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Essential genetic modifiers and their measurable impact in a community-recruited population analysis for non-severe hemoglobin E/β-thalassemia prenatal genetic counseling

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ABSTRACT

The study aimed to identify essential phenotype-modulating factors among the pre-existence of several important ones and clarify their measurable impact on the clinical severity of hemoglobin (Hb) E/β-thalassemia in a community-recruited population analysis. This prospective study was designed to compare modifiers between community- (less or no symptoms) and hospital-recruited individuals with Hb E/β-thalassemia. The formerly included couples previously assessed for prenatal thalassemia at-risk status at 42 community and 7 referral hospitals in Thailand through on-site investigations between June 2020 and December 2021. The control included Hb E/β-thalassemia patients undergoing transfusions. The Mahidol score classified disease severity. Beta-globin, α^0-thalassemia ($-^{SEA}$, $-^{THAI}$), α^+-thalassemia ($-\alpha^{3.7}$, $-\alpha^{4.2}$), Hb Constant Spring (α^{CS}) alleles, rs766432 in *BCL11A*, rs9399137 in *HBS1L-MYB*, and rs7482144-*Xmn*I were evaluated. Modifiers were compared between 102 community- and 104 hospital-recruited cases. Alleles of β^+, $-^{SEA}$, $-\alpha^{3.7}$, α^{CS}, and a minor allele of rs9399137 were prevalent in the community and mild severity groups ($p < 0.05$). Multiple linear regression analysis associated modulating alleles with −4.299 ($-^{SEA}$), −3.654 (β^+), −3.065 (rs9399137, C/C), −2.888 (α^{CS}), −2.623 ($-\alpha^{3.7}$), −2.361 (rs7482144, A/A), −1.258 (rs9399137, C/T), and −1.174 (rs7482144, A/G) severity score reductions ($p < 0.05$). Certain modifiers must be considered in routine prenatal genetic counseling for Hb E/β-thalassemia.

1. Introduction

Hemoglobin (Hb) E (HBB:c.79G > A)/β-thalassemia is the most common form of severe β-thalassemia worldwide [1]. However, owing to its wide disease severity spectrum and phenotypic destabilization associated with advanced age, prenatal genetic counseling for Hb E/β-thalassemia pregnancies is complicated [2–5]. Genetic factors that improve the globin chain balance in Hb production have positive impacts on clinical severity in Hb E/β-thalassemia [5–10]. This disease has remarkable clinical heterogeneity, and certain affected individuals require no therapeutic support for it. These individuals cannot be recruited for clinical studies, particularly those trying to identify genetic modifiers. The lack of asymptomatic subjects or those who present unidentified mild disease symptoms in hospital-based studies produces

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results that are marginally significant or even nonsignificant for several strong genetic modifiers. It is generally accepted that the β-thalassemia allele *in trans* to Hb E mutation is vital to β-globin chain production, which can alleviate the globin chain imbalance. However, earlier studies demonstrated only a limited role of β-thalassemia mutation in clinical disease severity [3,4,7,10]. In their findings, patients with Hb E/β-thalassemia with the mild phenotype usually had a serious β-thalassemia (β^0) mutation, which was also observed in patients with either mild or severe disease. Therefore, it was concluded that the mild β-thalassemia (β^+) mutation is only one of several genetic modifiers. In contrast, prior research on homozygous and compound heterozygous β-thalassemia demonstrated that the β-thalassemia allele is the strongest genetic modifier of disease severity [11]. Similar to that of the β^+-thalassemia allele, the clinical influence of the α^0-thalassemia mutation on Hb E/β-thalassemia severity in several hospital-based studies could not be demonstrated owing to the lack of α^0-thalassemia alleles (<1 %) in the study populations [3,8]. Therefore, community-recruited Hb E/β-thalassemia populations are necessary to compensate for this methodological weakness. However, the principal research obstacle is the identification of mild or asymptomatic Hb E/β-thalassemia cases outside of the hospital setting.

Couples at risk of carrying fetuses with Hb E/β-thalassemia are common in Thailand and other Southeast Asian countries [12]. Physicians must have a broad perspective on Hb E/β-thalassemia phenotypes to provide impartial genetic counseling. Unfortunately, milder or asymptomatic cases are seldom encountered in routine hospital practice. Despite the pre-existence of several important genetic modifiers, none are required by national guidelines to be screened in routine practice, except β-globin mutation for diagnosis confirmation. Therefore, the severity of Hb E/β-thalassemia in a fetus is predicted based on whether the β^0 or β^+-globin allele is inherited and depends upon the counselor's perspective of the disease. The aim of this study was to identify essential phenotype-modulating genetic factors to be considered for implementation in nationwide prenatal genetic counseling for Hb E/β-thalassemia.

2. Material and methods

Male and female community-recruited individuals were identified from a list of couples that had attended antenatal care (ANC) at either 1 university, 6 provincial hospitals, or 42 community hospitals in lower northern Thailand. In the ANC records, the prenatal fetal thalassemia risk status was determined for each couple at the Thalassemia Research Unit of the Naresuan University Hospital. The diagnostic center receives blood samples from its network hospitals and tests them for carrier status, which sometimes happens to be the disease condition. Using the university hospital database, husbands or wives diagnosed with Hb E/β-thalassemia between January 2008 and December 2021 were invited to participate in the study. Subjects with Hb E/β-thalassemia diagnosed before this period by other laboratories were eligible to participate and recruited using the ANC records from each network hospital. A mobile medical team from the university hospital made on-site visits at each hospital and collected clinical severity data and blood samples between June 2020 and December 2021. Equal numbers of patients with Hb E/β-thalassemia currently being treated in one university and two provincial hospitals during the same recruitment period comprised the control group. The diagnosis of Hb E/β-thalassemia for all subjects enrolled was confirmed using automated capillary zone electrophoresis (Minicap Flex; Sebia, Lisses, France) for Hb analysis and a PCR-based method [13]. Genotypes other than Hb E/β-thalassemia were excluded. The study was approved by the Institutional Ethical Committee of Naresuan University (approval No. 393/2019) and each provincial hospital. Written informed consent was obtained from all participants before their recruitment into the study.

Clinical disease severity was categorized following the Mahidol severity score [14]. The clinical parameters included: age at presentation and first transfusion, the requirement for transfusion, spleen size, percentile of growth development, and pre-transfusion Hb levels. The scoring system classified disease severity as mild (score 0–3.5), moderate (4–7), or severe (7.5–10). An automated blood cell counter (XN-10; Sysmex Corporation, Kobe, Japan) was used to obtain complete blood counts for individuals who had never been transfused. Medical records were reviewed for patients who had undergone prior transfusions.

The β^+-globin mutation, α^0-thalassemia [SEA ($-^{SEA}$) and Thai ($-^{THAI}$) deletions], α^+-thalassemia [3.7 kb ($-\alpha^{3.7}$) and 4.2 kb ($-\alpha^{4.2}$) deletions], Hb Constant Spring (α^{CS}) alleles, and the single nucleotide polymorphisms (SNPs) rs766432 in *BCL11A*, rs9399137 in *HBS1L-MYB*, and rs7482144-*Xmn*I in $^{G}\gamma$-*globin* were determined for each subject. The β-globin mutation was identified by high-resolution melting (HRM) analysis [13]. Deletional α-thalassemia and Hb Constant Spring mutations were detected by gap PCR and amplification refractory mutation system (ARMS) PCR, respectively. Tetra-primer ARMS-PCR was developed to detect all three SNPs [15,16]. Outer and inner primer pairs were constructed for each SNP. The inner primers were designed to match various alleles of a particular SNP, generate PCRs with their corresponding outer primers, and yield PCR products of different sizes. The outer pairs produced large PCR products that served as an internal control. The SNP genotyping was A-C for rs766432 (*BCL11A*), T-C for rs9399137 (*HBS1L-MYB*), and G-A (antisense strand) for rs7482144-*Xmn*I ($^{G}\gamma$-*globin*). Known DNA samples from DNA sequencing of each SNP were used in PCR optimization during method development. The primers and PCR product sizes for all three SNPs are listed in Table 1. Common β-globin deletions (3.4-kb and FIL deletions) and deletions causing $(\delta\beta)^0$-thalassemia and hereditary persistence of fetal Hb (HPFH) [$(\delta\beta)^0$-thalassemia, HPFH-6, Indian and Chinese ($^{A}\gamma\delta\beta)^0$-thalassemia], determined by PCR methods described elsewhere [17], were analyzed in samples with the HRM pattern of Hb E homozygote to identify Hb E/deletional β-thalassemia, Hb E/$(\delta\beta)^0$-thalassemia, and Hb E/HPFH or to confirm the diagnosis of a Hb E homozygote.

The sample size for the community-recruited population was estimated to be ≥100 subjects. The same sample size was intended for the control. Clinical parameters and genetic modifiers were compared

Table 1
Primers and sizes of single-nucleotide polymorphisms.

Locus	Primer	Amplicon (base pair)
rs766432	Outer forward: 5′-CTTCCTCACATACTCACCAGTACTC-3′	509
	Outer reverse: 5′-GGTGGTAGGTGGGGGTTCAGT-3′	–
	Inner forward (A): 5′-GTTTTGTTTCGCTTTAGCTTTATTAAGGTACAA-3′	236
	Inner reverse (C): 5′-CACTTAAAATGAATGACTTTTGTTGTATGTAGAG-3′	339
rs9399137	Outer forward: 5′-ATCACTGAGAAAAGCATAAGCCTG-3′	586
	Outer reverse: 5′-GGAGTACTACATAACAATCCACAGA-3′	–
	Inner forward (C): 5′-AATAATGTAATTAACTGAACATATGGTTATTC-3′	410
	Inner reverse (T): 5′-GCAGGGTTGCTTGTGAAAAAACTTTA-3′	233
rs7482144	Outer forward: 5′-CCCTTGAGATCATCCAGGTGCTTTA-3′	1002
	Outer reverse: 5′-CTTAAGAGATAATGGCCTAAAACCACAG-3′	–
	Inner forward (G): 5′-CCATGGGTGGAGTTTAGCCATGG-3′	441
	Inner reverse (A): 5′-TATCTCAATGCAAATATCTGTCTGAAACGTTT-3′	615

between the community- and hospital-recruited populations and among severity groups using χ^2/Fisher's exact tests for categorical data and Mann–Whitney *U*/Kruskal–Wallis/*t*-tests for continuous variables. The associations of individual SNPs with the community- and hospital-recruited populations, as well as various severity groups, were determined using codominant, dominant, and recessive genetic models and the χ^2 test. The lowest *p*-value for each model was selected as the reported value. Multiple linear regression models were used to determine the relationships between potential genetic modifiers and clinical severity scores among participants. For each SNP, the homozygote for the major frequency allele was considered the referent group in the codominant model. The statistical significance level was set at $p < 0.05$. All data were analyzed using Statistical Package for the Social Sciences (SPSS) v. 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

One hundred and fifteen community Hb E/β-thalassemia subjects were recruited. Four individuals carrying Hb E homozygotes with high Hb F, two with Hb E homozygotes, two with Hb E heterozygotes, two with β-thalassemia heterozygotes, one carrying a Hb E heterozygote with high Hb F, one with Hb E/$(\delta\beta)^0$-thalassemia, and one with Hb E/Chinese $(^A\gamma\delta\beta)^0$-thalassemia were excluded, leaving 102 subjects with Hb E/β-thalassemia for the analysis. The hospital-recruited group consisted of 104 patients with Hb E/β-thalassemia after exclusions. Two subjects with β-thalassemia homozygotes, two with AEBart's, one with EFBart's, one with β-thalassemia compound heterozygote, and one with a Hb E homozygote were excluded. Demographic data of the two groups are shown in Table 2. While all participants in the community group had ≥1 child, up to 30 patients in the hospital group (28.8 %) were able to have children despite having a higher clinical severity score. Of the 206 Hb E/β-thalassemia cases evaluated, 88 (42.7 %), 72 (35.0 %), and 46 (22.3 %) were mild, moderate, and severe, respectively, as determined by the clinical severity score.

Clinical parameters of the severity scoring system were compared between the community- and hospital-recruited groups and among the severity groups (Tables 2 and 3). In the community group, 12 cases (9 moderate, 2 severe, and 1 mild) had received regular blood transfusions. There were also 12 splenectomized subjects, of which 7 were moderate, 4 were severe, and 1 was mild. Six splenectomized patients were receiving regular blood transfusions. Of the 88 mild cases, 8 received regular blood transfusions and 2 were splenectomized. Six regularly transfused patients (75 %) had received their first transfusions after the age of 43 (range: 43–70 years).

Genetic modifiers were compared between the community- and hospital-recruited groups and among the severity groups (Tables 4 and 5) and included β^+-globin, α^0-thalassemia ($-^{SEA}$), α^+-thalassemia ($-\alpha^{3.7}$), Hb Constant Spring mutations, and the C allele of rs9399137 in *HBS1L-MYB*. These five markers were more prevalent in the community than in the hospital-recruited group and in mild than in moderate and severe cases ($p < 0.05$). Over 1/4 of all cases in the community-recruited group (28.4 %) inherited β^+-globin alleles. Of these, the −28 mutation (−28 ATA) was the most common. No α^0-thalassemia alleles were detected in the hospital-recruited group. All 88 mild cases exhibited ≥1 of the aforementioned genetic modifiers. All 32 cases of β^+-globin mutation and 6 cases with α^0-thalassemia co-inheritance were mild. No Thai or 4.2 kb deletions of α-thalassemia were detected here. The C allele of *BCL11A* (rs766432), C allele of *HBS1L-MYB* (rs9399137), and A allele of $^G\gamma$-*globin* (rs7482144) were considered minor. The SNP genotypes and allelic frequencies are listed in Table 6.

For the multiple linear regression analyses, all genetic modifiers except the two deletional α-thalassemia alleles ($-^{THAI}$ and $-\alpha^{4.2}$) could predict severity scores with statistical significance ($F = 19.463$, $p < 0.001$). The observed variability in 50 % ($R^2 = 0.500$) of the severity score could be explained by the genetic factors included. The regression coefficients indicated that in descending order of potency, the α^0-thalassemia ($-^{SEA}$), β^+-globin, Hb Constant Spring, α^+-thalassemia ($-\alpha^{3.7}$) mutations, and the two SNPs in *HBS1L-MYB* (rs9399137) and $^G\gamma$-*globin* (rs7482144) significantly reduced the predicted severity score ($p < 0.05$) (Table 7). Both dominant and recessive genetic models were subsequently applied in the SNP analysis of the regression model, and the results obtained were the same as those for the codominant model. The multiple linear regression equation for predicting the severity score is as follows:

Table 2
Demographic data and relative clinical parameters of community and hospital subjects.

Clinical parameter	Community $n = 102$	Hospital $n = 104$	*p*-value
Age (year) (median/range)	32 (18–64)	34 (15–82)	0.600[e]
Female (number/%)	67 (65.7)	54 (51.9)	0.045[f]
Clinical severity (number/%)			
Mild	78 (76.5)	10 (9.6)	<0.001[f]
Moderate	20 (19.6)	52 (50.0)	
Severe	4 (3.9)	42 (40.4)	
Age at presentation[a] (year) (median/range)	9.5 (0.5–62)	5 (0–70)	0.021[e]
Age at which first transfusion received[b] (year) (median/range)	15 (1–62)	7 (0.5–70)	0.004[e]
Asymptomatic[c] (number/%)	48 (47.1)	0 (0)	<0.001[f]
Never received any blood transfusion for thalassemia[d] (number/%)	53 (52.0)	0 (0)	<0.001[f]
Requirement for regular transfusion (number/%)	12 (11.8)	96 (92.3)	<0.001[f]
Impalpable spleen (number/%)	39 (38.2)	3 (2.9)	<0.001[f]
Splenectomy (number/%)	12 (11.8)	45 (43.3)	<0.001[f]
Growth and development >25th percentile (number/%)	94 (92.2)	65 (62.5)	<0.001[f]
Hemoglobin level before receiving transfusion[b] (g/dL) (mean ± SD)	7.14 ± 1.39	6.78 ± 1.16	0.101[g]
Hemoglobin level of subjects who never received any transfusion (g/dL) (mean ± SD)	10.54 ± 2.52	–	–
Severity score (median/range)	1 (0–9)	7 (2–10)	<0.001[e]

[a] Excludes asymptomatic subjects.
[b] Excludes cases who had never undergone any blood transfusion for thalassemia.
[c] Six asymptomatic subjects had blood transfusions for reasons other than thalassemia.
[d] Five subjects who never underwent blood transfusion were symptomatic.
[e] *p*-value determined by Mann–Whitney *U* test.
[f] *p*-value determined by χ^2 test.
[g] *p*-value determined by unpaired *t*-test.

Table 3
Comparative clinical parameters of disease severity groups.

Clinical parameter	Mild $n = 88$	Moderate $n = 72$	Severe $n = 46$	*p*-value
Age at presentation[a] (year) (median/range)	15 (1.5–70)	7 (0.5–51)	2 (0–10)	<0.001[e]
Age at which first transfusion received[b] (year) (median/range)	26 (6–70)	9 (0.7–51)	3 (0.5–10)	<0.001[e]
Asymptomatic[c] (number/%)	48 (54.5)	0 (0)	0 (0)	<0.001[f]
Never received any blood transfusion for thalassemia[d] (number/%)	53 (60.2)	0 (0)	0 (0)	<0.001[f]
Requirement for regular transfusion (number/%)	8 (9.1)	57 (79.2)	43 (93.5)	<0.001[f]
Impalpable spleen (number/%)	40 (45.5)	2 (2.8)	0 (0)	<0.001[f]
Splenectomy (number/%)	2 (2.3)	24 (33.3)	31 (67.4)	<0.001[f]
Growth and development >25th percentile (number/%)	83 (94.3)	53 (73.6)	23 (50.0)	<0.001[f]
Hemoglobin level before receiving transfusion[b] (g/dL) (mean ± SD)	7.93 ± 1.23	6.74 ± 1.12	6.36 ± 1.00	<0.001[g]
Hemoglobin level in subjects who never received any transfusion (g/dL) (mean ± SD)	10.54 ± 2.52	–	–	–
Severity score (median/range)	0.5 (0–3.5)	5.75 (4–7)	8 (7.5–10)	<0.001[e]

[a] Excludes asymptomatic subjects.
[b] Excludes cases who had never undergone any blood transfusion for thalassemia.
[c] Six asymptomatic subjects had blood transfusions for reasons other than thalassemia.
[d] Five subjects who never underwent blood transfusion were symptomatic.
[e] *p*-value determined by Kruskal–Wallis test.
[f] *p*-value determined by χ^2 test.
[g] *p*-value determined by *F*-test in ANOVA.

Table 4
Comparison of genetic modifiers between community- and hospital-recruited subjects.

Genetic modifier	Community $n = 102$	Hospital $n = 104$	p-value
β^+-globin mutation (number/%)	29 (28.4)	3 (2.9)	
• −28 ATA (A-G)	19	1	<0.001[a]
• −31 ATA (A-G)	4	1	
• Codon 147 (+AC)	3	0	
• Codon 19 (A-G)	2	1	
• Codon 126 (T-G)	1	0	
α^0-thalassemia mutation (number/%)			
• $-^{SEA}$	6 (5.9)	0 (0)	0.014[b]
• $-^{THAI}$	0 (0)	0 (0)	1.000[b]
α^+-thalassemia mutation (number/%)			
• $-\alpha^{3.7}$	21 (20.6)	6 (5.8)	0.002[a]
• $-\alpha^{4.2}$	0 (0)	0 (0)	1.000[b]
Hb constant spring (number/%)	9 (8.8)	2 (1.9)	0.028[a]
Single-nucleotide polymorphism (SNP) (allele frequency)			
• *BCL11A* (rs766432) (C allele)	0.23	0.16	0.120[a]
• *HBS1L-MYB* (rs9399137) (C allele)	0.51	0.43	0.013[a]
• *Xmn*I (rs7482144) (A allele)	0.46	0.38	0.073[a]

[a] *p*-value determined by χ^2 test.
[b] *p*-value determined by Fisher's exact test.

$$\begin{aligned}\text{Predicted severity score} = & 7.688 - \left(4.299 \times \left[- - ^{\text{SEA}}\ \text{factor}\right]\right) \\ & - \left(3.654 \times \left[\beta^+\ \text{factor}\right]\right) \\ & - \left(3.065 \times \left[\text{rs9399137, C/C factor}\right]\right) \\ & - \left(2.888 \times \left[\alpha^{\text{CS}}\ \text{factor}\right]\right) \\ & - \left(2.623 \times \left[- \alpha^{3.7}\ \text{factor}\right]\right) \\ & - \left(2.361 \times \left[\text{rs7482144, A/A factor}\right]\right) \\ & - \left(1.258 \times \left[\text{rs9399137, C/T factor}\right]\right) \\ & - \left(1.174 \times \left[\text{rs7482144, A/G factor}\right]\right) \\ & - \left(0.856 \times \left[\text{rs766432, C/C factor}\right]\right) \\ & + \left(0.038 \times \left[\text{rs766432, C/A factor}\right]\right)\end{aligned} \quad (1)$$

The severity score decreases by 4.299 in the presence of the α^0-thalassemia ($-^{SEA}$) allele when all other genetic factors are kept constant. Moreover, the same interpretation applies to the other genetic factors in Eq. (1).

Of the 48 asymptomatic subjects, 43 (89.6 %) carried at least one of the strong genetic modifiers (β- and α-thalassemia alleles and A/A homozygote of rs7482144-*Xmn*I). All 32 subjects with the β^+-globin allele were asymptomatic or presented with only mild symptoms. Their average Hb level was 10.74 ± 2.50 g/dL. Twenty-two subjects (68.8 %) had never received any blood transfusion for thalassemia. Two cases required regular transfusions after the age of 70 and 61. Twenty-three cases (71.9 %) had impalpable spleens. Of the six subjects with the α^0-thalassemia ($-^{SEA}$) allele, five were asymptomatic and had impalpable spleens, while one required a single blood transfusion every several years and had a palpable spleen 1 cm below the left costal margin. Their average Hb level was 11.83 ± 2.94 g/dL. The β^+-globin and α^0-thalassemia ($-^{SEA}$) mutations strongly modulated the clinical severity of Hb E/β-thalassemia; these were further demonstrated to be the two strongest genetic modifiers using multiple linear regression analysis.

4. Discussion

The recruitment of Hb E/β-thalassemia subjects from the ANC database can be used to select mild cases and supply abundant genetic modifiers for comparison. Most subjects recruited by this approach had mild symptoms or were asymptomatic. However, 18 inadvertently recruited cases were receiving regular blood transfusions or had undergone splenectomies. These community-recruited subjects were patients who were able to have children, being followed in hospitals other

Table 5
Comparison of genetic modifiers between different severity groups.

Genetic modifier	Mild $n = 88$	Moderate $n = 72$	Severe $n = 46$	*p*-value
β^+-globin mutation (number/%)	32 (36.4)	0 (0)	0 (0)	<0.001[a]
• −28 ATA (A-G)	20	0	0	
• −31 ATA (A-G)	5	0	0	
• Codon 147 (+AC)	3	0	0	
• Codon 19 (A-G)	3	0	0	
• Codon 126 (T-G)	1	0	0	
α^0-thalassemia mutation (number/%)				
• $-^{SEA}$	6 (6.8)	0 (0)	0 (0)	0.014[b]
• $-^{THAI}$	0 (0)	0 (0)	0 (0)	1.000[b]
α^+-thalassemia mutation (number/%)				
• $-\alpha^{3.7}$	24 (27.3)	2 (2.8)	1 (2.2)	<0.001[a]
• $-\alpha^{4.2}$	0 (0)	0 (0)	0 (0)	1.000[b]
Hb constant spring (number/%)	9 (10.2)	2 (2.8)	0 (0)	0.023[b]
Single-nucleotide polymorphism (SNP) (allele frequency)				
• *BCL11A* (rs766432) (C allele)	0.19	0.20	0.18	0.925[a]
• *HBS1L-MYB* (rs9399137) (C allele)	0.52	0.45	0.40	0.004[a]
• *Xmn*I (rs7482144) (A allele)	0.47	0.40	0.35	0.112[a]

[a] *p*-value determined by χ^2 test.
[b] *p*-value determined by Fisher's exact test.

Table 6
Genotype and allele frequency of single nucleotide polymorphisms.

Gene (chromosome)	SNP	Genotype			Allele			HWE[a] (*p*-value)
		Type	Frequency	Proportion	Type	Frequency	Proportion	
BCL11A	rs766432	C/C	6	0.03	C	80	0.19	0.734
(2.p16.2)		C/A	68	0.33	A	332	0.80	
		A/A	132	0.64				
HBS1L-MYB (6.q23.3)	rs9399137	C/C	9	0.04	C	194	0.47	<0.001
		C/T	176	0.85	T	218	0.53	
		T/T	21	0.10				
HBG2 (11.p15.5)	rs7482144-*Xmn*I	A/A	22	0.11	A	172	0.42	<0.001
		A/G	128	0.62	G	240	0.58	
		G/G	56	0.27				

HWE, Hardy–Weinberg equilibrium; SNP, single-nucleotide polymorphism.
[a] *p*-value determined by χ^2 test. $p > 0.05$ means that the alleles are in equilibrium.

Table 7
Multiple linear regression analysis of relationships between genetic modifiers and severity scores.

Genetic modifier	Coefficient	Standard error	*p*-value	95 % CI
Constant	7.688	0.601	<0.001	(6.503, 8.874)
β^+-globin mutation	−3.654	0.450	<0.001	(−4.541, −2.767)
α^0-thalassemia mutation (--^{SEA})	−4.299	0.956	<0.001	(−6.185, −2.414)
α^+-thalassemia mutation (-$\alpha^{3.7}$)	−2.623	0.482	<0.001	(−3.575, −1.672)
Hb constant spring	−2.888	0.725	<0.001	(−4.319, −1.458)
Single-nucleotide polymorphism (SNP)				
• *BCL11A* (rs766432, C/A)	0.038	0.342	0.911	(−0.636, 0.712)
• *BCL11A* (rs766432, C/C)	−0.856	1.054	0.418	(−2.936, 1.224)
• *HBS1L-MYB* (rs9399137, C/T)	−1.258	0.544	0.022	(−2.332, −0.185)
• *HBS1L-MYB* (rs9399137, C/C)	−3.065	0.927	0.001	(−4.893, −1.237)
• *Xmn*I (rs7482144, A/G)	−1.174	0.376	0.002	(−1.916, −0.432)
• *Xmn*I (rs7482144, A/A)	−2.361	0.584	<0.001	(−3.513, −1.209)

$F = 19.463$, $p < 0.001$, $R^2 = 0.500$.
CI, confidence interval.

than the one that provided ANC, and unwittingly enrolled in the community group. A re-analysis of the proportion of genetic modifiers between the groups after the exclusion of these cases yielded the same but more statistically significant results. Therefore, the methodology in this study is suitable for identifying clinically relevant genetic modifiers, especially the strongest ones; the number of strong modulators was adequate for excellent statistical analysis. To the best of our knowledge, this is the first community-recruited population study of Hb E/β-thalassemia using the reproductive ability of subjects for enrollment; half of the participants were asymptomatic. The proportions of the four modifying factors (β- and α-thalassemia alleles) identified in this study were higher in the community group and among the mild severity subjects than the prevalence of thalassemia carriers in the same area [12]. Hence, these factors were truly significant modulating alleles.

The second aim of the present study was to demonstrate the measurable effects of the genetic modifiers for providing practical and informative genetic counseling. To this end, we applied clinical severity scores as the measuring template to the dependent variables in multiple linear regression analyses. The magnitudes of the influences were indicated by regression coefficient values. The implementation of the high-impact α-thalassemia alleles observed in our study in national guidelines for prenatal genetic counseling in Thailand and other Southeast Asian countries would be practical owing to their high prevalence and could have a great impact on parents of Hb E/β-thalassemia fetuses. Based on the four genetic modifiers, the predicted severity scores for Hb E/β-thalassemia fetuses could be calculated as follows:

$$\text{Predicted severity score} = 5.536 - \left(4.719 \times \left[- -^{\text{SEA}} \text{ factor}\right]\right) - \left(3.899 \times \left[\beta^+ \text{ factor}\right]\right) - \left(2.787 \times \left[-\alpha^{3.7} \text{ factor}\right]\right) - \left(2.410 \times \left[\alpha^{\text{CS}} \text{ factor}\right]\right) \quad (2)$$

It was previously shown that the β^E-globin mutation in the Thai population is in linkage disequilibrium with the A allele of the *Xmn*I polymorphism. Therefore, the frequency of the A allele in this SNP could be higher than it is in other ethnic groups [7,18,19]. In contrast, there are no explanations at this time for the observed high frequency of the minor allele (C) of rs9399137 in *HBS1L-MYB*.

For the limitations of the study, the genetic factors selected for this investigation were all known common determinants that could alleviate disease severity. However, other potentially vital factors, particularly those outside the three loci (*BCL11A*, *HBS1L-MYB*, and $^G\gamma$-*globin*) that play a major role in increasing Hb F levels, such as rs2071348 in *HBBP1* and *KLF1* variations or rs13398071 associated with *C2orf71* involved in the premature termination of β-globin mRNA, were not included [20–23]. Not all the strong genetic modifiers (β- and α-thalassemia alleles and SNP homozygote) investigated here could be detected in the five subjects (10.4 %) with the asymptomatic phenotype. For this reason, other strong genetic modifiers such as rs2071348 in *HBBP1*, *KLF1* mutations, and rs13398071 associated with *C2orf71* might be identified in future research. In contrast, other well-known genetic determinants, such as rs766432 in *BCL11A*, may not strongly demonstrate statistical significance.

5. Conclusion

Strong genetic modifiers can be easily identified in terms of statistical significance with our community-recruited population analysis model, which has sufficient essential phenotype-modulating factors of

interest, and measurable levels of clinical influence for severity score reduction were substantially demonstrated. Certain genetic modifiers must be investigated and determined routinely in prenatal genetic counseling for parents of Hb E/β-thalassemia fetuses.

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CRediT authorship contribution statement

Peerapon Wong: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Thirabhat Chitsobhak:** Investigation. **Suporn Jittasathian:** Investigation. **Chonnigarn Sirichantharawat:** Investigation. **Naritsara Cherdchoo:** Investigation. **Weerapong Prangcharoen:** Investigation. **Patcharanapa Jongautchariyakul:** Investigation. **Katechan Jampachaisri:** Formal analysis. **Akamon Tapprom:** Investigation. **Rawisut Deoisares:** Investigation. **Piyatida Chumnumsiriwath:** Investigation.

Declaration of competing interest

None.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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