ยีนฮีโมโกบินอี มีความสัมพันธ์อย่างใกล้ชิดกับปัจจัยภายในกลุ่มของยีนโกลบินที่เกี่ยวข้องกับระดับฮีโมโกลบินเอฟสูง: กรณีศึกษาในครอบครัว HbE/β-thalassemia ชาวไทย

Closed relationship of β^E-allele and high HbF associated cis-loci : Case studies in Thai HbE/ β-thalassemia families

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Abstract

Introduction: Previous statistical analyses have demonstrated linkage disequilibrium of β^E-allele and surrounding *in-cis* sequences.

Objective: To confirm the linkage disequilibrium of β^E-allele and surrounding *in-cis* sequences.

Materials and Methods: Associations of the β^E-allele with the β-globin haplotype, $(AT)_xN_z(AT)_y$ motif of βLCR-HS2, *XmnI*-$^G\gamma$ polymorphism, pre-$^G\gamma$ framework, (TG)n motif in IVSII of $^A\gamma$-globin gene and *HBBP1 : rs2071348* polymorphism were analyzed in three Thai HbE/β^O-thalassemia families.

Results: The β^E-allele was always *in-cis* with the β-globin haplotype III, pre $^G\gamma$-framework 2, XmnI+, $(TG)_{13}$ motif within IVSII of $^A\gamma$- globin gene and "C" at *HBBP1 : rs2071348* locus, all previously shown to be associated with high HbF expression. Combination of the $(AT)_9N_{12}(AT)_{10}$ motif of βLCR-HS2 and these *cis*-acting factors seemed to be required for maximum HbF-expression.

Conclusion: This family study substantially confirmed the linkage between the β^E allele and *cis*-acting loci associated with high HbF phenotype. This study also highlighted the closed relationship between HbE and HbF as well as the need of combination of the analyzed *cis*-acting sequences in activating HbF expression in human.

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Keywords: HbE/β^O-thalassemia, HbF, β-globin haplotype, β-globin mutations, *cis*-acting loci

บทคัดย่อ

บทนำ: การวิเคราะห์ทางสถิติก่อนหน้านี้พบความเกี่ยวพันของ β^E-allele กับดีเอ็นเอบริเวณรอบๆ

วัตถุประสงค์: เพื่อแสดงหลักฐานเชิงประจักษ์ยืนยันความเกี่ยวพันของ β^E-allele กับดีเอ็นเอบริเวณรอบๆ

วัสดุและวิธีการ: ทำการศึกษาความเกี่ยวพันของ β^E-allele และ β-globin cluster haplotype, $(AT)_xN_z(AT)_y$ motif บน βLCR-HS2, *XmnI*-$^G\gamma$ polymorphism, pre-$^G\gamma$ framework, (TG)n motif ใน IVSII บน $^A\gamma$-globin gene และ *HBBP1 : rs2071348* polymorphism ในครอบครัวผู้ป่วยชาวไทยโรค HbE/β^O-thalassemia จำนวน 3 ครอบครัว

ผลการศึกษา: β^E-allele มักอยู่บนโครโมโซมข้างเดียวกับการเปลี่ยนแปลงของลำดับดีเอ็นเอที่เกี่ยวพันกับระดับฮีโมโกลบินเอฟที่สูงขึ้นซึ่งประกอบด้วย β-globin haplotype III, pre $^G\gamma$-framework 2, XmnI บวก, $(TG)_{13}$ motif ใน IVSII บน $^A\gamma$- globin gene และเบส “C” ตำแหน่ง rs2071348 บนยีน *HBBP1* และพบว่าการอยู่ร่วมกันของ $(AT)_9N_{12}(AT)_{10}$ motif บน βLCR-HS2 และ *cis*-acting factors ที่กล่าวข้างต้นพบมีความจำเป็นในการสร้างฮีโมโกลบินเอฟให้สูงขึ้น

สรุปผลการศึกษา: การศึกษาครั้งนี้สามารถยืนยันอย่างเป็นรูปธรรมความเกี่ยวพันของ β^E allele และดีเอ็นเอบริเวณรอบๆ ที่เกี่ยวข้องกับปริมาณฮีโมโกลบินเอฟที่เพิ่มมากขึ้น และนอกจากนั้นการศึกษานี้ยังแสดงให้เห็นถึงความสัมพันธ์ที่ใกล้ชิดของ HbE และ HbF อีกทั้งยังแสดงให้เห็นถึงความสำคัญของดีเอ็นเอบริเวณรอบๆ เหล่านี้ต่อการกระตุ้นการแสดงออกของ HbF ในมนุษย์

วารสารเทคนิคการแพทย์เชียงใหม่ 2558; 48(2): 88-99. Doi: 10.14456/jams.2015.14

คำสำคัญ: HbE/β^O-thalassemia, HbF, β-globin haplotype, β-globin mutations, *cis*-acting loci

Introduction

HbE ($\alpha_2\beta^E_2$) is abnormal hemoglobin arising from an assembly of mutated β^E (A-G at codon 26 altering glutamic to lysine) and normal α-globin chains. This hemoglobin variant is common in Southeast Asia, attaining a frequency of about 60% among the population of Thailand, Laos and Cambodia.[1,2] The β^E-mRNA was shown to be ineffectively synthesized owing to competitive utilization of an alternative splice site at codon 25[3] and the silencing effect of the $(AT)_9T_5$ motif in the β-globin promoter.[4] The reduction of β^E-mRNA leads to a reduced β^E-globin chain, which results in the β^+-thalassemia phenotype. Thus, compound heterozygote of the β^E-globin and β-thalassemia alleles leads to a syndrome referred to as HbE/β-thalassemia.[1]

Phenotypically, HbE/β-thalassemia presents in a broad range of clinical symptoms, ranging from transfusion-dependent β-thalassemia major to a mild form of β-thalassemia intermedia requiring only occasional blood transfusion.[2,5] Several factors have been shown to be responsible for this clinical heterogeniety, including types of the counterpart β-thalassemia alleles, co-existence of α-thalassemia and co-inheritance of high HbF genetic variants.[5] The *XmnI*-$^G\gamma$ site, a “T” substitution for “C” at nucleotide -158 in $^G\gamma$ promoter, was shown to be a major cis-acting genetic factor associated with increased HbF.[6-9] Recent statistical analyses in Thai HbE carriers, HbE homozygotes and HbE/β-thalassemia revealed association of the XmnI-$^G\gamma$ site and other surrounding *cis*-acting loci with β^E-allele.[8,10,11]

In this study, we attempted to confirm these previous findings by conducting family studies to determine associations of β^E-allele and cis sequence variations, including the *XmnI*-$^G\gamma$ polymorphism, β-globin haplotypes, $(AT)_xN_z(AT)_y$ motif of βLCR-HS2, pre-$^G\gamma$ framework (PGγF), (TG)n motif in IVSII of $^A\gamma$-globin gene and *HBBP1 : rs2071348* single nucleotide polymorphism. The information obtained from this study should confirm the previous linkage disequilibrium analyses and provide evidence of the close relationship between HbE and HbF.

Materials and methods

Subjects

This study was approved by the research ethics board of Faculty of Associated Medical Sciences, Chiang Mai University (Approval notice 205/2012). Three northern Thai families, each having one HbE/β^{O}-thalassemia member, were recruited. EDTA blood samples were collected. Hb typing was performed by cation exchange HPLC (The Primus Variant System 99, Kansas City, MO, USA). The β-thalassemia mutations in the first and second families were identified by multiplex allele-specific PCR[12] and that in the third family by automated fluorescent nucleotide sequencing.[13] Based on the results of Hb typing and β-globin genotyping, all volunteers were diagnosed. The first family had four members: HbE/ $\beta^{41/42(-TTCT)}$ -thalassemia proband, HbE-heterozygous father, β-thalassemia heterozygous mother and HbE-heterozygous brother. The second family had four members: HbE/ $\beta^{17(A-T)}$ -thalassemia proband, HbE-heterozygous mother, HbE-heterozygous brother and β-thalassemia heterozygous father. The third family had four members: HbE/$\beta^{IVS1-nt1\ (G-T)}$ -thalassemia proband, HbE/$\beta^{IVS1-nt1\ (G-T)}$ -thalassemia sister, HbE heterozygous father and β-thalassemia heterozygous mother.

Molecular analysis

Genomic DNA was prepared from leukocytes by the direct Chelex extraction method.[14] The *XmnI*-$^{G}\gamma$ polymorphism was determined by *XmnI* digestion of PCR products covering the position -158 on $^{G}\gamma$-promoter, following the procedure described previously.[15] Determination of A/C polymorphism of *HBBP1 : rs2071348* was performed by the tetra-primer ARMS-PCR, established previously in our laboratory.[15] The RFLP haplotypes (*HindII-*ε*, HindIII-*$^{G}\gamma$*, HindII-*$^{A}\gamma$*, HindII-5'*$\psi\beta$*, HindII-3'* $\psi\beta$*, AvaII-*β *and HinfI-*β) were generated following the procedure described previously.[16,17] Sanger's direct nucleotide sequencing was employed to genotype the $(AT)_xN_z(AT)_y$ motif of βLCR-HS2, PGγF, (TG)n motif in IVSII of $^{A}\gamma$-globin gene.[18-20]

Results

Association of β^{E}-allele and sequence variations on β-globin gene cluster

Five β-globin haplotypes (*HindII-*ε*, HindIII-*$^{G}\gamma$*, HindII-*$^{A}\gamma$*, HindII-5'* $\psi\beta$*, HindII-3'*$\psi\beta$*, AvaII-*β *and HinfI-*β) were observed in these three families, including haplotypes I (+ - - - -++), III (- + - + + + -), V (+ - - - - + -), VI (- + + - - - +) and VII (+ - - - - - +) (Table 1). β^{E}-allele was *in-cis* with only haplotype III.

Five (AT)xNz(AT)y motifs of βLCR-HS2 were found. These included $(AT)_9N_{12}(AT)_{10}$, $(AT)_{10}N_{12}(AT)_{10}$, $(AT)_{11}N_{12}(AT)_{10}$, $(AT)_8N_{12}(AT)_{11}$ and $(AT)_9N_{12}(AT)_{11}$ (Figure 1 and Table 1). The β^{E} allele was predominately *in-cis* with the $(AT)_9N_{12}(AT)_{10}$ motif.

According to Pissard & Beuzard, the combination of SNPs at positions -1450, -1280 and -1225 related to $^{G}\gamma$-globin gene cap site formed three pre-$^{G}\gamma$ frameworks (PGγF).(21) These included PGγF1 (TGA), PGγF2 (TAG) and PGγF4 (TGG). Interestingly, combinations of SNPs at positions -1067, -807, -369 and -309 formed two sub-PGγFs (TCCA and AACA) and were shown to be PGγF-specific. The "TCCA" was specific to PGγF 2, whereas the "AACA" was always *in-cis* to the PGγFs 1, 4. The β^{E}-allele was *in-cis* with only PGγF2 (Table 1).

The *XmnI*+ and *XmnI*- sites were equally distributed in these three families. The $\beta^{17(A-T)}$, $\beta^{41/42(-TTCT)}$ and $\beta^{IVS1-nt1(G-T)}$ were *in-cis* with the *XmnI*- site. The β^{A}-allele was *in-cis* with both the *XmnI*+ and *XmnI*- sites. However, the β^{E}-allele was *in-cis* with only the *XmnI*+ site (Table 1).

Two (TG)n motifs within IVS2 of $^{A}\gamma$-globin gene were observed, including $(TG)_{13}$ and $(TG)_9(CG)_5(TG)_8$ motifs (Figure 2). Combination of single nucleotide polymorphisms (SNPs) loci *rs 2187608*, *rs 28379094, rs 28440105* and *rs 3841756* formed three sub-haplotypes within this region, including "GGGA", "CAGA" and "CAG delA". The $(TG)_{13}$ motif was *in-cis* with only the "GGGA" sub-haplotype. The $(TG)_9(CG)_5(TG)_8$ motif was *in-cis* with either the "CAGA" or "CAG delA" sub-haplotypes. The β^{E}-allele was *in-cis* with only the $(TG)_{13}$ motif within this region (Figure 2 and Table 1).

In cases of A-C polymorphism of the *HBBP1: rs 2071348* locus, the polymorphic "C" allele was less frequent than wild type "A" allele. However, the β^E-allele was *in-cis* with only the polymorphic "C" allele (Table 1).

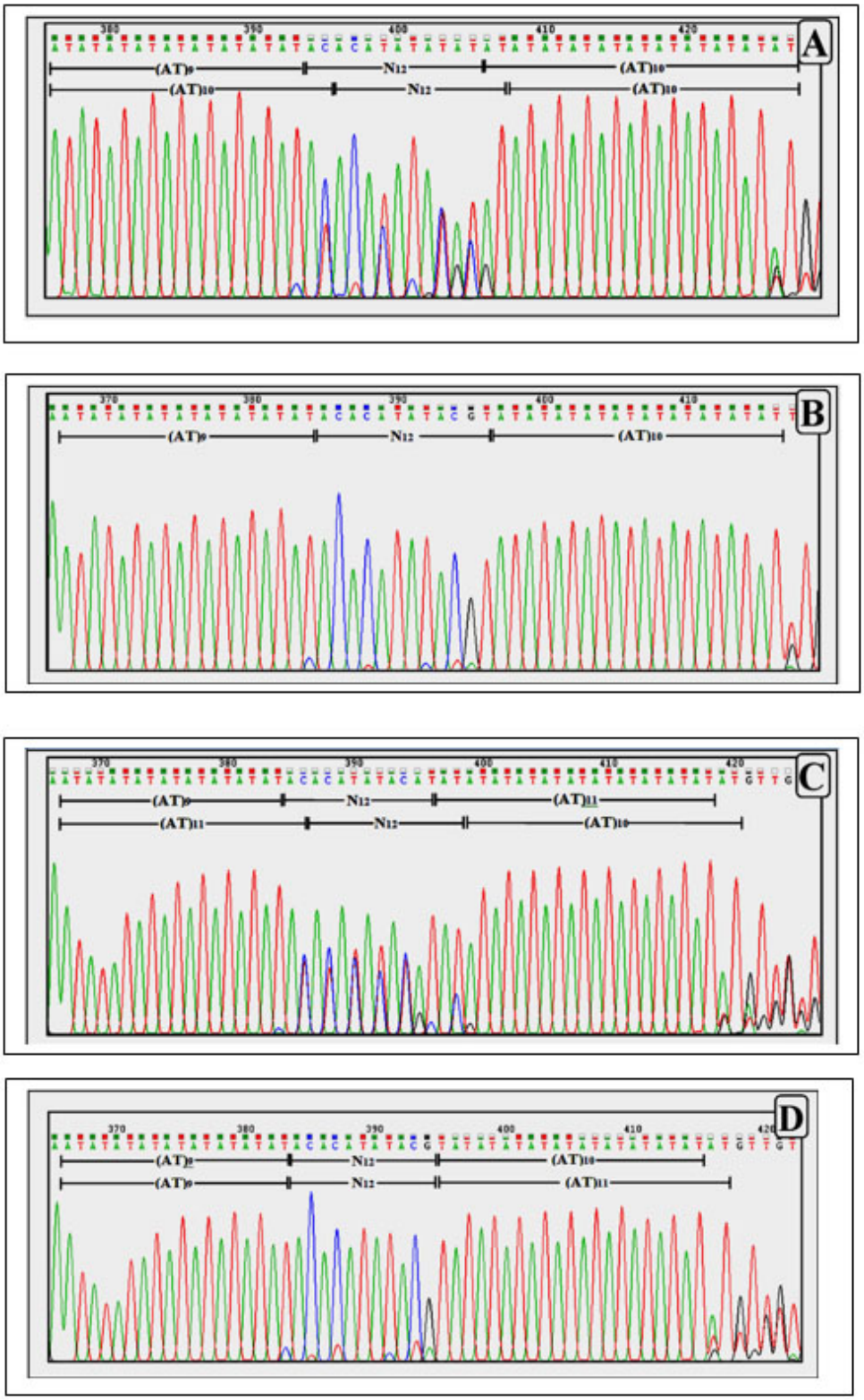


Figure 1 *Electropherograms of the (AT)xNz(AT)y motif of βLCR-HS2. Boundery of (AT)x, Nz and (AT)y repeats are shown as the lines above peaks within each electropherogram.*

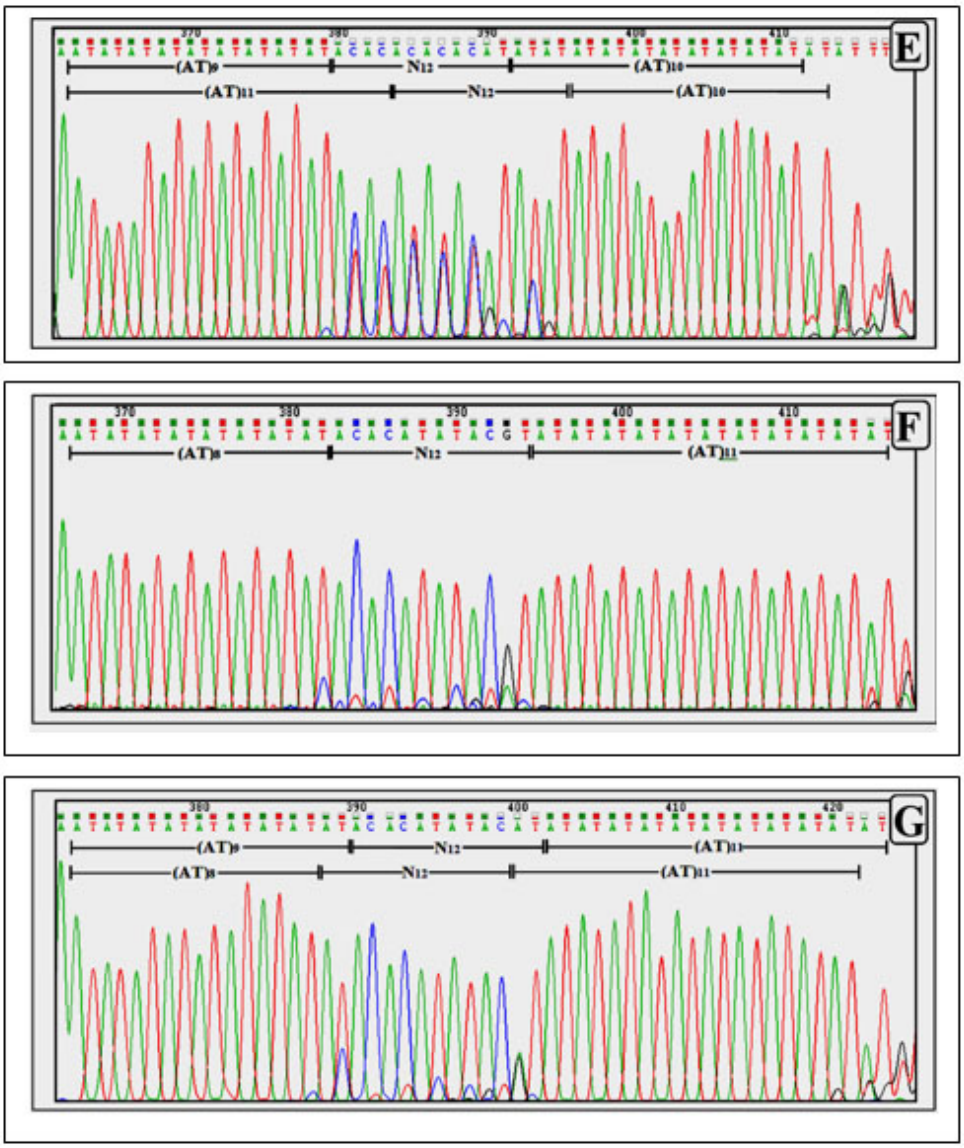


Figure 1 *Electropherograms of the (AT)xNz(AT)y motif of βLCR-HS2. Boundery of (AT)x, Nz and (AT)y repeats are shown as the lines above peaks within each electropherogram. (continued)*

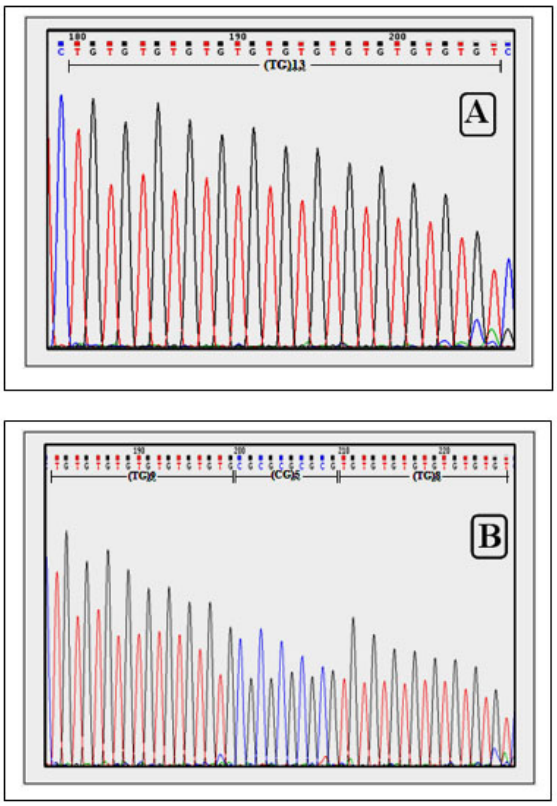
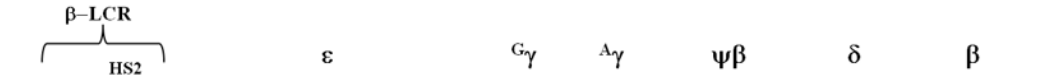


Figure 2 *(TG)n motif within IVS2 of $^{A\gamma}$-globin gene. A, B are the $(TG)_{13}/(TG)_{13}$ and $(TG)_9(CG)_5(TG)_8/(TG)_9(CG)_5(TG)_8$, respectively.*

Table 1. β-globin haplotypes and sequence variations of β-globin cluster in three Thai HbE/β^0-thalassemia families.

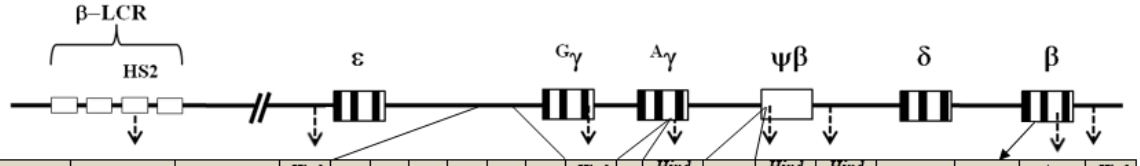


Family I	(AT)xNz(AT)y	β-haplotypes	*Hind* II	PG*γF	-1067	-807	-369	-309	-158	*Hind* III		*Hind* III	rs207 1348	*Hind* II	*Hind* II		β^{mutant}	*Ava* II	*Hinf* I
I-I	9-12-10	III	-	2	T	C	C	A	+	+		-	C	+	+		β^E	+	-
	10-12-10	VI	-	2	T	C	C	A	+	+		+	A	-	-		β^A	-	+
I-II	10-12-10	VII	+	1	A	A	C	A	-	-		-	A	-	-		$\beta^{41/42}$	-	+
	9-12-10	V	+	1	A	A	C	A	-	-		-	A	-	-		β^A	+	-
II-I*	10-12-10	VII	+	1	A	A	C	A	-	-		-	A	-	-		$\beta^{41/42}$	-	+
	9-12-10	III	-	2	T	C	C	A	+	+		-	C	+	+		β^E	+	-
II-II	9-12-10	V	+	1	A	A	C	A	-	-		-	A	-	-		β^A	+	-
	10-12-10	VI	-	2	T	C	C	A	+	+		+	A	-	-		β^A	-	+

Family I	(TG)n	rs2187608(C>G)	rs28379094(G>A)	rs28440105(T>G)	rs3841756(delA)
I-I	(TG)13	G	G	G	A
	(TG)13	G	G	G	A
I-II	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)9(CG)5(TG)8	C	A	G	A
II-I*	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)13	G	G	G	A
II-II	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)13	G	G	G	A

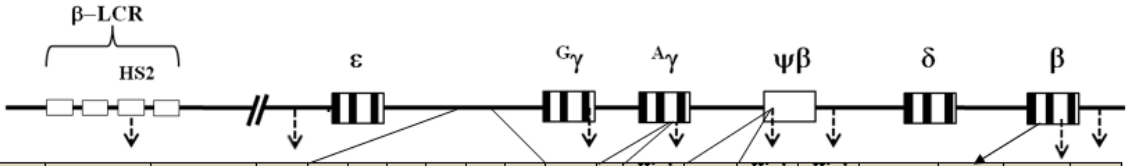
(AT)xNz(AT)y is the repeat motif of βLCR-HS2. β-haplotypes are the RFLP-haplotypes of β-thalassemia chromosome. PG$^*\gamma$F stands for $^{G^*}\gamma$ framework. *rs2071348* is the SNP (C/A) in *HBBP1* locus. Numbers preceded by the minus symbol indicate locations of SNPs in promoter region of $^{G\gamma}$-globin gene. "-158" is the *XmnI*-$^{G\gamma}$ site. " + " indicates presence of cutting sites and " - " indicates absence of cutting sites for the restriction enzymes.

Table 1. β-globin haplotypes and sequence variations of β-globin cluster in three Thai HbE/β^0-thalassemia families. (continued)



Family II	(AT)xNz(AT)y	β-haplotypes	*Hind* II	PGγF	-1067	-807	-369	-309	-158	*Hind* III	*Hind* III	rs207 1348	*Hind* II	*Hind* II	β^{mutant}	*Ava* II	*Hinf* I
I-I	11-12-10	VII	+	1	A	A	C	A	-	-	-	A	-	-	β^{17}	-	+
	9-12-11	I	+	1	A	A	C	A	-	-	-	A	-	-	β^{A}	+	+
I-II	9-12-10	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{A}	+	-
	9-12-10	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{E}	+	-
II-I	9-12-11	I	+	1	A	A	C	A	-	-	-	A	-	-	β^{A}	+	+
	9-12-10	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{E}	+	-
II-II*	11-12-10	VII	+	1	A	A	C	A	-	-	-	A	-	-	β^{17}	-	+
	9-12-10	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{E}	+	-

Family II	(TG)n	rs2187608(C>G)	rs28379094(G>A)	rs28440105(T>G)	rs3841756(delA)
I-I	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)9(CG)5(TG)8	C	A	G	A
I-II	(TG)13	G	G	G	A
	(TG)13	G	G	G	delA
II-I	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)13	G	G	G	delA
II-II*	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)13	G	G	G	delA



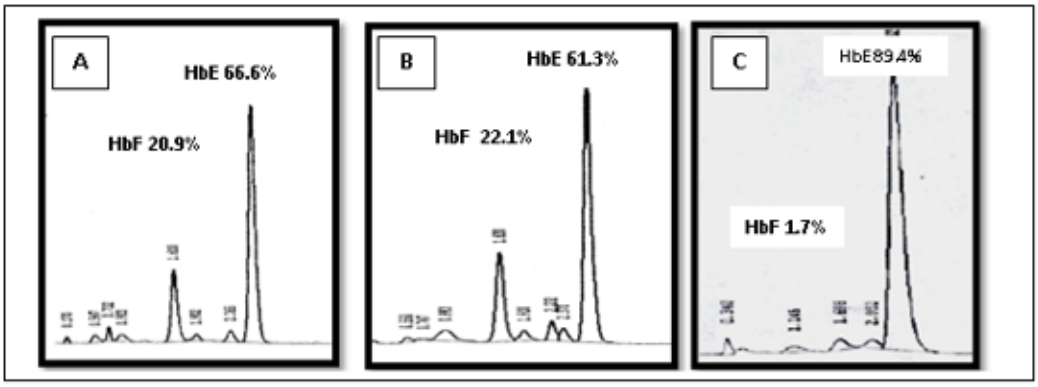
Family III	(AT)xNz(AT)y	β-haplotypes	*Hind* II	PGγF	-1067	-807	-369	-309	-158	*Hind* III	*Hind* III	rs207 1348	*Hind* II	*Hind* II	β^{mutant}	*Ava* II	*Hinf* I
I-I	8-12-11	I	+	4	A	A	C	A	-	-	-	A	-	-	β^{A}	+	+
	8-12-11	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{E}	+	-
I-II	8-12-11	VII	+	1	A	A	C	A	-	-	-	A	-	-	β^{A}	-	+
	9-12-11	I	+	1	A	A	C	A	-	-	-	A	-	-	β^{IVS1-1}	+	+
II-II*	8-12-11	III	-	2	T	C	C	A	+	+	-	C	+	+	β^{E}	+	-
	9-12-11	I	+	1	A	A	C	A	-	-	-	A	-	-	β^{IVS1-1}	+	+

Family III	TG repeated	rs2187608(C>G)	rs28379094(G>A)	rs28440105(T>G)	rs3841756(delA)
I-I	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)13	G	G	G	A
I-II	(TG)9(CG)5(TG)8	C	A	G	A
	(TG)9(CG)5(TG)8	C	A	G	delA
II-II*	(TG)13	G	G	G	A
	(TG)9(CG)5(TG)8	C	A	G	delA

(AT)xNz(AT)y is the repeat motif of βLCR-HS2. β-haplotypes are the RFLP-haplotypes of β-thalassemia chromosome. PGγF stands for $^{G}\gamma$ framework. *rs2071348* is the SNP (C/A) in *HBBP1* locus. Numbers preceded by the minus symbol indicate locations of SNPs in promoter region of $^{G}\gamma$-globin gene. "-158" is the *XmnI*-$^{G}\gamma$ site. " + " indicates presence of cutting sites and " - " indicates absence of cutting sites for the restriction enzymes.

Extended β-globin haplotypes and HbF synthesis in Thai HbE/β^{O}-thalassemia

Extended haplotypes of the β-globin gene cluster in three HbE/β-thalassemia; two with Hbs EF and another with Hb EE, were analyzed and compared. The first patient (II-I in family I, as shown in Table 1 and A in Figure 3), with 66.6% HbE and 20.9% HbF, was compound heterozygous for β^{E} and $\beta^{41/42(-TTCT)}$ alleles. The second patient (II-II in family II, as shown in Table 1 and B in Figure 3), with 61.3% HbE, 22.1% HbF, was compound heterozygote for β^{E} and $\beta^{17(A-T)}$. The third patient (II-II in family III, as shown in Table 1 and C in Figure 3), with 1.7% HbF and 89.4% HbE, was heterozygous for β^{E} and $\beta^{IVS1\text{-}nt1\ (G-T)}$ (Figure 3). As shown in Figure 3, the β^{E}-allele in the two patients ("A" and "B") with high HbF levels was in-cis with the $(AT)_9N_{12}(AT)_{10}$, haplotype III, PGγF2, $(TG)_{13}$ and "C" at the *HBBP1: rs 2071348* locus. In contrast, the β^{E}-allele in the patient ("C") with a lower HbF level than patients "A", "B" was also *in-cis* with haplotype III, PGγF2, $(TG)_{13}$ and "C" at the *HBBP1: rs 2071348* locus. However, the (AT)xNz(AT)y motif of the βLCR-HS2 in patient "C" was $(AT)_8N_{12}(AT)_{11}$.



	β-alleles	(AT)xNz(AT)y	β-haplo	PGγF	*Xmn*I-$^{G}\gamma$	$^{A}\gamma$ IVS2-(TG)n	*HBBP1 rs2071348*
A	β^{E}	*9-12-10*	*III*	*2*	*C*	$(TG)_{13}$	*C*
	$\beta^{41/42}$	*9-12-10*	*VII*	*1*	*T*	$(TG)_9(CG)_5(TG)_8$	*A*
B	β^{E}	*9-12-10*	*III*	*2*	*C*	$(TG)_{13}$	*C*
	β^{17}	*11-12-10*	*VII*	*1*	*T*	$(TG)_9(CG)_5(TG)_8$	*A*
C	β^{E}	*8-12-11*	*III*	*2*	*C*	$(TG)_{13}$	*C*
	$\beta^{IVS1nt1}$	*9-12-11*	*I*	*1*	*T*	$(TG)_9(CG)_5(TG)_8$	*A*

Figure 3 *Hb typing by HPLC (top panel) and cis-acting sequence variations (lower panel) in HbE/β^{O}-thalassemia patients "A", "B" and "C" as mentioned in the text. β^{17} represents $\beta^{17(A-T)}$, $\beta^{41/42}$ represents $\beta^{41/42(-TTCT)}$, β^{IVS1nt} represents $\beta^{IVS1\text{-}nt1(G-T)}$, β-haplo represents β-haplotypes and PGγF represents pre-$^{G}\gamma$ framework.*

Discussion

The β^E-allele mutates from the normal β^A-allele by missense mutation of codon 26 of the β-globin gene, altering the codon for glutamic acid to that for lysine.[1] This mutation is common in Southeast Asia.[22,23] This high incidence was shown to be due to natural selection against malaria, particularly *Plasmodium falciparum*.[24-26]

Recent pairwise LD analyses in Thai HbE/β^O-thalassemia by Ma *et al*[8] and in Thai homozygous HbE (EE), heterozygous HbE (AE) and normal (A_2A) individuals by Ohashi *et al*[10] indicated significant linkage disequilibrium of the β^E-allele and surrounding *in-cis* sequence variations. These studies assumed that malarial selection was involved. The family studies undertaken in this study found that the β^E-allele was always *in-cis* with the $(AT)_9N_{12}(AT)_{10}$ motif, β-globin haplotype III, PGγF 2, $(TG)_{13}$ motif within IVS2 of $^A\gamma$-globin gene, XmnI+ and "C" allele of *HBBP1 : rs2071348*, confirming those previous statistical assumptions. Interestingly, all of these *in-cis* sequence variations have been shown to be associated with increased HbF expression.[15,21,27-35] Hence, closed relation between HbE and *cis*-acting genetic factors associated with increased HbF expression observed in this study was most likely a result of positive selection against malaria, which is endemic in Thailand. Red blood cells containing Hbs E and F retarded the growth of *P. falciparum,* probably due to increased oxidative stress.[36,37] Thus, individuals inheriting both HbE and HbF-activating loci are likely to survive malarial infection.

Analysis in 3 HbE/β-thalassemia patients having typical EF ("A", "B" in Figure 3) and atypical EE ("C" in Figure 3) phenotypes highlighted the importance of combination of the $(AT)_9N_{12}(AT)_{10}$ motif, β-globin haplotype III, PGγF 2, $(TG)_{13}$ motif within IVS2 of $^A\gamma$-globin gene, *XmnI*+ and "C" allele of *HBBP1 : rs2071348* in activating HbF production. The $\beta^{17(A-T)}$, $\beta^{41/42\ (-TTCT)}$ and $\beta^{IVS1\text{-}nt1(G-T)}$-alleles in these three patients were *in-cis* to the β-globin haplotype I & VII, PGγF I, $(TG)_9(CG)_5(TG)_8$ motif within IVS2 of $^A\gamma$-globin gene, *XmnI*- and "A" allele of *HBBP1 : rs2071348* previously shown to be involved with low HbF expression.[15,33,34] Thus, HbF expression in these three patients was likely the result of icreased expression of the γ-globin gene on the β^E-chromosome. However, in patient "C" (Figure 3), the $(AT)_xN_z(AT)_y$ motif in the β^E-chromosome was $(AT)_8N_{12}(AT)_{11}$, which has been previously shown to be involved in normal HbF expression[33] and should be responsible for the atypically low HbF in patient "C". The $(AT)_xN_z(AT)_y$ motif of βLCR-HS2 has been shown to be a classical enhancer for $^G\gamma$-globin gene expression via opening chromosomal domain, thus increasing accessibility of RNA polymerase.[38] The $(AT)_8N_{12}(AT)_{11}$ motif might have less impact on the chromosome opening activity at γ-globin promoter, resulting in minimal γ-globin gene expression. This study was, however, the first to describe this unusual phenomenon.

In conclusion, this family study confirmed the linkage of the β^E allele and cis-acting loci associated with high HbF phenotype. The close relationship between HbE and HbF as well as the need of combination of the analyzed *cis*-acting sequences in activating HbF expression was highlighted.

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Confilict of interest

None

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