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ORIGINAL ARTICLE

Effect of Swiss-type heterocellular HPFH from *Xmn*I-$^{G}\gamma$ and *HBBP1* polymorphisms on HbF, HbE, MCV and MCH levels in Thai HbE carriers

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Abstract Relationships of Swiss-type heterocellular HPFH as functions of *Xmn*I-$^{G}\gamma$ and *HBBP1*:rs2071348 polymorphisms and HbF, HbE, MCV and MCH in HbE carriers were evaluated in 52 non-anemic and α-thalassemia-free Thai HbE carriers. HbF and HbE levels were measured using cation-exchange HPLC. MCV and MCH were determined using an automated blood counter. The *Xmn*I-$^{G}\gamma$ polymorphism was identified by *Xmn*I digestion of amplified products, and the *HBBP1*:rs2071348 polymorphism by tetra-ARMS-PCR. HbF levels in HbE carriers were higher than those in normal individuals. HbF levels >0.8 % indicated the Swiss-type heterocellular HPFH in these subjects, rendering a prevalence of 40.4 %. The *Xmn*I-$^{G}\gamma$ (+) and *HBBP1*:rs2071348 (C) alleles were modestly positively correlated with elevated HbF, elevated MCH and lowered HbE values. This study thus confirms the influence of the *Xmn*I-$^{G}\gamma$ and *HBBP1*:rs2071348 polymorphisms on HbF production. The present study demonstrates the association of *Xmn*I-$^{G}\gamma$ and *HBBP1*:rs2071348 with HbF, HbE, MCV and MCH in HbE carriers for the first time, and highlights the effect of elevated HbF production on HbE levels.

Keywords *Xmn*I-$^{G}\gamma$ · *HBBP1* · HbF · HbE · Erythrocytic indices

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Introduction

HbF ($\alpha_2\gamma_2$) is the hemoglobin predominantly produced in fetuses and newborn babies. A small level of this hemoglobin is observed in adult blood, being confined to subpopulations of red blood cells termed "F cells" [1]. Less than 1 % HbF, corresponding to <4.5 % F cells, occurs in adults [2, 3]. HbF levels, however, can be elevated in adult life via either an acquired or inherited condition [4]. In the inherited form, hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by higher than normal levels of HbF and F cells in peripheral blood as a result of defects in the β-globin gene cluster that favor increased expression of γ-globin genes. HPFH is classified into two types, pancellular and heterocellular HPFH according to the intra-erythrocytic distribution of HbF. Swiss-type HPFH is a subgroup of heterocellular HPFH that is fairly common in the general population (10–20 %) [5–7] and is characterized by its modest elevation of HbF [7, 8].

Several *in-cis* and *in-trans* genetic loci have been shown to be involved in HbF and F cell production in Swiss-type HPFH [2]. The *Xmn*I-$^{G}\gamma$ polymorphism is the *in-cis* loci that has been shown to influence HbF and F cell production in the Swiss-type HPFH [9, 10]. The presence of *Xmn*I-$^{G}\gamma$ site (*Xmn*I-$^{G}\gamma$ (+)) is associated with increased HbF and F cell levels. In a UK twin study, the *Xmn*I-$^{G}\gamma$ polymorphism was shown to have a significant effect on F cell levels,

accounting for approximately 13 % of F cell variance [11]. This single nucleotide polymorphism (SNP) as well as the *rs2071348* of the ψβ-globin gene (*HBBP1*) have recently been analyzed in the Thailand population affected by HbE/β-thalassemia, and was shown to be strongly associated with mild clinical symptoms and elevated HbF production [12–14].

HbE is a β-structural hemoglobin variant resulting from assembly of a normal α-globin chain and a mutated β-globin chain, β^{E}, which originates from the G-A substitution at codon 26. HbE is commonly found across the Kingdom of Thailand, with 10–60 % frequency of carriers depending on the region [15]. Mating of HbE and β-thalassemia carriers has 25 % chance of producing a life-threatening HbE/β-thalassemia. Thus, population-based detection of the carriers of HbE is essential. The heterozygous state of HbE, however, is clinically asymptomatic with characteristically minimal morphological abnormalities of red blood cells [16]. A recent survey in Thailand showed that mild inherited HbE/β-thalassemia is associated with elevated HbF production [13]. This indicated that high HbF determinants should exist in HbE carriers who are parents of these HbE/β-thalassemia patients. However, the prevalence and impact of high HbF in Thai HbE carriers has to date not been investigated.

Herein, we demonstrate the presence of Swiss-type heterocellular HPFH in Thai HbE carriers. The *Xmn*I-$^{G}\gamma$ and the *HBBP1*: *rs2071348* polymorphisms were identified and shown to be in complete linkage disequilibrium (LD). They were involved in increased HbF and MCH levels, but with decreased HbE values. However, the MCV was not influenced by these two SNPs. This is the first report concerning the effects of the *Xmn*I-$^{G}\gamma$ and the *HBBP1*: *rs2071348* polymorphisms in HbE carriers. We also discuss the influence of high HbF levels on those of HbE.

Materials and methods

Determination of hematologic parameters

Blood samples of 52 HbE carriers were collected from a routine laboratory without traceable identification. Hemoglobin concentration (Hb; g/L), mean corpuscular volume (MCV; fL) and mean corpuscular hemoglobin (MCH; pg) were determined using an automated blood counter (Beckman Coulter LH 750, Beckman Coulter, Inc. California, USA). HbE (with HbA_2 in %) and HbF (%) levels were determined by weak cation-exchange high-performance liquid chromatography (HPLC) (Bio-Rad VARIANT^{TM} Hemoglobin Analysis System, CA).

DNA preparation and identification of common α-thalassemia determinants

Genomic DNA was prepared from buffy coat using the modified Chelex^{TM} DNA extraction technique initially described by Polski et al. [17, 18]. Three common α-hemoglobinopathies, including SEA-α thalassemia 1, 3.7-kb α-thalassemia 2 and Hb Constant Spring, were determined using the multiplex allele-specific PCR protocol established in our laboratory [18].

Identification of the *Xmn*I-$^{G}\gamma$ polymorphism

The *Xmn*I-$^{G}\gamma$ polymorphism at position −158 on $^{G}\gamma$-globin gene promoter was determined by the *Xmn*I restriction of PCR products initially described by Craig et al. [19] with some modifications. The PCR was performed in a total volume of 25 μl containing 1.5 μg of genomic DNA, 100 μM of dNTPs, 2 M of betaine, 0.05 units of DNA polymerase (*iTaq*; iNtRON Biotechnology, Inc. Kungki-Do, Korea), 0.2 μM of primers [5′-GG-1 primer: 5′-AAC TGT TGC TTT ATA GGA TTT TTC A-3′, 3′-AG-1 primer: 5′-GTC TGG ACT AGG AGC TTA TTG AT-3′], in 10 mM Tris–HCl pH 8.5, 50 mM KCl and 2.5 mM $MgCl_2$. A total of 35 thermal cycles were carried out with each cycle comprising DNA denaturation at 95 °C for 1 min, primer annealing at 58 °C for 2 min and primer extension at 72 °C for 1 min. The initial denaturation time was extended to 5 min, while the final extension time was prolonged to 5 min. The 665-bp amplified product was produced and subsequently digested with the *Xmn*I restriction enzyme (New England BioLabs Inc., MA, USA) using conditions suggested by the manufacturer. Successful digestion yielded the digested fragments shown in Fig. 3.

Identification of A/C polymorphism at the *rs2071348* allele of the *HBBP1* gene

Detection of A/C polymorphism at the *rs2071348* allele in ψβ-globin gene (*HBBP1*) was done by the tetra-ARMS-PCR protocol initially introduced by Ye et al. [20] and slightly modified in our laboratory. Briefly, the 25 μl PCR reaction contained 0.6 μg genomic DNA, 200 μM of dNTPs, 0.05 units of DNA polymerase (*iTaq*; iNtRON Biotechnology, Inc. Kungki-Do, Korea), 0.25 μM each of "rs2071348-AF" primer (5′-TTG CAC TAT GGG TAC TTT TGA AAG CAG-3′) and "rs2071348-CR" primer (5′-CCT CCA GGA CTA TGC AGA AAA GTG ACT A-3′), 0.2 μM of "rs2071348-CF" primer (5′-AAC TAA AAT TTG GTA GAG CAA GGA CTA CGC), 0.1 μM of "rs2071348-AR" primer (5′-CAA ATG GTA AGT GGC CTT CCA TTG TT-3′) in 10 mM Tris–HCl pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$ (Fig. 4a). A total of 35

thermal cycles were carried out with each cycle comprising DNA denaturation at 94 °C for 1 min, primer annealing at 65 °C for 1 min and primer extension at 72 °C for 1 min. The initial denaturation time was extended to 5 min, while the final extension time was prolonged to 5 min. The 218-bp amplified products generated from "rs2071348-CF" and "rs2071348-CR" primers were produced in the presence of the "C" allele. The 170-bp product was produced from "rs2071348-AF" and "rs2071348-AR" primers in the presence of "A" allele. The 332-bp fragments produced from "rs2071348-AF" and "rs2071348-CR" were used as internal control (Fig. 4b).

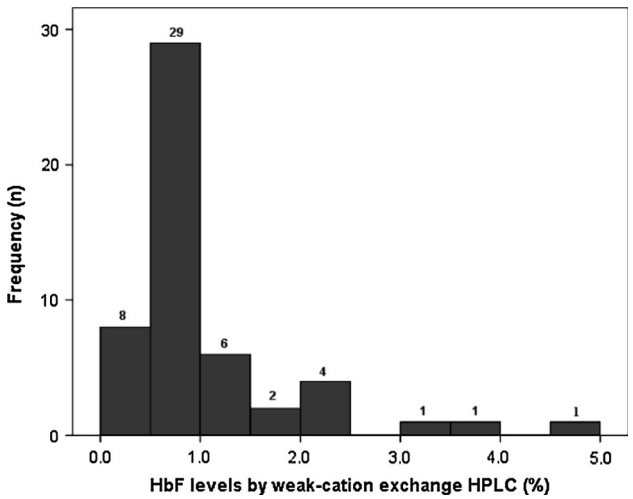


Fig. 1 Distribution of HbF levels in 52 Thai HbE carriers after cation-exchange HPLC determination. The positively skewed distribution is clearly shown with 40 % of the subjects being Swiss-type heterocellular HPFH as indicated by HbF levels >0.8 %. The numbers of subjects are placed on the *top of each bar*

Statistical analysis

Means and standard deviations of HbF, HbE, MCV and MCH were calculated. Correlation coefficient (r) and coefficient of determination (r^2) of *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* genotypes with HbF, HbE, MCV and MCH values were determined by Pearson's correlation coefficient analysis. All calculations were performed using statistical software.

Results

Demographic data of HbE carriers

Blood samples were taken from 52 HbE carriers aged 3–90 years. Hb concentrations ranged from 101 to 157 g/L, MCV from 68.9 fL to 85.9 fL and MCH from 16.0 to 29.5 pg. HbE levels were in the 20.6–31.4 % range. HbF spanned 0.0–4.7 % with the distribution positively skewed (Fig. 1). None of these samples had SEA-α-thalassemia 1, 3.7-kb α-thalassemia 2 or Hb Constant Spring alleles. Figure 2 shows the HPLC histogram of HbE carriers with normal and high HbF levels.

*Xmn*I-$^{G}\gamma$, *HBBP1*: r*s2071348* polymorphisms and HbF levels

The *Xmn*I digested products of the 665-bp amplified products covering the nucleotides at position −158 to Cap on the $^{G}\gamma$-promoter are shown in Fig. 3. Locations of the tetra-ARMS primers as well as the corresponding amplified products are shown in Fig. 4. Both *Xmn*I-$^{G}\gamma$ and *HBBP1*: *rs2071348* polymorphisms were found in complete linkage disequilibrium. The *Xmn*I-$^{G}\gamma$ (+) was always seen with the "C" allele of the *HBBP1*: *rs2071348*. The *Xmn*I-$^{G}\gamma$ (-) was

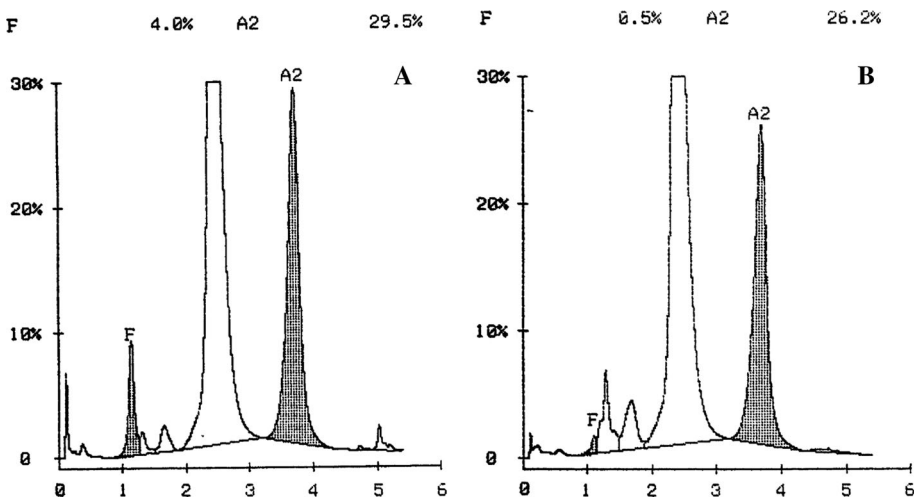


Fig. 2 Cation-exchange HPLC histograms of hemoglobin forms in HbE carriers. Three hemoglobin forms, Hbs A_0, A_2 and F, are normally observed. Hbs F and A_2 peaks are highlighted in *black with names labeled* at the summit. The HPLC histogram of HbE carriers with high HbF level (4.0 %) is shown in (**a**) and that with normal HbF level (0.5 %) in (**b**). Peaks labeled A_2 in the histogram are HbA_2/E which accounts for 29.5 % in (**a**) and 26.2 % in (**b**)

always observed with the "A" allele of the *HBBP1*: *rs2071348*. Homozygotes for the presence of the *Xmn*I-$^{G}\gamma$ site (+/+) and *HBBP1*: *rs2071348* (C/C) were observed in 11 samples (21.1 %). Homozygotes for the absence of the *Xmn*I-$^{G}\gamma$ site (-/-) and *HBBP1*: *rs2071348* (A/A) were seen in another 11 samples (21.1 %). The heterozygotes of *Xmn*I-$^{G}\gamma$ site (±) and *HBBP1*: *rs2071348* (A/C) were present in 30 samples (57.8 %). The allele frequencies of the *Xmn*I-$^{G}\gamma$ (+) and *HBBP1*: *rs2071348* (C) in these HbE carriers were identical (0.50). HbF levels in those having *Xmn*I-$^{G}\gamma$ site (+/+ and ±) and *HBBP1*: *rs2071348* (C/C and A/C) were higher than those having *Xmn*I-$^{G}\gamma$ site (-/-) and *HBBP1*: *rs2071348* (A/A). HbE carriers had higher mean HbF levels than normal subjects within the same *Xmn*I-$^{G}\gamma$ and *HBBP1*: *rs2071348* genotypes. HbF levels above 0.8 % in 21 HbE carriers (40.4 %) seemed to be influenced by the *Xmn*I-$^{G}\gamma$ site (+/+ or ±) or *HBBP1*: *rs2071348* polymorphism (C/C or A/C) (Table 1; Fig. 5).

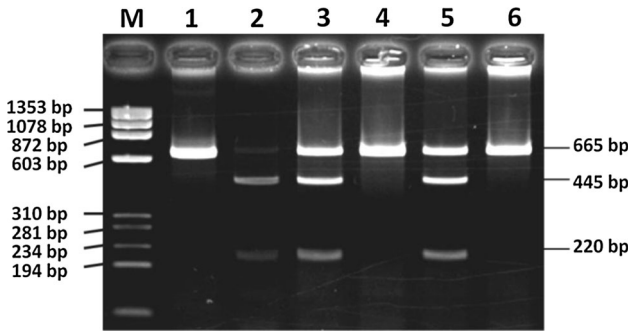
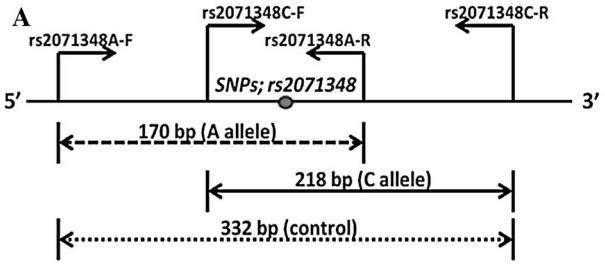


Fig. 3 Determination of the *Xmn*I-$^{G}\gamma$ polymorphism by *Xmn*I digestion of the 665-bp PCR products covering the C–T substitution at the nucleotide −158 on $^{G}\gamma$-promoter. After separating in 2.0 % agarose gel electrophoresis, the *Xmn*I-$^{G}\gamma$ (-/-) shows only a 665-bp undigested band, the *Xmn*I-$^{G}\gamma$ (+/+) shows 2 bands of 445 and 220 bp, and the *Xmn*I-$^{G}\gamma$ (±) shows 3 bands of 665, 445 and 220 bp. *Lane "M"* is ϕX 174 DNA size standard marker; *lanes 1, 4* and *6* are of *Xmn*I-$^{G}\gamma$ (-/-) genotype; *lanes 3 and 5* of *Xmn*I-$^{G}\gamma$ (±) genotype; *lane 2* of *Xmn*I-$^{G}\gamma$ (+/+) genotype

Influence of *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* polymorphisms on HbF, HbE, MCV and MCH levels in HbE carriers

Since the *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* were closely associated and the presence of these SNPs tended to increase HbF levels, we evaluated their effects on HbF as well as HbE, MCV and MCH. HbF was most influenced by these SNPs, followed by HbE and MCH, respectively. These SNPs were responsible for approximately 6.0, 6.4 and 2.3 % of the variation of HbF, HbE and MCH levels, respectively. MCV, however, was not influenced by these SNPs (Fig. 6).



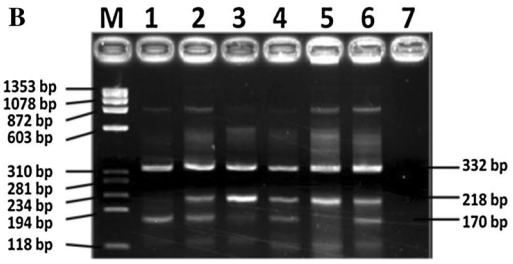


Fig. 4 Detection of the A/C polymorphism at the *rs2071348* allele of *HBBP1*gene by the tetra-ARMS PCR. **a** Graphic representation of primer localizations and sizes of amplified products. **b** PCR products in 2 % agarose gel electrophoresis. The 170-bp PCR product is specific for the "A" allele, and 218-bp product for the "C" allele. The 332-bp product is the internal control. *Lane 1* is the homozygote for A/A; *lanes 3* and *5* are homozygotes for C/C; *lanes 2, 4* and *7* heterozygotes for A/C; *lane 7* the no-DNA blank control. *Lane "M"* is a ϕX-174 DNA size standard

Table 1 HbF levels in HbE carriers and normal subjects with different *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* polymorphisms

	*Xmn*I-$^{G}\gamma$ (-/-) *rs2071348* (A/A)	*Xmn*I-$^{G}\gamma$ (±) *rs2071348* (A/C)	*Xmn*I-$^{G}\gamma$ (+/+) *rs2071348* (C/C)
HbE carriers Mean ± SD (*n*)	0.65 ± 0.30 (11)	1.01 ± 0.90 (30)	1.33 ± 1.21 (11)
Normal Mean ± SD (*n*)	0.18 ± 0.29 (6)	0.38 ± 0.83 (11)	NA

NA not available

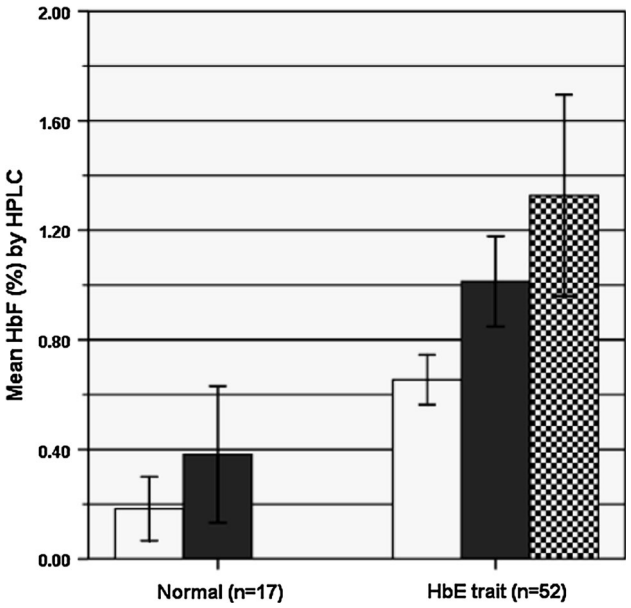


Fig. 5 HbF levels (determined by cation-exchange HPLC) in normal and HbE carriers with different *Xmn*I-$^{G}\gamma$ genotypes. The *white bars* represent the *Xmn*I-$^{G}\gamma$ (-/-) or *HBBP1*: *rs2071348* (A/A). *Dark gray bars* represent the *Xmn*I-$^{G}\gamma$ (±) or *HBBP1*: *rs2071348* (A/C). *Black-spotted bars* represent the *Xmn*I-$^{G}\gamma$ (+/+) or *HBBP1*: *rs2071348* (C/C). The HbF levels in HbE carriers are higher than those in normal individuals in all categories. Note that none of the subjects in the normal group had the *Xmn*I-$^{G}\gamma$ (+/+) or *HBBP1*: *rs2071348* (C/C) genotype

Discussion

HbF levels in healthy adults vary widely. The distributions are positively skewed, with 10–20 % of individuals having 0.8–5 % HbF levels residing at the right margin of the distribution curve [5–7]. The HbF distribution observed in Thai HbE carriers analyzed in this study was also positively skewed, resembling those previously documented. This observation highlighted the presence of the Swiss-type heterocellular HPFH in Thai HbE carriers. HbE carriers had higher HbF levels than normal individuals. The same findings have been shown in β-thalassemia carriers [21]. HbF levels may be increased in situations accompanied by erythroid expansion due to selective survival of erythroid precursors that contain high HbF [22, 23]. Both β-thalassemia and HbE carriers have some degree of erythroid expansion [16, 24]. Thus, HbF levels were undoubtedly increased in HbE carriers. However, HbE carriers having the *Xmn*I-$^{G}\gamma$ (± or +/+) and *HBBP1: rs2071348* (C/A or C/C) had elevated HbF levels. This indicated that these two SNPs were involved in increasing HbF production in these subjects and thus exacerbating high HbF levels.

A prevalence of approximately 40 % of Swiss-type heterocellular HPFH was observed in this cohort, which was higher than those reported in European and Japanese subjects. This might be explained by the fact that Thailand is in the malarial endemic area and those having high HbE and high HbF are naturally selected. Erythrocytes containing HbE and HbF have been shown to retard growth of *P. falciparum* due to induction of oxidative stress [25]. Cyto-adherance of HbF-containing erythrocytes is impaired, thus rendering lower parasite densities and less clinical burden [26]. Morbidity rate in those having both high HbE and HbF levels should thus be decreased.

The *Xmn*I-$^{G}\gamma$ polymorphism was shown to be the major *cis*-acting genetic factor associated with increased HbF/F cell levels. It had an allele frequency of 0.32–0.35 in British twins and accounted for approximately 1/3 of F cell variation [11]. This SNP has been studied by several authors, and its association with increased $^{G}\gamma$-globin gene expression and elevated HbF/F cell levels in both normal and β-hemoglobinopathies have been demonstrated [6, 9, 10, 27–32]. The effect of the *Xmn*I-$^{G}\gamma$ site becomes pronounced when it is accompanied by stress erythropoiesis in several conditions, e.g., β-hemoglobinopathies [2, 33]. HbE carriers having the *Xmn*I-$^{G}\gamma$ site (*Xmn*I-$^{G}\gamma$ (+)) in both heterozygous and homozygous states had increased levels of HbF compared to those lacking this site. This should thus be a result of the combined effects of stress erythropoiesis occurring in HbE carriers and the *Xmn*I-$^{G}\gamma$ site.

The *HBBP1* polymorphism was studied by Nuinoon et al. [13] who found that the SNP *rs2071348* was significantly associated with clinical diversity as well as high HbF levels in HbE/β-thalassemia patients. This SNP seemed to be in close linkage disequilibrium with the *Xmn*I-$^{G}\gamma$ polymorphism in these HbE carriers, as shown by their completely identical allele and genotype frequencies. This was not surprising, since these two SNPs are localized in the β-globin gene cluster and approximately 10 kb apart (NG_000007.3). This short genomic distance combined with natural selection against malarial infection could explain this phenomenon. However, more studies with larger sample groups would be needed to confirm this hypothesis concerning linkage disequilibrium.

The *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* polymorphisms accounted for almost the same magnitude of HbF and HbE variation, as shown in Fig. 6a and b. However, they accounted for only 6 % of the variation of these hemoglobins, suggesting that other factors were involved, particularly for HbF production. Interestingly, their relationships to HbF and HbE levels were inversely correlated. While the *Xmn*I-$^{G}\gamma$ (+) and *HBBP1: rs2071348* (C) polymorphisms were associated with high HbF levels, they were mostly accompanied by low levels of HbE. The preferential assembly of α-globin chains to γ-globin chains rather than to the β^{E}-globin chains might be the most plausible explanation [34, 35]. This finding strongly suggests that low levels of HbE are found when HPFH co-

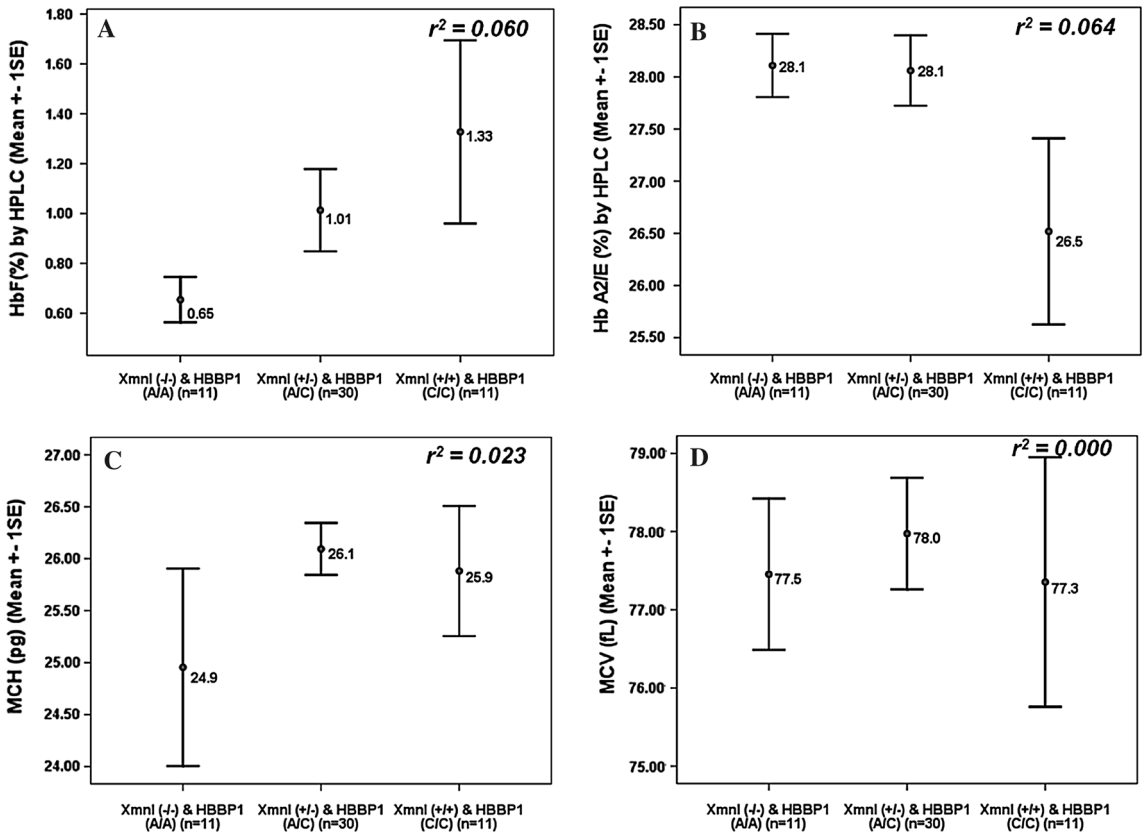


Fig. 6 Relationships of the *Xmn*I-$^{G}\gamma$ and *HBBP1: rs2071348* polymorphisms and HbF (**a**), HbE (**b**), MCH (**c**) and MCV (**d**) levels. *Bars* in individual boxes represent mean ± SE of the corresponding parameters. Mean values are shown at the *middle of each bar*. R^2 values at the *top right corner* indicate the extent of variations of the parameters on the *Y-axis* that are explained by the SNP genotypes on the *X-axis*

exists in HbE carriers. This should trigger awareness when interpreting hemoglobin typing and making diagnosis of HbE carriers in subjects showing increased HbF levels.

Heterocellular HPFH, as a function of 6q-QTL, was shown to be significantly associated with reduced HbA_2 levels, elevated MCV and MCH values in β-thalassemia carriers [36]. The underlying mechanism of decreased HbA_2 levels in these β-thalassemia carriers is likely to be due to the preferential binding of α-globin chains to the γ-globin ones rather than to the δ-globin chains [34, 35]. This finding should raise awareness in β-thalassemia screening in individuals with the HPFH phenotype. In this study, the heterocellular HPFH, as a function of the *Xmn*I-$^{G}\gamma$ (+) and *HBBP1: rs2071348* (C) polymorphisms seemed to be associated with reduced HbE levels and elevated MCH values, but not MCV values. We postulated that high HbF-erythrocytes in HbE carriers are well provided with hemoglobin, with unaltered volumes, compared to those having normal HbF content. This should also be taken into account when HbE levels and red blood cell indices are used to screen HbE carriers in individuals having high HbF levels.

In conclusion, we have shown that high HbF levels do exist in Thai HbE carriers. This high HbF phenotype was certainly of the Swiss-type heterocellular HPFH. We also demonstrated that the Swiss-type heterocellular HPFH as a function of the *Xmn*I-$^{G}\gamma$ (+) and the *HBBP1: rs2071348* (C) polymorphisms was associated with increased HbF, increased MCH and reduced HbE levels. Although these results were derived from a relatively small sample size, they provided novel insights and should raise awareness when Hb typing and red cell indices are interpreted for diagnosis of HbE carriers. Further investigation among more HbE carriers is required to corroborate these findings.

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Conflict of interest The authors declare no conflict of interest.

Statement of human rights The authors declare that the procedures followed were approved by a relevant institutional ethics committee on human experimentation.

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