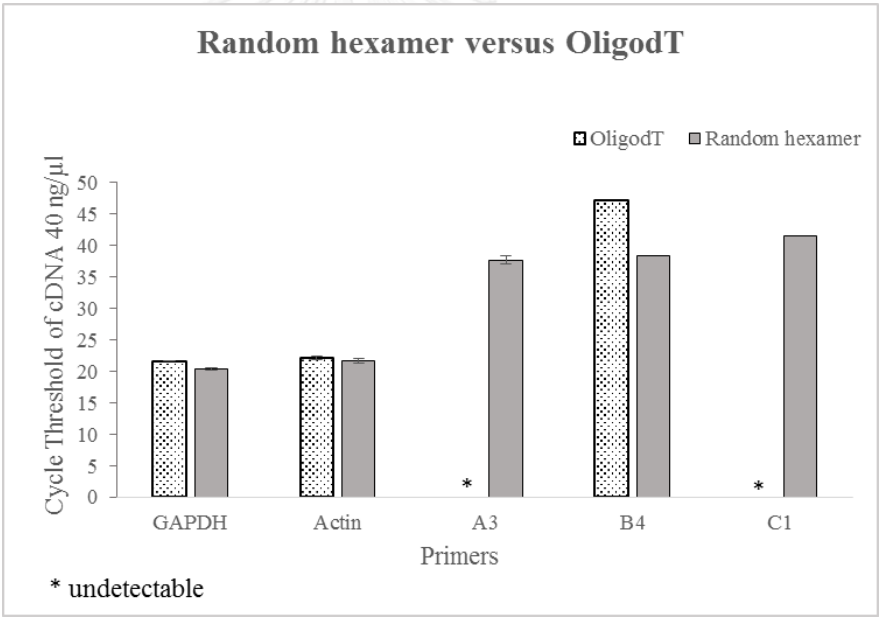

Figure 22. Amplification of HMIR transcripts using random hexamer compared to oligo-dT primer.
cDNA synthesized from oligo-dT primer could not amplify region A3 and C1. Both GAPDH and actin can be amplified when used either random hexamer or oligo-dT for cDNA synthesis. Low Ct values refer to high amount of gene products.

5. CBC and Hb typing results of β-thalassemia/HbE patients

Nine β-thalassemia/HbE patients were included to determine expression levels of HMIR transcripts compared to 5 normal subjects. The β-thalassemia/HbE patients carry mutation at β-globin gene including mutation at codon 17 (A>T) and deletion at codon 41/41 (-TCTT) together with mutation at codon 26 (G>A). The patients have low hemoglobin, MCV, MCH, MCHC and high RDW (Table 5), indicating hypochromic microcytic red blood cell. Severe patients have hemoglobin level and number of red blood cells lower than that of mild patients (Table 5). Higher HbF levels were found in mild pateints (38 %) as compared to severe cases (18 %).

Table 5. Hb typing, CBC and DNA analysis of nine β-thalassemia/HbE patients.

Parameters	**Mild patients (n=4)**	**Severe patients (n=5)**
Gender (M/F)	(3:1)	(2:3)
Age	(10-54)	(7-34)
Hb (g/dl)	8.125 ± 0.6	5.9 ± 0.5
MCV (fl)	59.075 ±2.97	61.44 ± 4.86
MCH (pg)	18.35 ± 1.11	17.52 ± 1.21
MCHC (g/dl)	31.125 ± 1.83	28.72 ± 3.11
%RDW	25.15 ± 2.16	28.36 ± 3.17
RBC ($x10^6$ cell/µl)	4.3525 ± 0.40	3.312 ± 0.40
WBC ($x10^3$ cell/µl)	9.2175 ± 0.71	9.31 ± 4.84
%HbA	3.875 ± 3.57	25.46 ± 29.64
%HbA2, E	53 ± 10.17	53.34 ± 24.49
%HbF	37.8 ± 8.37	17.92 ± 5.42
Non-Splenectomy	4	3

6. Differentiation of cultured erythroblasts

Normal and β-thalassemia erythroblasts were cultured to increase the cell number and induce differentiation of erythroid cells. The differentiation of cultured erythroblasts was monitored by flow cytometry using CD71 (transferrin receptor) and glycophorin A as markers of differentiation. Four erythroblast regions were gated. Proerythroblasts ($GlyA^{low}CD71^{high}$) were predominantly observed in day 6 of culture. The basophilic erythroblasts ($GlyA^{high}CD71^{high}$) were predominantly observed in day 8 with showed increased signal of glycophorin A. Polychromatic erythroblasts ($GlyA^{high}CD71^{medium}$) were predominantly at day 11 and orthochromatic erythroblasts ($GlyA^{high}CD71^{low}$) were predominantly at day 13 (Figure 24).

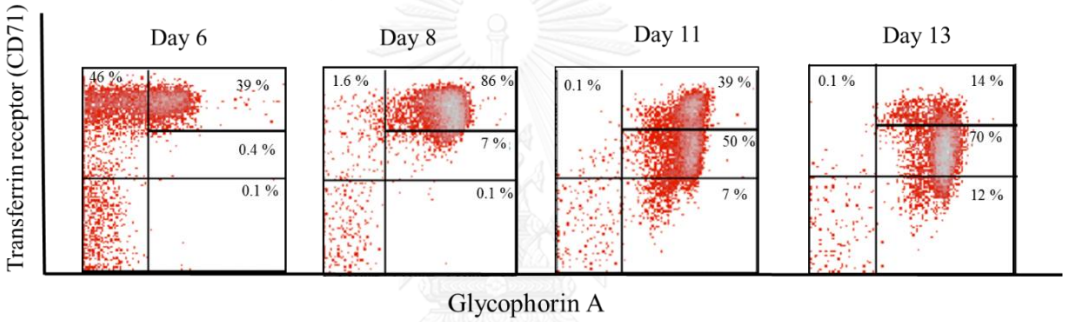

Figure 23. Differentiation profile of cultured erythroblasts.

Differentiation of cultured erythroblasts at day 6, day 8, day 11 and day 13 were analyzed by flow cytometry. Differentiation of erythroid cells was determined using CD71 PE and GlycophorinAPC.

7. Expression of HMIR during erythroid maturation

The expression level of HMIR in each stage of cultured erythroblasts was investigated. The HMIR transcripts were measured in erythroblasts at culture day 6, 8, 11 and 13. The expression levels of GAPDH were used as a normalizer. QPCR analysis revealed that the level of HMIR transcripts increased when cells undergo differentiation from proerythroblasts to early erythroblasts (highly expressed), but decrease in late erythroblasts (Figure 25). Hence, the analysis of HMIR in the next experiment was performed from day 8 erythroblasts.

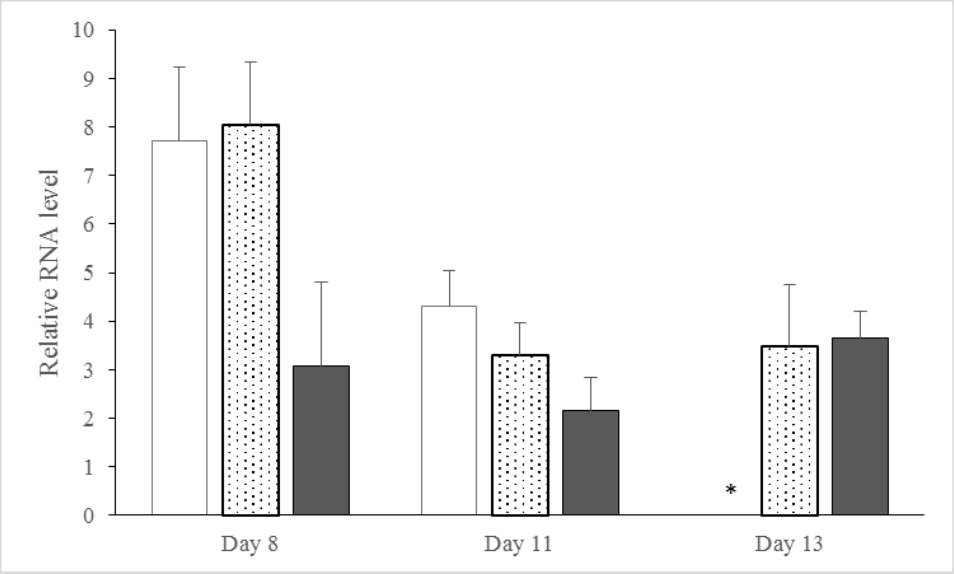

Figure 24. HMIR amplifying of each day of culture erythroblasts.
HMIR transcript levels in normal erythroblasts at culture day 8, day 11 and day 13 were monitored by relative RNA level normalized to day 6 culture erythroblasts (-ΔΔCt). HMIR transcripts were amplified using primers of region A3 (white bar), region B4 (dot bar) and region C (gray bar). (*) represents undetectable signal.

8. Down-regulation of HMIR in β-thalassemia/HbE erythroblasts

We determined the level of HMIR transcripts derived from the erythroblasts from β-thalassemia/HbE patients and normal individuals. In general, the signals from the patients are relatively lower by several cycles compared to those of normal controls. However, the caveat is the signals from non-coding transcripts is extremely low or absent in certain cases. The biggest difference between the patients and normal controls is the C3 transcript. It is worth noting that this primer set gives the highest signal to the level of Ct cycles from the coding gene controls. Reduction in C3 transcripts in patients provides the biggest difference and is the best example from all non-coding transcripts (Figure 26).

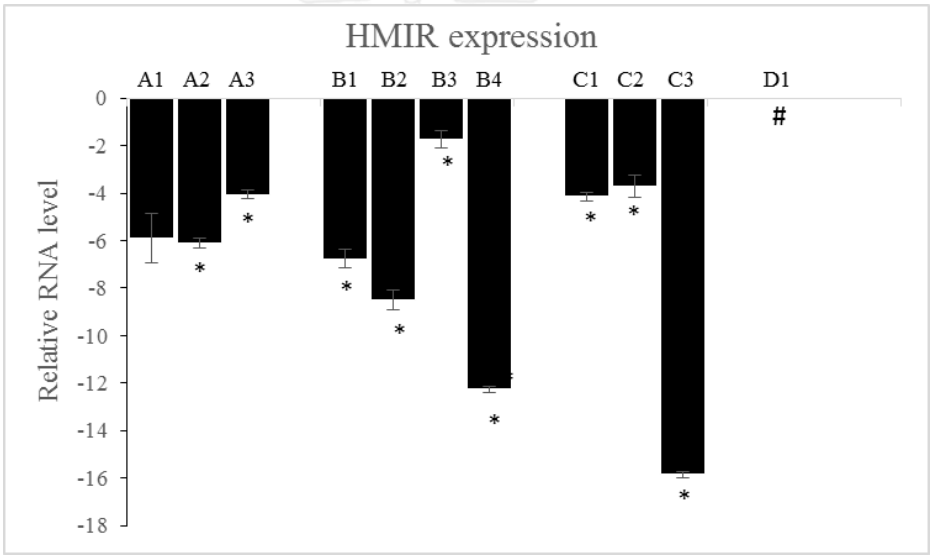

Figure 25. HMIR expression in K562 erythroid cell line, β-thalassemia/HbE and normal erythroblasts.

HMIR transcripts were amplified with specific primer in β-thalassemia/HbE and normal erythroblasts. Analysed data was shown in relative RNA level of β-thalassemia/HbE which compared to that of normal erythroblasts following this calculation formular $-\Delta\Delta Ct = -(\Delta Ct_{\beta\text{-Thalassemia (HMIR-GAPDH)}} - \Delta Ct_{\text{normal (HMIR-GAPDH)}})$. GAPDH was used as a reference gene. Black bar represent the level of HMIR transcripts in K562 cell. p-value(*) < 0.05.(#) are undetectable.

9. Gamma (γ) globin level in β-thalassemia/HbE

cDNA samples of β-thalassemia/HbE and normal erythroblasts were used for determining the copy number of γ-globin. Absolute quantification of γ-globin was calculated based on a standard curve of gamma globin plasmid (Figure 27). The average γ-globin in normal erythroblasts is $0.25 \times 10^5 \pm 0.09 \times 10^5$ copy number/ng. total RNA which is lower than that of β-thalassemia/HbE erythroblasts ($3.56 \times 10^5 \pm 2.49 \times 10^5$ copy number/ng. total RNA) (Figure 28).

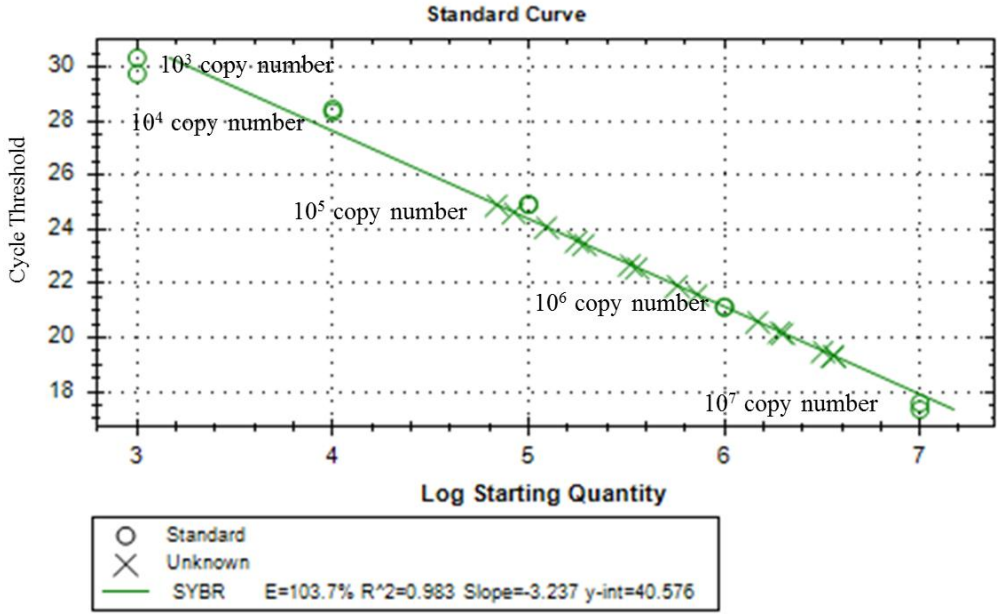

Figure 26. Standard curve of γ-globin.

Copy numbers of γ-globin were standardized by 5 concentration of plasmid DNA including 10^7, 10^6, 10^5, 10^4, 10^3 copy number/µl. The concentrations of standardized plasmid were analyzed in duplication reactions.

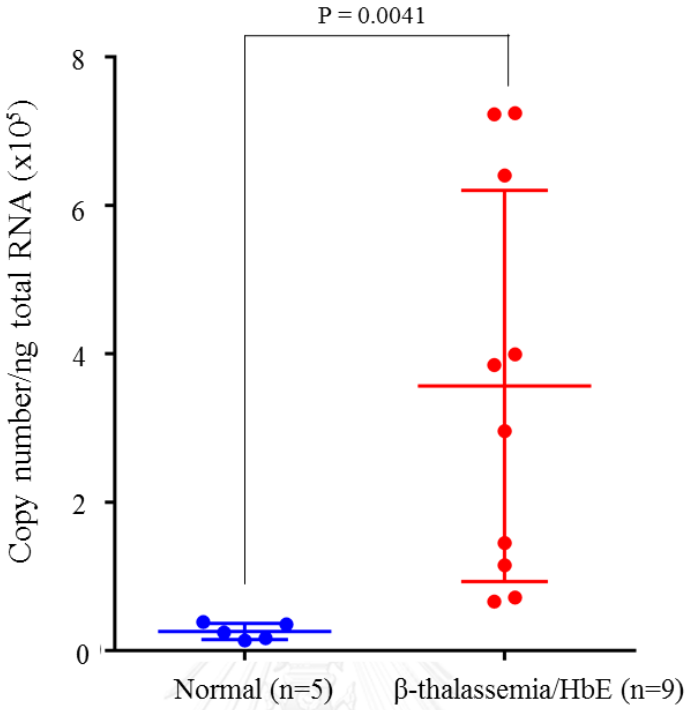

Figure 27. Comparative γ-globin mRNAs in normal and β-thalassemia/HbE erythroblasts.

cDNA of erythroblasts derived five normal (white bar) and nine β-thalassemia/HbE erythroblasts (black bar) were compared for the level of γ-globin. The two groups has statistically significant different level of gamma globin with *P*-value = 0.0041, tested by unpaired t-test with Welch's correction.

10. Association of HMIR and HbF levels

To prove that whether the amounts of γ-globin in pateints associated with level of HMIR expresion, HMIR levels in β-thalassemia/ HBE with high HbF level (HbF > 40%) and low HbF level (20%<HbF<40%) were compared. The levels of HMIR transcripts in both comparative groups were normalized to HMIR transcripts in normal erythroblasts. We found that the levels of HMIR transcripts in high-HbF and low-HbF β-thalassemia/ HbE erythroblasts are lower than that of normal erythroblasts, however, the HMIR transcripts in high-HbF patients are not significantly different from that of low-HbF β-thalassemia/ HbE patients (Figure 29). Correlations between the HMIR transcripts and gamma globin expression were found only with non-coding transcripts from region B1 and B3 (Figure 30).

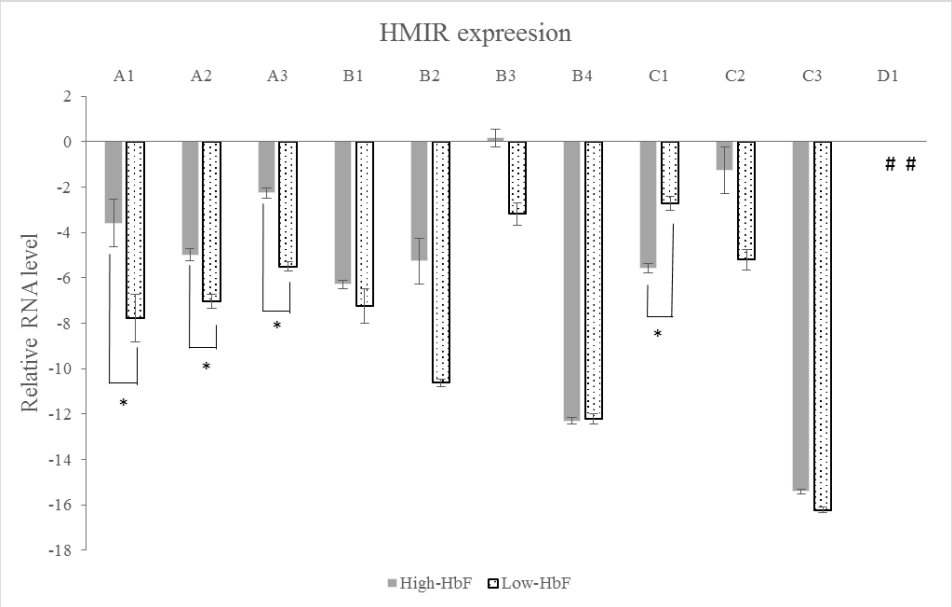

Figure 28. HMIR expresion in high HbF and low HbF β-thalassemia/ HbE.

HMIR levels were compared between the two group of patients. Data was shown in relative RNA level of β-thalassemia/HbE which compared to that of normal erythroblasts $-\Delta\Delta Ct = -(\Delta Ct\beta\text{-thal/HbE}_{(HMIR\text{-}GAPDH)} - \Delta Ct\ normal_{(HMIR\text{-}GAPDH)})$. GAPDH was used as a reference gene. Gray bar represent the level of HMIR transcripts in high HbF β-thalassemia, dot bar from low HbF β-thalassemia/pateints. P-value(*) < 0.05. (#) are undetectable.

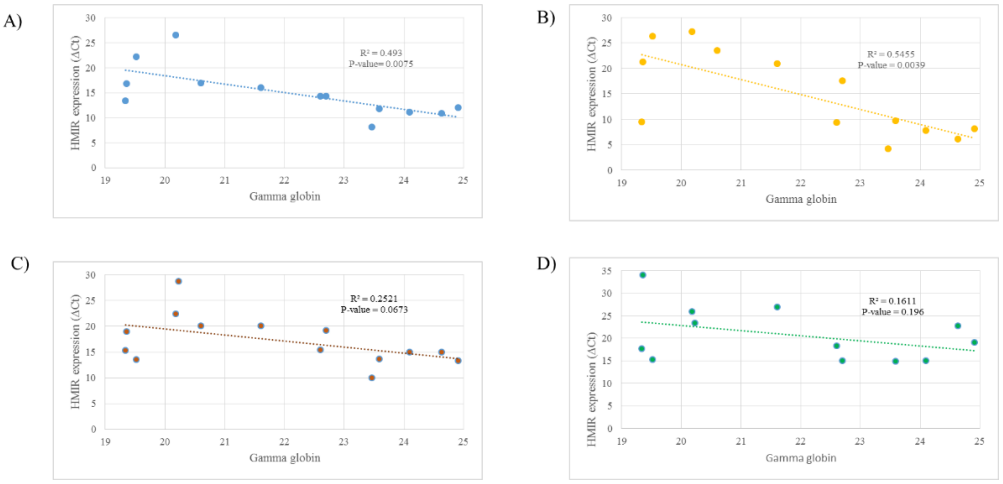

Figure 29. Relationship between HMIR expression and gamma globin in normal erythroblasts and β-thalassemia erythroblasts.
A) and B) show significant correlations (p value <0.05) between amount of HMIR transcripts and gamma globin expressin in region B1 and B3, respectively. C) and D) show no correlation between amount of HMIR transcripts and gamma globin expressin in region A1 and C1, respectively.

11. HMIR transcripts characterized by RACE Analysis

In order to determine whether the HMIR non-coding RNAs are intact long-noncoding RNA, 5′ RACE analysis was performed to define the nature of the HMIR transcripts. Nested PCR with outer primer could not detect any PCR product therefore, the inner primer was required in this experiment. The inner primer could amplify the HMIR PCR product and subsequently used for the next PCR step. After finishing two rounds of PCR process in 5′ RACE analysis, one band with size of 800 bp from region A, 800 bp and 100 bp from region B and 400 bp from region C were detected (Figure 31).

The 3′-RACE reaction using inner primer was performed with the PCR product amplified using outer primer as template. We detected a larger PCR product from region A (1200 bp) and a productsize of 300 bp from region B. Interestingly, we observed two bands from region C (size 500 bp and 300 bp) (Figure 32).

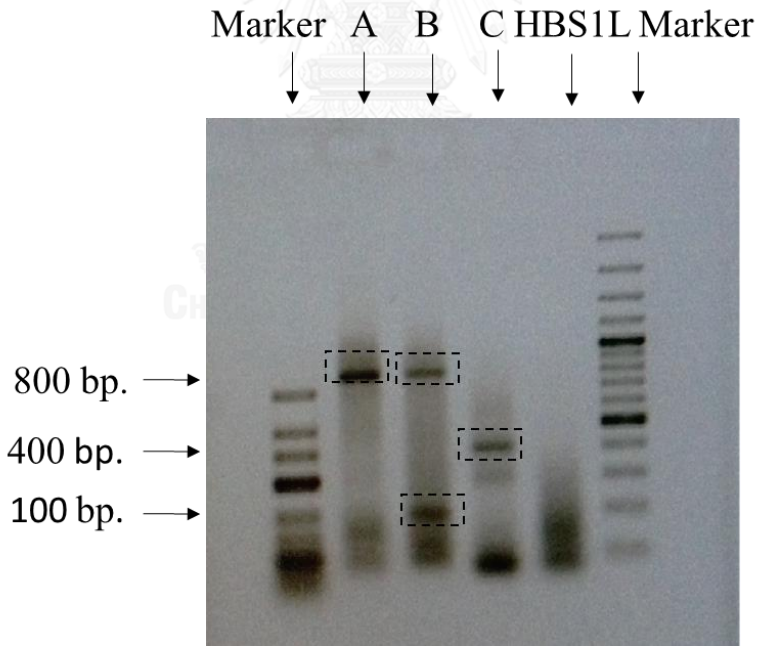

Figure 30. 5′ RACE product amplified from the inner primer.

PCR products amplified by the outer primer were used as template to the second round of nested PCR reaction. The PCR products were separated on 1.5% agarose gel electrophoresis and visualized by EtBr staining.

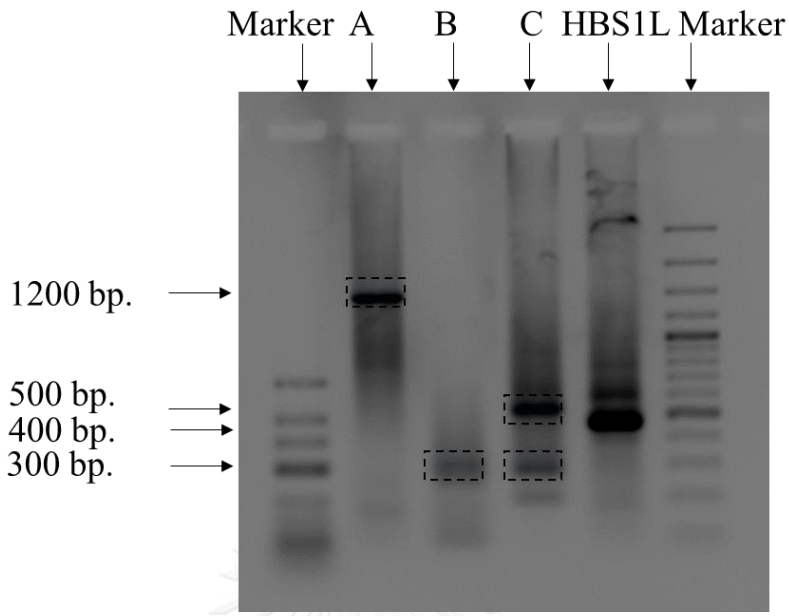

Figure 31. 3′ RACE product amplified from the inner primer.
The PCR products were separated on 1.5% agarose gel electrophoresis and visualized by EtBr staining.

12. Nucleotide sequence of RACE product

The sequences of 5′ RACE and 3′ RACE products were verified by sequencing analysis. PCR products from agarose gel were cut and purified to reduce non-specific interfering. The PCR fragments were inserted in to pGEM-T easy vector using T/A overhang cloning. The clones were selected for DNA sequencing and blast to sequence database. The results are listed in Table 6. The sequences of RACE products were matched to mRNAs in various chromosome regions but not matched with the HMIR sequences.

Table 6. Result of sequencing from RACE product.

Region	Sequencing
5′A3 region	Homo sapiens P1 protein (Cdc42/Rac)-activated kinase2 pseudogene (LOC646214), ncRNA)
3′A3 region	Homo sapiens LYR motif containing 9 (LYRM9)
5′B4 region	Homo sapiens uncharacterized LCO105377857, transcript variant X2, ncRNA
	Homo sapiens 45s ribosomal RNA
3′B4 region	Homo sapiens tripartite motif containing 8 (TRI8), transcripts variant X2, mRNA
5′C3 region	Homo sapiens chromosome 11, mRNA
	Homo sapiens chromosome 3, mRNA
3′C3 region	Homo sapiens chromosome 3, mRNA
	Homo sapiens chromosome 21, mRNA
	Homo sapiens chromosome 7, mRNA

CHAPTER V
DISCUSSION

The clinical heterogeneity of β-thalassemia/HbE present a challenge in patient management. Increase level of HbF would be a improve the quality of life of β-thalassemia/HbE patients (44). Understanding the impact of HbF associated factors will be important in defining their biological roles and for the development of new therapies. HMIR was revealed by a series of GWAS studies and was found to have the impact on disease severity (49). HMIR polymorphism are highly associated with HbF in β-thalassemia carriers. In this study, the levels of non-coding HMIR transcripts were determined and we found the lower expression of HMIR in β-thalassemia/HbE erythroblasts as compared to normal erythroblast. It also suggests a putative mechanism for adjusting γ-globin expression, probably via MYB.

HMIR is located near the *MYB* gene that produces a transcription factor and plays role in erythropoiesis as repressor of the gamma globin gene (14). It is possible that HMIR functions as an enhancer for activating *MYB* leading to decrease the level of γ-globin. This knowledge is consistent to higher *HMIR* expression in comparison to lower level of γ-globin copy number in normal erythroblasts. It is important to determine the function of this region and the underlying mechanism of the non-coding transcripts role in adjusting disease severity.

The work presented here is originated from RNA-Seq showing non-coding RNA (ncRNA) from *HMIR*. In general, non-coding RNAs are transcribed by RNA polymerase II. Their transcriptional machineries are consistent to those transcribing coding genes, which allow the identification of transcription signature using histone modification sites such as H3K4me3 and H3K27Ac (17). Non-coding RNAs were proposed to play a functional role of recruiting transcriptional factors to turn the genes on or off. The best example in that category is the role of non-coding RNA in X-inactivation (76).

Recently, several enhancer elements were found to be associated with their own transcriptional activity. Enhancer RNA (eRNA) is a short transcript associated with the enhancer region. eRNAs were found to be generated from many enhancer regions, and

the transcripts from HMIR might be a novel eRNA group. They are not contiguously long transcripts like long non-coding RNA. They are transcribed by RNA polymerase II as well. eRNA was shown to bind transcription factor and enrich the proteins close to the promoter region (66). The enrichment by eRNA bring necessary transcription factors to the promoter of choice.

The analyses as presented in the dissertation confirmed that the *HMIR* non-coding transcripts exist in K562 and erythroblast cells. They are detected by using qRT-PCR with the pattern consistent to the RNA-seq data. The amount of HMIR transcripts is associated with fetal hemoglobin in normal group. No association pattern was observed in β-thalassemia/HbE.

However, the ends of the transcripts were not mapped by using the RACE techniques. Even though the primers were sequentially moved to locate the RNA, no clone with matching sequences to *HMIR* were observed. One possibility is that the non-coding RNA might not be capped and polyadenylated similar to that of the conventional coding transcripts (17). The 5´-RACE technique requires intact 7-methylguanosine to keep the RNA intact for enzymatic ligation to the adaptor. Without the cap, the 5´ phosphate will be enzymatically cleaved and prevent the sequential RACE reactions (77). At present, there is no concrete data on the capping process of lncRNA and eRNA.

For The 3´-RACE technique, the reaction requires the polyadenylation process of targeted RNA in order to get recognized by oligo-dT. Interestingly, our own experiment already showed that some non-coding RNAs from HMIR lack polyadenylation as indicated by the difference between random hexamer and oligo-dT in the RT reactions.

Alternatively, the nature of non-coding RNAs can be revealed by using primer extension (78). Primer extension can identify the end of non-coding RNA by using a specific oligo as a primer for the RT reaction. The extended transcript is demarcated the end of the transcripts. The technique also does not require 7-methylguanosine and polyadenylation. The extended transcripts are detected by ^{32}P labeled primers or nucleotide triphosphate. Size limitation is also not a factor since the products are resolved and visualized in polyacrylamide gel. However, this approach requires extensive usage of radioactive material.

Here the level of non-coding HMIR RNAs is linked to thalassemia patients. This is consistent with the genetic linkage between HMIR and the severity of thalassemia. It is necessary to functionally test the effect of non-coding HMIR RNAs on the amount of fetal hemoglobin. The HMIR transcripts can be exogenously expressed. The effect of transgene on globin gene expression will be a good indicator for the influence of non-coding RNA on the gamma globin gene. However, it is possible that the non-coding HMIR RNA transcripts might not affect disease severity by means of fetal globin. MYB itself is a major transcription factor with broad activity. It might not exclusively adjust disease severity via globin gene expression.

A caveat for testing the role of non-coding RNAs is that the transcripts alone might work

exogenously. It may require the transcript to be expressed endogenously from the locus. An example is in the action of eRNA. The RNAs generated from active transcriptional machineries are located at the transcription sites near the promoter region. Exogenous RNA expression will not naturally be enriched at the site. One possible solution is to insert more copies of non-coding RNA near the promoter using the CRISPR/Cas9 technology. The technique will introduce the transgene without an unnecessary marker, which allows the effect of the non-coding RNAs to be studied. endogenously.

CHAPTER VI
CONCLUSION

A qRT-PCR-based method for detecting non-coding transcripts from *HBS1L-MYB* intergenic region (HMIR) in human erythroid leukemic K562 cell line has now developed. These transcripts appear to not be polyadenylated and match with regions enriched with histone H3 Lysine 4 trimethylation sites.

This study characterized the HMIR transcripts using qRT-PCR and RACE (Rapid Amplification cDNA ends) analysis. We found that β^0-thalassemia/HbE erythroblasts with relatively high HbF appears to have lower level of HMIR transcripts as compared to normal erythroblasts. The result suggested a new modifying factor for improving β-thalassemia/HbE disease severity. The result could be applied for developing a new treatment for the patients.

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APPENDIX

APPENDIX A
DOCUMENT PROOF OF ETHICAL CLEARANCE

AF 01-12

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย
254 อาคารจามจุรี 1 ชั้น 2 ถนนพญาไท เขตปทุมวัน กรุงเทพฯ 10330
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COA No. 113/2559

ใบรับรองโครงการวิจัย

โครงการวิจัยที่ 209.1/58	:	การถอดรหัสดีเอ็นเอที่อยู่ระหว่างยีน HBS1L และ MYB มีความสัมพันธ์กับระดับฮีโมโกลบินเอ็ฟไนเม็ดเลือดแดงตัวอ่อนโรคเบต้าธาลัสซีเมีย-ฮีโมโกลบินอี
ผู้วิจัยหลัก	:	นางสาวดวงกมล เลิศบรรจือชัย
หน่วยงาน	:	คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย ได้พิจารณา โดยใช้หลัก ของ The International Conference on Harmonization – Good Clinical Practice (ICH-GCP) อนุมัติให้ดำเนินการศึกษาวิจัยเรื่องดังกล่าวได้

ลงนาม.....
(รองศาสตราจารย์ นายแพทย์ปริวรรต ทัศนประดิษฐ)
ประธาน

ลงนาม.....
(ผู้ช่วยศาสตราจารย์ ดร.นันทวิ ชัยชนะวงศาโรจน์)
กรรมการและเลขานุการ

วันที่รับรอง : 25 พฤษภาคม 2559 **วันหมดอายุ** : 24 พฤษภาคม 2560

เอกสารที่คณะกรรมการรับรอง

1) โครงการวิจัย
2) ข้อมูลสำหรับกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัยและใบยินยอมของกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย
3) ผู้วิจัย
4) แบบสอบถาม

ลายที่โครงการวิจัย 209.1/58
วันที่รับรอง 25 พ.ค. 2559
วันหมดอายุ 24 พ.ค. 2560

เงื่อนไข

1. ข้าพเจ้ายินยอมร่วมเป็นกลไกหนึ่งในการ พิจารณาดำเนินการเก็บข้อมูลการวิจัยก่อนได้รับการอนุมัติจากคณะกรรมการพิจารณาจริยธรรมการวิจัยฯ
2. หากใบรับรองโครงการวิจัยหมดอายุ การดำเนินการวิจัยต้องยุติ เมื่อต้องการต่ออายุต้องขออนุมัติใหม่ล่วงหน้าไม่น้อยกว่า 1 เดือน พร้อมส่งรายงานความก้าวหน้าการวิจัย
3. ต้องดำเนินการวิจัยตามที่ระบุไว้ในโครงการวิจัยอย่างเคร่งครัด
4. ใช้เอกสารข้อมูลสำหรับกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย ใบยินยอมของกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย และเอกสารอื่นๆ ที่ใช้ในการวิจัย ที่มี **เฉพาะที่ประทับตรารับรองของคณะกรรมการเท่านั้น**
5. หากเกิดเหตุการณ์ไม่พึงประสงค์ร้ายแรงในสถานที่เก็บข้อมูลที่ขออนุมัติจากคณะกรรมการ ต้องรายงานคณะกรรมการภายใน 3 วันทำการ
6. หากมีการเปลี่ยนแปลงการดำเนินการวิจัย ให้ส่งคณะกรรมการพิจารณารับรองก่อนดำเนินการ
7. โครงการวิจัยไม่เกิน 1 ปี ส่งรายงานสิ้นสุดโครงการวิจัย (AF 05-12) และยุติส่งผลการวิจัยภายใน 30 วัน เมื่อโครงการวิจัยเสร็จสิ้น สำหรับโครงการวิจัยที่เป็นวิทยานิพนธ์ให้ส่งยุติส่งผลการวิจัย ภายใน 30 วัน เมื่อโครงการวิจัยเสร็จสิ้น

AF 02-12

The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University
Jamjuree 1 Building, 2nd Floor, Phyathai Rd., Patumwan district, Bangkok 10330, Thailand.
Tel/Fax: 0-2218-3202 E-mail: eccu@chula.ac.th

COA No. 113/2016

Certificate of Approval

Study Title No. 209.1/58 : *HBS1L - MYB* INTERGENIC REGION EXPRESSION IS ASSOCIATED WITH FETAL HEMGLOBIN LEVEL IN ß-THALASSEMIA/HbE ERYTHROBLASTS

Principal Investigator : MISS DUANGKAMON LOESBANLUECHAI

Place of Proposed Study/Institution : Faculty of Allied Health Sciences, Chulalongkorn University

The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP).

Signature: ..... Signature: .....

(Associate Professor Prida Tasanapradit, M.D.) (Assistant Professor Nuntaree Chaichanawongsaroj, Ph.D.)

Chairman Secretary

Date of Approval : 25 May 2016 Approval Expire date : 24 May 2017

The approval documents including

1) Research proposal
2) Patient/Participant Information Sheet and Informed Consent Form
3) Researcher
4) Questionnaire

Protocol No. 209.1/58
Date of Approval 25 MAY 2016
Approval Expire Date 24 MAY 2017

The approved investigator must comply with the following conditions:

1. *The research/project activities must end on the approval expired date of the Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University (RECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the RECCU approval expired date.*
2. *Strictly conduct the research/project activities as written in the proposal.*
3. *Using only the documents that bearing the RECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available).*
4. *Report to the RECCU for any serious adverse events within 5 working days.*
5. *Report to the RECCU for any change of the research/project activities prior to conduct the activities.*
6. *Final report (AF 03-12) and abstract is required for a one year (or less) research/project and report within 30 days after the completion of the research/project. For thesis, abstract is required and report within 30 days after the completion of the research/project.*
7. *Annual progress report is needed for a two- year (or more) research/project and submit the progress report before the expire date of certificate. After the completion of the research/project processes as No. 6.*

APPENDIX B
RAPID AMPLIFICATION OF cDNA ENDS (RACE) PROTOCOL

1. 5′ RACE protocol

1.1 RNA processing method

1.1.1 Treat DNase with RNA to remove DNA contamination

1) Set up the DNase digestion reaction as follows

- RNA (450 ng)	20 µl
-DNaseI	2 µl
-10x reaction buffer	2 µl

2) Incubate at 37°C for 30 minutes.

3) Add 1µl of EDTA to terminate the reaction.

4) Incubate at 65°C for 10 minutes to inactivate the DNase.

1.1.2 Treat with CIP to remove free 5’ phosphates from molecules

1) Assemble the following components in a tube

- DNase treated RNA (1 µg)	16 µl
- 10x CIP buffer	2 µl
- Calf Intestine Alkaline Phosphatase (CIP)	2 µl
- Nuclease – free water	- µl

2) Mix gently, spin briefly

3) Incubate at 37 °c for 1 hour

4) Put Ammonium acetate solution for termination CIP reaction

1.1.3 Extract RNA with phenol: chloroform and precipitate RNA

1) Add Acid phenol: chloroform 150 µl and 115 µl nuclease-free water, vortex thoroughly, then centrifuge 5 minutes at 14,000 x g at room temperature.

2) Transfer aqueous phase (top layer) to a new tube. Put 150 µl 0f choloform, vortex thoroughly, and centrifuge 5 minutes at room temperature at 14,000 x g.

3) Transfer aqueous phase to anew tube.

4) Precipitate with 150 µl isopropanol. Chill on ice 10 minutes, then centrifuge at 14,000 x g for 20 minutes, remove isopropanol.

5) Rinse pellet with 0.5 ml col 70 % ethanol, centrifuge 5 minutes at 14,000 x g, discard ethanol and air dry the pellet

6) Resuspend pellet in nuclease free water 11 µl

1.1.4 Treat with TAP to remove cap structure

1) Put the components in a tube

- CIP treated RNA	4 µl
- 10X TAP buffer	1 µl
- Tobacco acid pyrophosphatase	2 µl
- Nuclease-free water	2 µl

2) Mix gently, spin briefly

3) Incubate at 37 °c for 1 hour

1.1.5 Ligate 5’ RNA with 5’ RACE adapter

1) Assemble the following components in a tube

- CIP/TAP treated RNA	2 µl
- 5’ RACE adapter	1 µl
- 10X RNA ligase buffer	1 µl
- T4 RNA ligase (2.5 U/ µl)	2 µl
- Nuclease-free water	4 µl

2) Mix gently, spin briefly

3) Incubate at 37 °c for 1 hour

1.1.6 Convert RNA to cDNA

1) Assemble the following in a tube

- Ligated RNA	2 µl
- dNTP mix	4 µl
- Random decamer	2 µl
- 10X RT buffer	2 µl
- RNase Inhibitor	1 µl
- M-MLV reverse transcriptase	1 µl
- Nuclease-free Water	8 µl

2) Mix gently, spin briefly

3) Incubate at 42 °c for 1 hour

1.2 Nested PCR for 5′RACE

1.2.1 Methods (5′ outer RLM-RACE)

1) Put the components in PCR tubes on ice

- cDNA from RT reaction	1 µl
- 10x PCR buffer	5 µl
- Q solution	2.5 µl
- dNTP mix	2.5 µl
- 5′ RACE outer primer (10 µM)	2 µl
- HMIR-forward primer	2 µl
- DNA polymerase (5 U/µl)	0.25 µl
- Nuclease-free water	9.75 µl
Total volume	25 µl

2) Mix gently and spin down

3) Use PCR cycling profile as such

- Initial denaturation	98 °C 5 min
- Amplification 35 cycles	98 °C 30 sec.
	58 °C 90 sec.*
	72 °C 135 sec.
- Final extension	72 °C 5 min

1.2.2 5′ inner RLM-RACE

1. Put the components in PCR tubes on ice

- cDNA from RT reaction	1 µl
- 10x PCR buffer	5 µl
- Q solution	2.5 µl
- dNTP mix	2.5 µl
- 3’ RACE inner primer (10 µM)	2 µl

- HMIR-forward primer	2	µl
- DNA polymerase (5 U/µl)	0.25	µl
- Nuclease-free water	9.75	µl
Total volume	25	µl

2. Mix gently and spin down
3. Use PCR cycling profile as such

- Initial denaturation	98 °C 5 min
- Amplification 35 cycles	98 °C 30 sec.
	58 °C 90 sec.*
	72 °C 135 sec.
- Final extension	72 °C 5 min

2. 3′ RACE

2.1 Convert RNA to cDNA

1. Put the reagents in a tube

- RNA		
- dNTP Mix	4	µl
- 3'RACE adapter	2	µl
- 10X RT buffer	2	µl
- RNase Inhibitor	1	µl
- M-MV reverse transcriptase	1	µl
- Nuclease-free water		

2. Mix gently, spin down
3. Incubate at 42 °c for 1 hour

2.2 Nested PCR for 3′RACE

2.2.1 3′ outer RLM-RACE

1. Put the components in PCR tubes on ice

- cDNA from RT reaction	1	µl
- 10x PCR buffer	5	µl
- Q solution	2.5	µl
- dNTP mix	2.5	µl
- 3′ RACE outer primer (10 µM)	2	µl
- HMIR-forward primer	2	µl
- DNA polymerase (5 U/µl)	0.25	µl
- Nuclease-free water	9.75	µl
Total volume	25	µl

2. Mix gently and spin down

3. Use PCR cycling profile as such

- Initial denaturation	98 °C 5 min
- Amplification 35 cycles	98 °C 30 sec.
	58 °C 90 sec.*
	72 °C 135 sec.
- Final extension	72 °C 5 min

2.2.1 3′ inner RLM-RACE

1. Put the components in PCR tubes on ice

- cDNA from RT reaction	1 µl
- 10x PCR buffer	5 µl
- Q solution	2.5 µl
- dNTP mix	2.5 µl
- 3′ RACE inner primer (10 µM)	2 µl
- HMIR-forward primer	2 µl
- DNA polymerase (5 U/µl)	0.25 µl
- Nuclease-free water	9.75 µl
Total volume	25 µl

2. Mix gently and spin down

3. Use PCR cycling profile as such

- Initial denaturation	98 °C	5 min
- Amplification 35 cycles	98 °C	30 sec.
	58 °C	90 sec.*
	72 °C	135 sec.
- Final extension	72 °C	5 min

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PUBLICATION

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