GENETIC FACTORS INFLUENCING HEMOGLOBIN F LEVEL IN β-THALASSEMIA/Hb E DISEASE

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Abstract. Genetic factors influencing Hb F content in adult red blood cells include β-thalassemia genotypes, co-inheritance of α-thalassemia traits and single nucleotide polymorphisms (SNPs). Genotyping of α- and β-thalassemia and five SNPs in β-globin gene cluster previously identified in genome-wide association studies as being markers of elevated Hb F in β-thalassemia were performed in 81 subjects diagnosed with β-thalassemia/Hb E. Hb F levels are higher (0.9-7.1 g/dl) in subjects (n = 57) with the severe compared to mild β-thalassemia (0.8-2.5 g/dl) (n = 4) genotypes, and are similarly low (0.7-3.5 g/dl) in those (n = 15) with α-thalassemia co-inheritance. Hb F levels in non-thalassemia controls (n = 150) range from 0 to 0.15 g/dl. The presence of homozygous minor alleles of the 5 SNPs are significant indicators of β-thalassemia/Hb E individuals with high Hb F (> 4 g/dl), independent of their thalassemia genotypes. Given that re-activation of γ-globin genes leads to amelioration of β-thalassemia severity, understanding how genetic factors up-regulate Hb F production may lead to possible therapeutic interventions, genetically or pharmacologically, of this debilitating disease in the not too distant future.

Keywords: globin gene, Hb F, single nucleotide polymorphism, α-thalassemia, β-thalassemia/Hb E disease

INTRODUCTION

β-Thalassemia is an autosomal recessive blood disease common in Southeast Asia (and other regions of the world where malaria is or has been prevalent) caused mainly by substitutions/indels in β-globin gene, resulting in impaired β-globin biosynthesis (Thein *et al*, 1990). The carrier frequency in Thailand is 1%-9% (Fucharoen and Winichagoon, 1992). Hemoglobinopathy is usually found among Thais with β-thalassemia associated hemoglobin E (Hb E: $\alpha_2\beta_2^{26E>K}$) (β-thalassemia/Hb E). Anemia of β-thalassemia stems from ineffective erythropoiesis and reduced life span of the circulating thalassemic red blood cells resulting from toxicity of unmatched α-hemoglobin molecules (Weatherall, 2001).

In certain circumstances, severity of anemia in β-thalassemia is ameliorated by a concomitant inheritance of α-thalassemia 1 (--/αα genotype) (Winichagoon *et al*, 1985) or by elevated Hb F production (HPHF) (Fessas *et al*, 1961), conditions whereby the red cell burden of unmatched α-hemoglobins is reduced. In HPFH-6

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and $\delta\beta^0$-thalassemia (due to deletion of both β- and δ-globin genes), level of Hb F expression is up-regulated more in HPFH-6 due to the removal of BCL11A binding site at the upstream region of δ-globin gene (Prakobkaew *et al*, 2013). BCL11A is the major γ-globin gene repressor and its loss is associated with increase in Hb F level (Ghedira *et al*, 2013). A number of single nucleotide polymorphisms (SNPs) in the β-globin gene cluster has been reported also to be strongly associated with elevated Hb F levels in β-thalassemia/Hb E (Ma *et al*, 2007).

Herein we report the associations of β-thalassemia genotypes, α-thalassemia co-inheritance and SNPs in the β-globin gene cluster with Hb F levels in β-thalassemia/Hb E subjects.

MATERIALS AND METHODS

Blood samples

Blood samples from 81 subjects diagnosed with β-thalassemia/Hb E and 150 non-thalassemia individuals of > 1 year of age were obtained from the Blood Disease Diagnostic Laboratory, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Blood samples were identified as β-thalassemia/Hb E or non-thalassemia using blood cell parameters, hemoglobin typing and DNA analysis (Thedsawad *et al*, 2012). Complete blood count was conducted using Sysmex XS-1000i analyzer (Kobe, Japan). Hb F content was quantified using a Variant-II high performance liquid chromatography (HPLC) system equipped with β-thalassemia Short Program software (Bio-Rad, Hercules, CA). DNA was extracted from 300 ml of EDTA whole blood using a Maxwell-16 Blood DNA Purification kit (Promega, Madison, WI) for SNP genotyping.

The study protocol was approved by the Committee on Human Rights Related to Research Involving Human Subjects of Ramathibodi Hospital (MURA 2011/619), and written informed consents were obtained from subjects or legal guardian prior to enrollment in the study.

Thalassemia genotyping

Genotyping of α- and β-thalassemias were performed using PCR-based methods as previously described (Thedsawad *et al*, 2012; Siriworadechkul *et al*, 2014). In brief, common α-thalassemia 1 ($--^{SEA}$, $--^{THAI}$ and $-^{FIL}$ deletions), α-thalassemia 2 ($-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions), Hb Constant Spring (CS) ($-\alpha^{CS}$) and Hb Pakse ($-\alpha^{Pakse}$) were detected using multiplex Gap-PCR and amplification refractory mutation system (ARMS) PCR. β-thalassemia mutations were identified by multiplex ARMS-PCR.

SNP genotyping

Five SNPs in the non-coding regions of the β-globin gene cluster were selected from previous genome-wide association studies (GWAS) based on significant associations with β-thalassemia/Hb E disease severity (p-value < 10^{-8}) and percent Hb F ($p < 0.05$) (Table 1) (Nuinoon *et al*, 2010; Sherva *et al*, 2010). Three SNPs are located on up-stream and down-stream regulatory regions of $^{G}\gamma$-globin gene (rs2855122, rs2855125 and rs7482144) and two between ψβ- and δ-globin genes (rs2071348 and rs4910543) (Fig 1). SNP genotypes were determined employing a multiplex ARMS-PCR with primers listed in Table 2. ARMS-PCR was carried out in 30-μl volume containing 5 μl of 5X PCR buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$, 0.05 U *Taq* DNA polymerase (Promega, Madison, WI), 6.7 mM dithiotreitol, 27-107 nM primers, 50 nM β-actin primers (internal control) and 100-150 ng of DNA sample. Thermocy-

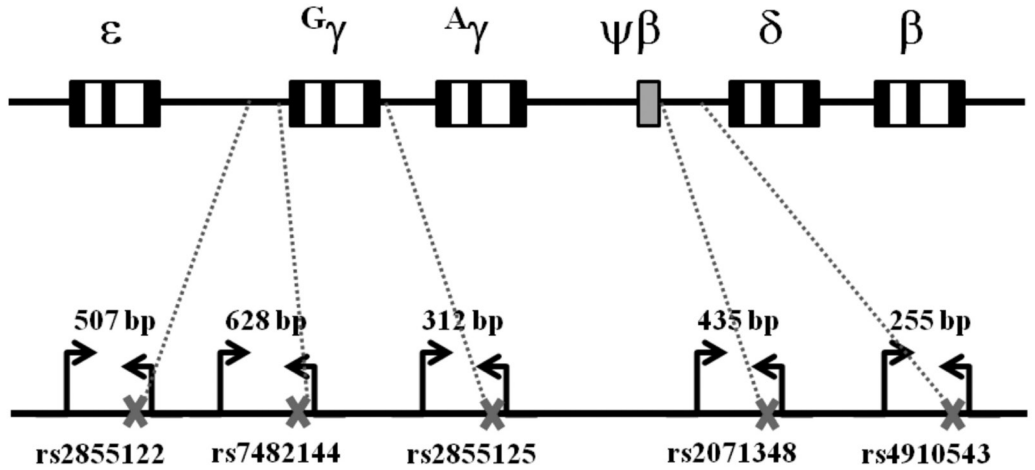

Fig 1–Schematic diagram of the positions of SNPs in β-globin gene cluster. Arrows depict primer locations. Black and white band in gene depicts exon and intron region, respectively. X, SNP location.

cling (performed in PCR thermal cycler; Eppendorf, Germany) conditions were as follows: 95°C for 5 minutes; followed by 30 cycles of 97°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 minute; with a final step at 72°C for 5 minutes. Amplicons were analyzed by 2% agarose gel-electrophoresis and ethidium bromide staining, then were extracted from gel using NucleoSpin^{Ò} Gel and PCR Clean Up kit (Machery-Nagel, Germany) and sequenced (1st Base Laboratory; Selangor, Malaysia).

Statistical analysis

Hardy-Weinberg equilibrium and allele frequency were analyzed using Haploview software (Broad Institute, Camgridge, MA). Associations of SNPs in β-thalassemia/Hb E and non-thalassemic control samples were analyzed using chi-square or Fisher's exact test. Hb F amounts associated with SNPs were compared employing Mann-Whitney *U* test with SPSS software version 16 (IBM, Armonk, NY). Statistical significance is accepted at $p < 0.05$.

RESULTS

Association of β-thalassemia genotypes with Hb F and total Hb levels

Of the 61 β-thalassemia/Hb E samples (without concomitant α-thalassemia allele), there were 46 cases of β^0-thalassemia [19 with cd41/42 (-TTCT), 20 with cd17 (A>T), 2 with IVSI-1 (G>T), 2 with cd71/72 (+A), and 1 each with cd27/28 (+C), cd26 (G>T) and cd43 (G>T) mutations], 11 of $\beta^{+severe}$-thalassemia [IVSII-654 (C>T) mutation] and 4 of β^+-thalassemia (nt-28 (A>G) mutation]. The ranges of Hb F and total Hb levels in β^0-thalassemia/Hb E, $\beta^{+severe}$-thalassemia/Hb E, and β^+-thalassemia/Hb E samples were 0.9-7.1 (median = 2.5) and 4.9-12.1 (median = 7.2), 1.5-6.1 (median = 2.4) and 5.0-10.1 (median = 6.7), and 0.8-2.5 (median = 1.1) and 8.4-9.5 (median = 9.2) g/dl, respectively. Statistically significant higher Hb F amounts are found in subjects with β^0- and $\beta^{+severe}$- than β^+-thalassemia genotype, $p = 0.017$ and 0.040, respectively. No difference in Hb F level is found between β^0- and $\beta^{+severe}$-thalassemia

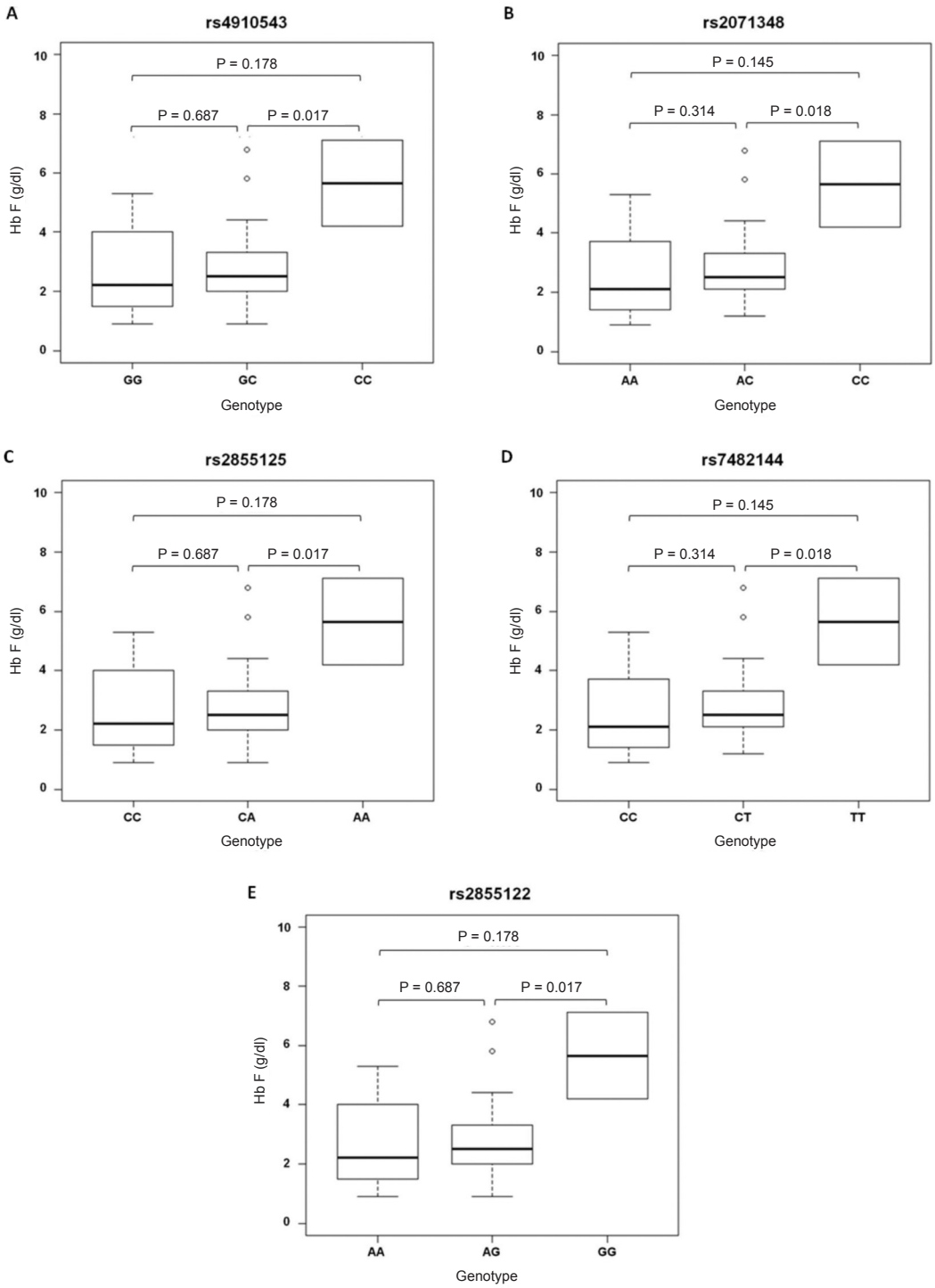

Fig 2–Box plots of Hb F levels associated with SNP genotypes in β^0-thalassemia/Hb E subjects (n = 46). Box represents 25% and 75% interval. Bar in box is median, and the upper and lower bars represent maximum and minimum values. P = p-value, with statistical significance at < 0.05.

Table 1
SNPs in β-globin gene cluster investigated in the study.

SNP ID	Position on chromosome 11[a]	Gene	Association (*p*-value)[b]	
			Disease severity	Percent Hb F
rs4910543	5277723	HBBP1-HBD	2.82E-11	1.05E-18
rs2071348	5264146	HBBP1	2.96E-13	3.03E-19
rs2855125	5273687	HBG2	8.80E-09	0.01
rs7482144	5276169	HBG2	4.40E-09	0.04
rs2855122	5277236	HBG2	2.80E-10	0.17

[a]NCBI build 37.2 and NC_000011.9. [b]Nuinoon *et al* (2010); Sherva *et al* (2010).

Table 2
Primers used in multiplex-ARMS-PCR detection of SNPs.

Primer	Sequence (5′ →3′)	Tm (°C)	GC content (%)	Amplicon size (bp)
F1	AGTTGACTTCCATTCTAACCCAC	57.1	43.5	255
R1M	GAGGTCAGAGGTTAGAAATCAGAG	61.0	45.8	
R1mi	GAGGTCAGAGGTTAGAAATCAGAC	61.0	45.8	
F2	TGAAAGAGGGATTAGCCCG	58.7	52.6	507
R2M	TGGTAAGTGGCCTTCCATTATT	56.5	40.9	
R2mi	TGGTAAGTGGCCTTCCATTATG	58.4	54.8	
F3	GGGGCAATACTATTTCCAACG	57.9	47.6	312
R3M	GCTGACTTGTGAGCTTCTGCG	61.8	57.1	
R3mi	GCTGACTTGTGAGCTTCTGCT	59.8	52.4	
F4	GGGTGCCTACATACATACCTGAA	60.6	47.8	628
R4M	GGTGGAGTTTAGCCAGGGAC	61.4	60.0	
R4mi	GGTGGAGTTTAGCCAGGAAC	59.3	55.0	
F5	GATCCCCTATCTTAAAGAGACCCTA	61.3	44.0	435
R5M	ATCCATGACCTTGGTAGATTATGAT	58.1	36.0	
R5mi	ATCCATGACCTTGGTAGATTATGAC	59.7	40.0	

genotype, $p = 0.848$. Hb F levels in 150 non-thalassemic control individuals were 0-0.15 g/dl.

Co-inheritance of α-thalassemia genotypes and Hb F and total Hb levels in β-thalassemia/Hb E

As Hb F and total Hb levels in β-thalassemia/Hb E have been shown to be affected by concomitant inheritance of α-thalassemia traits (Sripichai *et al*, 2008), the presence of the latter genotypes among the test subjects were identified. There were 13 β^0-thalassemia/Hb E subjects with co-inheritance of α-thalassemia 2 trait (12 with $-\alpha^{3.7}$ and 1 with $-\alpha^{4.2}$) and one subject with heterozygous Hb CS, having Hb F level of 0.7-3.0 (median = 1.6) and 3.5 g/dl, respectively and total Hb level of 6.5-9.9

Table 3
Genotype and minor allele frequencies of SNPs in non-thalassemia and β-thalassemia/Hb E subjects.

SNP ID	Genotype	Non-thalassemia ($N = 150$)			β-thalassemia/Hb E ($N = 81$)		
		n	GF	MAF	n	GF	MAF
rs4910543	G/G	81	0.540		13	0.160	
	G/C	55	0.367		59	0.728	
	C/C	14	0.093	0.277	9	0.112	0.475
rs2071348	A/A	111	0.740		19	0.235	
	A/C	32	0.213		57	0.703	
	C/C	7	0.047	0.153	5	0.062	0.414
rs2855125	C/C	81	0.540		13	0.160	
	C/A	55	0.367		60	0.741	
	A/A	14	0.093	0.277	8	0.099	0.469
rs7482144	C/C	106	0.707		19	0.235	
	C/T	38	0.253		57	0.703	
	T/T	6	0.040	0.167	5	0.062	0.414
rs2855122	A/A	78	0.520		13	0.160	
	A/G	58	0.387		60	0.741	
	G/G	14	0.093	0.287	8	0.099	0.469

GF, genotypic frequency; MAF, minor allele frequency; N and n, number of subjects.

(median = 8.5) and 8.5 g/dl, respectively. Statistically significant lower Hb F levels are observed in β^0-thalassemia/Hb E subjects with α-thalassemia trait having high (> 8.0 g/dl; $n = 12$) and moderate (6-8 g/dl; $n = 3$) total Hb level compared with those with no concomitant α-thalassemia trait (11, 26 and 9 having total Hb level > 8.0, 6-8 and < 6 g/dl, respectively) ($p = 0.009$).

Association of SNP genotypes and Hb F levels in β-thalassemia/Hb E subjects

Hardy-Weinberg equilibrium values among all 5 SNPs genotypes in non-thalassemia samples are not statistically different (data not shown). Frequencies of the 5 SNPs in β-thalassemia/Hb E are significantly different from those in non-thalassemia individuals ($p < 0.001$) (Table 3). Homozygosity in minor allele frequencies (MAFs) and heterozygous genotypes are higher in β-thalassemia/Hb E, whereas homozygosity of the major alleles is found mainly in non-thalassemia subjects. Irrespective of the thalassemia genotypes, statistically significant higher Hb F levels are present in β-thalassemia/Hb E subjects with homozygosity of minor alleles for all 5 SNPs ($p < 0.05$) (Fig 2). All those carrying homozygous minor alleles have total Hb level > 8.0 g/dl and the majority (81%-83%) of the cases with heterozygous minor alleles have mild (total Hb level > 8 g/dl) to moderately severe (total Hb level of 6-8 g/dl) anemia.

DISCUSSION

It is well accepted that HPHF can decrease β-thalassemia severity, but the mechanisms by which the two γ-globin genes are activated remain unclear (El-

Beshlawy *et al*, 2009). However Hb F level can be affected by many conditions other than those considered in this study, such as age < 1 year, pregnancy and recent blood transfusion (Boyer *et al*, 1975; Edoh *et al*, 2006; Mosca *et al*, 2009), and so such individuals were excluded from the study.

Three genetic factors influencing Hb F levels in adult red blood cells, namely, β-thalassemia genotype, α-thalassemia co-inheritance and 5 SNPs in β-globin gene cluster were investigated in 61 β-thalassemia/Hb E subjects. Higher levels of Hb F were detected in β-thalassemia/Hb E samples with severe β-thalassemia genotypes compared to the less severe forms and this phenomenon may be due to a compensatory effect. This is consistent with reports of low Hb F levels present in β^0-thalassemia/Hb E individuals whose anemia severity are ameliorated by co-inheritance of α-thalassemia 2 trait or Hb CS (Winichagoon *et al*, 2000; Sripichai *et al*, 2008).

Independent of the thalassemia genotypes, homozygosity of the minor alleles of all 5 SNPs located in β-globin gene cluster, chosen for their significant association with high Hb F level and disease severity from a previous GWAS (Nuinoon *et al*, 2010; Sherva *et al*, 2010), are significant genetic markers of high Hb F. Remarkably, one case of β^0-thalassemia/Hb E [cd43(G>T)] with homozygous minor alleles for all 5 SNPs had normal total Hb level (12 g/dl) and high Hb F level (7.1 g/dl). The rs7482144 (-153 $^{G}\gamma$ *XmnI* polymorphism) is known as a quantitative trait locus for high Hb F production in β-thalassemia (Thein *et al*, 2009; Nuinoon *et al*, 2010; Oberoi *et al*, 2011), and the other SNPs may be linked (haplotype) or are independent informative genetic markers of HPHF.

In summary, among β-thalassemia/Hb E subjects in this study higher levels of Hb F are associated with β-thalassemia/Hb E carrying severe than mild β-thalassemia mutations, co-inheritance of α-thalassemia trait or heterozygous Hb CS. Homozygosity of the five minor SNP alleles located in the β-globin gene cluster are independent genetic markers of elevated Hb F levels in β-thalassemia/Hb E individuals. An understanding of these and other genetic (and epigenetic) factors underlying HPHF should lead to possible therapeutic interventions, either genetically or pharmacologically, to ameliorate the severity of this debilitating hereditary anemic disease that affects a sizeable population of Southeast Asia.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Faculty of Medicine Ramathibodi Hospital, Mahidol University. The authors thank Prof Prapon Wilairat for correcting the English of the manuscript.

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