Genetic predictions of life expectancy in southern Thai patients with β^0-thalassemia/Hb E

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Abstract. The types of β-thalassemia mutations, α-thalassemia interactions, and Hb F-associated SNPs have been described in association with variable disease phenotypes. This study aimed to determine the updated spectrum of β-thalassemia mutations and evaluate the contribution of primary and secondary genetic modifiers and SNPs to disease severity, age at onset, and predicted life expectancy in southern Thai β-thalassemia patients. A total of 181 β-thalassemia patients were enrolled and 135 β^0-thalassemia/Hb E patients without α-thalassemia interactions were divided into three categories according to disease severity, age at onset, and predicted life expectancy. A total of 16 β-thalassemia mutations were identified in this study, and the three most common β-thalassemia mutations accounted for 61.4% of all mutations. It was also found that the *Xmn*I polymorphism and rs2071348 were associated with age at onset and the predicted life expectancy. More than 82% of β^0-thalassemia/Hb E patients with CC genotype (*Xmn*I) were 3 years old or younger at onset. Additionally, >90% of the higher predicted life expectancy in β^0-thalassemia/Hb E patients had the T allele of *Xmn*I. Therefore, genetic prediction for age at onset and life expectancy is beneficial and practical during prenatal diagnosis or newborn screening for better genetic counseling and optimal management.

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Abbreviations: Hb E, hemoglobin E; Hb F, fetal hemoglobin; NBS, newborn screening; PND, prenatal diagnosis; SNPs, single nucleotide polymorphisms

Key words: β-thalassemia mutations, disease severity, predicted life expectancy, genetic modifiers, single nucleotide polymorphisms

Introduction

β-Thalassemia and hemoglobin E (Hb E), two globin gene defects characterized by β-globin gene mutations, lead to reduced (β^+), absent (β^0), or abnormal (β^E) β-globin chain synthesis. In Thailand, β-thalassemia and Hb E are very common, with frequencies varying from 3-9 and 10-60%, respectively (1,2). In Southeast Asia, particularly in Thailand, β-thalassemia/Hb E and homozygous β-thalassemia are common β-thalassemia diseases (3). Phenotypic variations in disease severity have been observed in β-thalassemia disease ranging from mild to severe clinical phenotypes (4-6). Clinical presentation of severe cases (β-thalassemia major-like phenotype) occurs between 6 and 24 months (7). Genetic factors affecting unbalanced globin chain synthesis in β-thalassemia disease are primary and secondary genetic modifiers of disease severity such as the type of β-thalassemia mutation (primary modifier) and coinheritance of α-thalassemia and polymorphisms associated with Hb F levels (secondary modifiers) (8-10).

The type of β-thalassemia mutation represents β^+ or β^0. In Thailand, in addition to Hb E, the three most common β-thalassemia mutations reported are as follows: Codons 41/42 (-TTCT), codon 17 (A>T) and IVS II-654 (C>T) (3,10). In the

southern Thai population, IVS I-5 (G>C), codon 19 (A>G), and Hb Malay were also common after codons 41/42 (-TTCT), which are the most common in all regions of Thailand (11-14). Additionally, genetic variations at three major loci (*HBB* cluster, *HBS1L-MYB*, and *BCL11A*) have been associated with fetal hemoglobin levels and disease phenotypes in β-thalassemia disease (15-17). In Thailand, several SNPs located in the *HBB* cluster, *HBS1L-MYB*, and *BCL11A* have been identified by two genome-wide association studies with different platforms (17,18). Several informative SNPs for predicting disease severity in Thai and Malaysian β^0-thalassemia/Hb E patients have recently been developed (19). In Thailand, the average life expectancy in β-thalassemia/Hb E patients is ~30 years (20,21). Several genetic and environmental factors as well as the treatment and management of each patient have been associated with life expectancy. Cardiovascular complications are a common cause of death in β-thalassemia major due to iron overload (21,22). According to the current management of patients with safe blood transfusion and iron chelation, the life expectancy in thalassemia major was comparable with that in thalassemia intermedia (23). Therefore, proper management could be considered at the age of presentation (age at onset) of each patient to extend the life expectancy of severe cases. This study aimed to determine the updated spectrum of β-thalassemia mutations to predict the contribution of genetic modifiers to disease severity, age at onset, and predicted life expectancy in southern Thai β-thalassemia patients.

Materials and methods

Ethical statement. Ethical clearance of the study protocol was obtained from the Institutional Review Board of Walailak University (Nakhon Si Thammarat, Thailand; approval no. 12/030). Written informed consent was obtained from all patients/guardians. All experiments were performed in accordance with relevant guidelines and regulations.

Study population. A cross-sectional study was conducted on β-thalassemia patients enrolled from thalassemia clinics, pediatric departments (child patients), and internal medicine departments (adult patients) from 6 different provinces between July 2012 and August 2014. All patients were diagnosed with β-thalassemia/Hb E (Hb types of EF or EFA) or homozygous β-thalassemia (Hb types of A_2F or A_2FA) based on the clinical manifestations, a complete blood count, and hemoglobin analysis. DNA analyses were then performed for confirmation of β-thalassemia/Hb E and homozygous β-thalassemia. Disease severity was classified using a scoring system according to 6 independent parameters as follows: the hemoglobin level at steady state, the age at first blood transfusion, a requirement for blood transfusion, the spleen size (or splenectomy status), the age at disease presentation and growth development (24).

Hematological analysis. A complete blood count was performed using the Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation). Hemoglobin analysis was performed using an automated high-performance liquid chromatography (HPLC-Variant II β-thalassemia short program, Bio-Rad Laboratories, Inc.).

DNA extraction and measurement of the concentration and purity of the extracted genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes using the Genomic DNA Extraction Kit (Geneaid) according to the manufacturer's instructions. The concentration and purity of gDNA were measured at wavelengths of 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Characterization of globin gene mutations. β-Thalassemia mutations were characterized using polymerase chain reaction (PCR)-based methods. Common β-globin gene mutations were first identified by PCR-reverse dot blot hybridization (PCR-RDB) (25), all probe sequences are listed in Table SI. or a multiplex amplification refractory mutation system (MARMS) (26) followed by multiplex gap-PCR (deletion type) (14). The Hb E allele was confirmed by real-time PCR-high resolution melting (HRM) analysis as described previously (27). Mutational characterization of DNA samples with negative results from PCR-RDB or MARMS-PCR and other PCR-based methods were further identified by automated DNA sequencing (Solgent Co., Ltd) of the whole *HBB* gene as described previously (28), the additional forward and reverse primer sequences were 5'-CGGCTGTCATCA CTTAGACC-3' and 5'-GCAGCTTGTCACAGTGCAG-3', respectively (product size, 598 bp). Common α-globin gene deletions, including α-thalassemia 1 alleles ($--^{SEA}$ and $--^{THAI}$) and α-thalassemia 2 alleles ($-\alpha^{3.7}$ and $-\alpha^{4.2}$), were characterized by multiplex gap-PCR whereas Hb constant spring and Hb Pakse alleles were identified by allele-specific PCR as described in previous studies (29,30). All primer sequences are shown in Table SII.

Single nucleotide polymorphism (SNP) genotyping. Four SNPs [rs7482144 (*Xmn*I), rs2071348 (*HBBP1*) rs766432 and rs9376074] from three representative regions (*HBB* cluster, *BCL11A* and *HBS1L*) were selected for genotyping. PCR-restriction fragment length polymorphism (RFLP) was used to characterize the genotypes of rs7482144, rs2071348 and rs766432 as described previously and the primer sequences for SNP genotyping were as follows: rs7482144 forward primer, 5'-GGCCTAAAACCACAGAGAGT-3' and reverse primer, 5'-CCAGAAGCGAGTGTGTGGAA-3'; rs2071348 forward primer, 5'-GGCACCTTTGCTACACTG AG-3' and reverse primer, 5'-TCATCATTCGGAGGGAAA CA-3', and rs766432 forward primer, 5'-AAAATCTCAGAA TACAAAGGGC-3' and reverse primer, 5'-GTTAGGGAA GGGGATTGAC-3' (27,31,32). Additionally, SNP genotyping of rs9376074 was performed using PCR-HRM and the primer sequences were: Forward, 5'-GAAGATGAAGCTAAGGTT TGG-3' and reverse, 5'-TCTGACTCCTCAAATGCC-3' (27).

Statistical analysis. Descriptive statistics were used to describe the spectrum of β-globin gene mutations, disease severity score/grouping, and hematological parameters of the patients. Clinical and hematological data from different severities of patients were compared using a χ^2 test for categorical variables and the Kruskal-Wallis test for continuous variables (non-normally distributed data) between the mild, moderate and severe groups using SPSS (version 26.0. IBM Corp.). Single

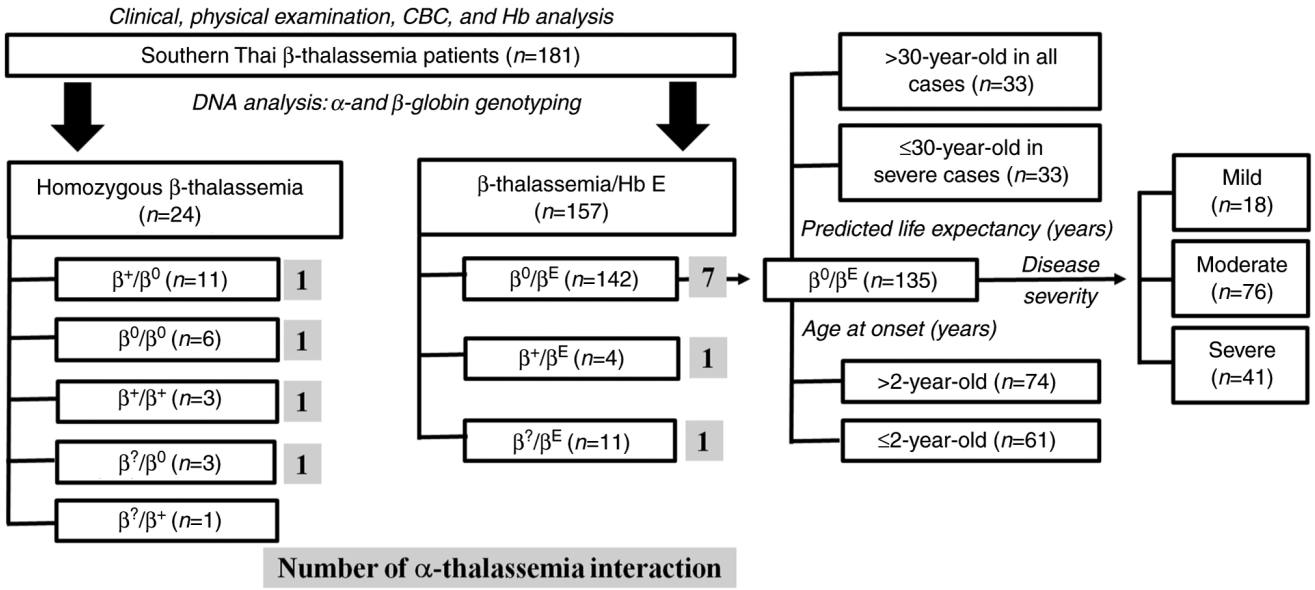

Figure 1. Schematic flow of patient enrollment and classification according to β-globin genotypes, α-thalassemia interactions, age at onset, disease severity and predicted life expectancy. $\beta^?$, uncharacterized β-globin gene mutation; CBC, complete blood count; Hb, hemoglobin.

SNP association analyses for disease severity, age at onset, and predicted life expectancy were performed in the recessive and allelic models using a Pearson's χ^2 test and/or Fisher's exact test. The P-value, odds ratios (OR), and 95% conference intervals (CIs) were calculated to compare genotype and allele frequencies using 2x2 contingency tables in publicly accessible statistical software (http://vassarstats.net/odds2x2.html). P<0.05 was considered to indicate a statistically significant difference. The clustered bar and the 100% stacked column were constructed using Infogram (https://infogram.com/) and Microsoft Excel, respectively.

Results

Patient classification according to α- and β-globin genotypes and disease phenotypes. A total of 181 β-thalassemia patients were enrolled and classified according to their β-globin genotypes, including 24 homozygous β-thalassemia and 157 β-thalassemia/Hb E patients. Clinical data, physical examination, complete blood count, and hemoglobin analysis were used for evaluating disease phenotypes. In addition, α-thalassemia interactions were found in 4 homozygous β-thalassemia and 9 β-thalassemia/Hb E patients. A total of 135 β^0-thalassemia/Hb E without α-thalassemia interactions were divided into 3 categories according to their predicted life expectancy, disease severity, and the age of onset. According to an average life expectancy of β^0-thalassemia/Hb E patients (30 years of age), the 135 β^0-thalassemia/Hb E patients were divided into two groups according to age: ≤30 (33 patients from severe cases who were predicted to have a lower life expectancy) and >30 (33 patients from all cases who were predicted to have a higher life expectancy). The second category was grouped according to disease severity, including 18 mild cases, 76 moderate cases, and 41 severe cases. The third category was grouped according to age at onset, including 61 cases with an age at onset ≤2 years old and 74 cases with an age at onset >2 years old (a threshold of 2 years of age was selected as this is the cutoff point between thalassemia major and thalassemia intermedia), as shown in Fig. 1.

Disease severity and primary and secondary genetic modifiers in southern Thai β-thalassemia patients. The 181 patients with β-thalassemia were classified as 34 mild cases, 95 moderate cases, and 52 severe cases and further subdivided into 6 groups according to the β-globin genotypes (Table I). Among the 181 patients with β-thalassemia, β^0-thalassemia/Hb E accounted for 78% and was grouped into 21 mild cases, 80 moderate cases, and 41 severe cases. All β-thalassemia patients with β^+/β^+ and β^+/β^E genotypes were grouped as the mild disease phenotype and demonstrated that the primary modifier, the type of β-globin mutation, can predict disease severity. Additionally, the effect of the secondary genetic modifier, α-thalassemia interaction, was demonstrated as β-thalassemia patients who carry α-thalassemia 2 ($-\alpha^{3.7}/\alpha\alpha$); 50% had mildly affected and 50% had moderately affected phenotypes. Moreover, one patient with β-thalassemia who carried Hb CS heterozygote had a mildly affected phenotype. In contrast, homozygous β^0-thalassemia patients were mostly scored as a severely affected phenotype. A homozygous β^0-thalassemia patient coinheritance with α-thalassemia 2 heterozygote had a mildly affected phenotype. Rarely did a patient with compound heterozygosity for IVSII-837, T>G (unclear β^+ or β^0), and Hb E have a moderate disease phenotype. Among 14 patients (8%), only one β-thalassemia mutation could be identified leaving 14 uncharacterized β-thalassemia alleles.

Clinical and hematological characteristics of southern Thai β^0-thalassemia/Hb E patients in different age groups. Several patient characteristics were significantly different between the 2 age groups (≤30 years old and >30 years old), such as age, age at presentation, age at first blood transfusion, frequency of blood transfusion, spleen status, and growth development (P<0.05). Approximately 97% of the ≤30-year-old group required regular blood transfusion. A greater number of splenectomized patients was highly observed in the ≤30-year

Table I. Primary (β-thalassemia mutations) and secondary modifiers (α-thalassemia mutations) of disease severity in the southern Thai β-thalassemia cohort.

β-globin gene genotype	Disease severity (score range)			Total, n
	Mild (0.0-3.5)	Moderate (4.0-7.0)	Severe (7.5-10.0)	
β^+/β^+ or β^+/β^E				
nt -28, A>G/nt -28, A>G	2	0	0	2
nt -28, A>G/Codon 19, A>G	1 [1][b]	0	0	1
nt -28, A>G/Codon 26, G>A (Hb E)	4 [1][a]	0	0	4
Total, n (%)	7 (100%)	0 (0%)	0 (0%)	7
β^0 (or β^+severe form)/β^+				
3.5-kb *HBB* deletion/Codon 19, A>G	0	1	0	1
Codons 41/42, -TTCT/Codon 19, A>G	1 [1][a]	2	2	5
Codon 17, A>T/Codon 19, A>G	0	0	2	2
IVS I-1, G>T/Codon 19, A>G	0	0	1	1
IVS II-654, C>T/Codon 19, A>G	0	1	1	2
Total, n (%)	1 (9%)	4 (36%)	6 (55%)	11
β^0 (or β^+severe form)/β^E				
Codons 8/9, +G/Hb E	0	2	0	2
Codon 17, A>T/Hb E	1	14	8	23
IVS I-1, G>T/Hb E	4	5	2	11
IVS I-5, G>C/Hb E	5 [1][a]	26 [1][a]	15	46
Codon 35, C>A/Hb E	0	1	1	2
Codon 41 (-C), TTC>TT-/Hb E	1 [1][a]	2	1	4
Codons 41/42, -TTCT/Hb E	5	22 [3][a]	12	39
Codon 43, G>T/Hb E	0	1	0	1
Codons 71/72, +A/Hb E	1 [1][a]	0	0	1
IVS II-654, C>T/Hb E	1	4	2	7
105-bp *HBB* deletion/Hb E	0	2	0	2
3.5-kb *HBB* deletion/Hb E	3	1	0	4
Total, n (%)	21 (15%)	80 (56%)	41 (29%)	142
β^0 (or β^+severe form)/β^0				
Codon 17, A>T/Codon 17, A>T	0	0	1	1
Codon 17, A>T/IVS I-1, G>T	0	0	1	1
Codon 17, A>T/Codons 41/42, -TTCT	0	0	1	1
Codons 41/42, -TTCT/Codons 41/42, -TTCT	0	1	1	2
IVS I-5, G>C/3.5-kb *HBB* deletion	1 [1][a]	0	0	1
Total, n (%)	1 (17%)	1 (17%)	4 (66%)	6
$\beta^{+\text{ or }0}/\beta^E$ (rare type, unclear β^+ or β^0)				
IVS II-837, T>G/Hb E	0	1	0	1
Total, n (%)	0 (0%)	1 (100%)	0 (0%)	1
β^{Unch}/β^E, β^{Unch}/β^0 and β^{Unch}/β^+				
Uncharacterized mutation/Hb E	3	7 [1][a]	0	10
Uncharacterized mutation/105 bp *HBB* deletion	0	1 [1][a]	0	1
Uncharacterized mutation/IVS I-5, G>C	0	1	0	1
Uncharacterized mutation/Codon 15, -T	0	0	1	1
Uncharacterized mutation/Codon 19, A>G	1	0	0	1
Total, n (%)	4 (29%)	9 (64%)	1 (7%)	14
β^+-Thalassemia (2 alleles), n (%)	7 (100%)	0 (0%)	0 (0%)	7
α-Thalassemia interaction/Hb CS, n (%)	7 (54%)	6 (46%)	0 (0%)	13
Total β-thalassemia patients, n (%)	34 (19%)	95 (52%)	52 (29%)	181

[a]Heterozygous α-thalassemia 2 ($-\alpha^{3.7}/\alpha\alpha$) was observed in 6 mild cases and 6 moderate cases. [b]Heterozygous Hb CS ($\alpha^{CS}\alpha/\alpha\alpha$) was characterized in 1 mild case. Numbers in square brackets [] represent the number of samples with heterozygous α-thalassemia 2 or heterozygous Hb CS. bp, base pair; Hb, hemoglobin; *HBB*, β-globin gene; Hb CS, Hb Constant Spring; IVS, intervening sequence; kb, kilobase; nt, nucleotide; Unch, uncharacterized.

Table II. Baseline, clinical, and hematological profiles of 66 southern Thai β^0-thalassemia/Hb E patients without α-thalassemia interactions categorized by age groups.

Patient characteristics	Age group		P-value
	≤30-year-old, severe cases, n=33	>30-year-old, all cases, n=33	
Sex, n (%)			0.210[d]
Male	11 (33)	16 (48)	
Female	22 (67)	17 (52)	
Age (years), mean ± SD	14.1±4.73	46.2±12.63	<0.0001[c,e]
Baseline Hb (g/dl), mean ± SD	6.7±0.99	6.9±1.32	0.705[e]
Age at presentation (years), mean ± SD	1.4±0.93	18.6±18.15	<0.0001[c,e]
Age at first transfusion (years), mean ± SD	1.8±1.48	22.8±19.94	<0.0001[c,e]
Requirement for regular blood transfusion, n (%)	32 (97)	19 (58)	0.0001[c,d]
Spleen size (cm), mean ± SD	7.2±4.80	7.1±5.52	0.966[e]
Splenectomy, n (%)	23 (70)	12 (36)	0.007[a,d]
Growth development: Height, n (%)			0.0003[b,d]
≤P3-10	23 (70)	8 (25)	
≥P10-25	10 (30)	24 (75)	
Growth development: Weight, n (%)			0.0013[a,d]
≤P3-10	22 (67)	9 (27)	
≥P10-25	11 (33)	24 (73)	

[a]P≤0.01, [b]P≤0.001, [c]P≤0.0001. [d]χ^2 test. [e]Mann-Whitney U test. P, percentile.

age group. According to the standard Thai growth chart, the >30-year-old group was found mostly (>73%) in the 10th-25th percentile, whereas in the ≤30 years age group it was mostly observed (>67%) in the 3rd-10th percentile (Table II). Obvious differences in all clinical and hematological findings between the 3 disease severity groups were observed (Table SIII).

An updated β-thalassemia mutational spectrum in southern Thai β-thalassemia patients. In the present study, 181 patients with β-thalassemia including, 24 with homozygous β-thalassemia and 157 with β-thalassemia/Hb E disease, were recruited. In total, 362 β-globin alleles from 181 β-thalassemia patients and 16 different mutations were identified, among which 3 common mutations accounted for 61.4% (Hb E was not included) as follows: Codons 41/42, -TTCT; IVS I-5, G>C and codon 17, A>T with frequencies of 23.9, 23.4, and 14.1%, respectively. All 3 of the most common mutations were categorized as β^0 (codons 41/42, -TTCT and codon 17, A>T) or the severe form of β^+ (IVS I-5, G>C). A total of 14 alleles of the β-globin gene, from 14 β-thalassemia patients were not successfully characterized in either allele of the β-globin gene, and these patients were grouped as having uncharacterized β-globin gene mutations (Fig. 2).

Associations between SNPs and disease severity and age at onset. The associations between the 4 SNPs and the disease severity of β^0-thalassemia/Hb E patients using mild and severe disease severity groups. The *Xmn*I polymorphism showed a strong association with the disease severity (P=0.004; OR, 3.20; 95% CI, 1.42-7.22) (Table SIV). To predict the age at onset of southern Thai β^0-thalassemia/Hb E patients according to the SNP genotypes from 3 independent regions, the CC genotype of *Xmn*I (rs7482144) was a strong predictor and showed a significantly increased risk for younger age at onset (P=0.004; OR, 3.13; 95% CI, 1.40-7.00). In contrast, there was no association in *BCL11A* (rs766432) and *HBS1L-MYB* (rs9376074)

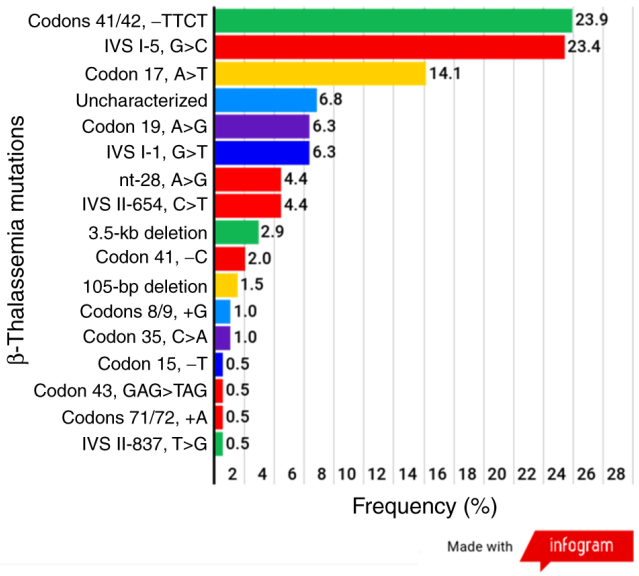

Figure 2. Clustered bar of frequency of β-globin gene mutations among 181 southern Thai β-thalassemia patients.

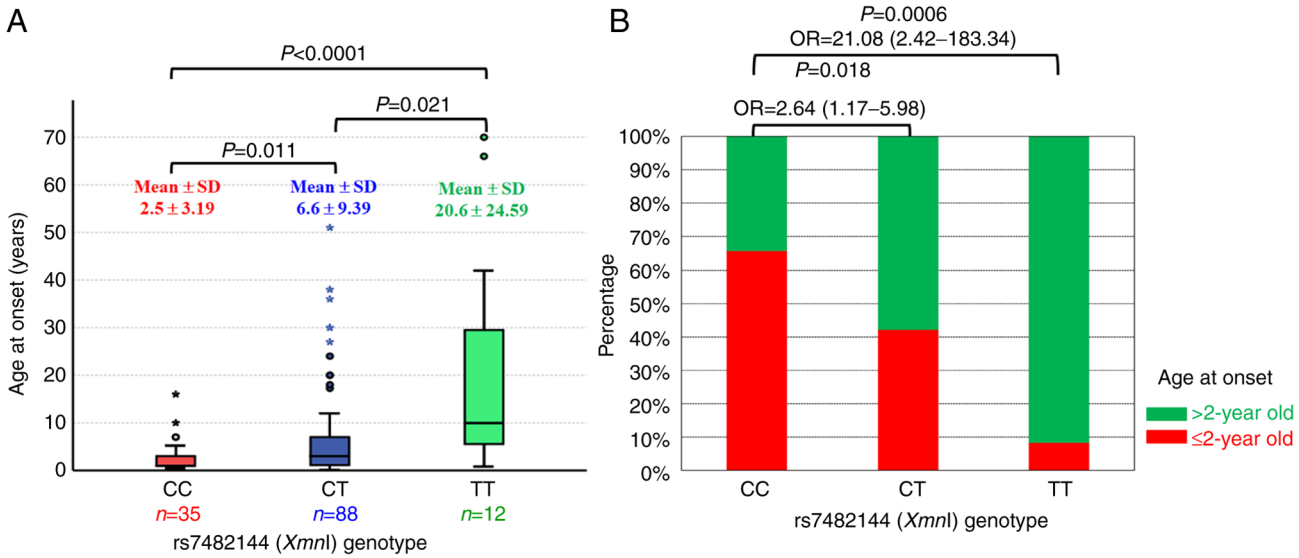

Figure 3. Genotype-phenotype correlation. (A) Box plot displaying the age at onset with the three different genotypes of rs7482144 (*Xmn*I). (B) Stacked bar chart of the percentage of patients by age at onset (cutoff at 2 years of age) according to the 3 different genotypes. OR, odds ratio; SD, standard deviation.

regions (Table SV). Among the 3 genotypes of *Xmn*I, the mean and standard deviation of age at onset (2.5±3.19, 6.6±9.39, and 20.6±24.59 years) were increased according to the number of T alleles (CC, CT, and TT, respectively). In addition, the comparisons of the mean age at onset from each genotype were significant (P<0.05) (Fig. 3A). To apply the *Xmn*I genotypes for predicting the age at onset, the TT genotype was observed in >90% of individuals in the >2 years of age group, and the CC genotype was observed in >60% of the individuals in the ≤2 years of age group. The frequency of the TT genotype in the >2 years of age group was higher than that in the ≤2 years of age group (P=0.0006; OR, 21.08; 95% CI, 2.42-183.34) (Fig. 3B).

Associations between SNPs and the predicted life expectancy. The associations between the 4 candidate SNPs from 3 independent regions and the predicted life expectancy of β⁰-thalassemia/Hb E patients were next assessed. The *Xmn*I (rs7482144) polymorphism showed a strong association with the predicted life expectancy (P=0.004; OR, 6.50; 95% CI, 1.64-25.80). The CT or TT genotype of *Xmn*I was associated with a higher predicted lifespan than those with the CC genotype. In addition, rs2071348 also exhibited an association with the predicted life expectancy (P=0.016). In contrast, rs766432 and rs9376074 demonstrated no association with the predicted life expectancy (P=0.458 and 0.438, respectively) (Table III).

Cascade genetic testing of β⁰-thalassemia/Hb E patients for phenotype predictions. According to the overall results, the age at onset, predicted life expectancy, and disease severity were assigned as phenotypic variations in β-thalassemia patients. Phenotypic variations were then classified into 2 groups: Low or high predicted life expectancy. The genotyping of β-thalassemia mutations, α-thalassemia interactions, and *Xmn*I genotypes were sequentially recommended for phenotype prediction; for example, the CT or TT genotype of *Xmn*I was observed in 90.9% of β⁰-thalassemia/Hb E patients with high predicted life expectancy (Fig. 4).

Discussion

β-thalassemia and Hb E are very common in Thailand, in which the frequency of the β-thalassemia trait varies from 3 to 9%, and the frequency of Hb E is 13% on average and varies from region to region. The frequency of Hb E is very high at the junction of Thailand, Laos, and Cambodia at 50-60% (33). In Thailand, the number of patients with compound heterozygotes for β-thalassemia and Hb E is higher than that for homozygous β-thalassemia because the frequency of Hb E is much higher than that for β-thalassemia (3,20,34). β-thalassemia/Hb E disease showed diverse disease phenotypes ranging from mild to severely affected patients (6,21). The variation in disease severity in β-thalassemia patients could be explained by β-thalassemia mutations (10), α-thalassemia interactions (35,36), and genetic determinants of Hb F production (15,17,18,37,38), and other factors related to the pathophysiology of β-thalassemia (39-41). Several genetic modifiers associated with disease severity and fetal hemoglobin levels in β-thalassemia/Hb E patients have been well studied in the Thai population (10,17,18). Factors affecting life expectancy in β-thalassemia patients were a subset of disease severity-associated genetic factors and proper treatments such as safe blood transfusion, iron chelation, and other supportive therapies can decrease disease complications. However, there is no report of SNP frequency data and some rare β-thalassemia mutations in southern Thai β⁰-thalassemia/Hb E patients. According to different genetic backgrounds and migration, the mutational spectrum of β-thalassemia and SNP frequency in southern Thai differed in other parts of Thailand. This phenomenon has also been observed in various countries such as India (42-44), Malaysia, China (45,46), and other countries (47). Therefore, the predictive performance of the β-thalassemia mutations and SNPs would differ in each region in the same country.

According to the primary modifier, the present study demonstrated that all β⁺/β⁺ and β⁺/β^{E} patients were scored as mildly affected due to the primary modifier (10,17). The

Table III. Association of the 4 SNPs in 3 independent regions with the predicted life expectancy in southern Thai β^0-thalassemia/Hb E patients without α-thalassemia interactions.

SNP info	Genotype/ allele	Age Range Status: ≤30-year-old, severe cases, n=33	Age Range Status: >30-year-old, all cases, n=33	P-value[b]	Odds ratio (95% Confidence interval)	Risk Genotype/ Allele[a]
rs7482144 (C/T), *HBG2*						
Genotype	CC	13 (0.394)	3 (0.091)	0.004	6.50 (1.64-25.80)	CC
	CT+TT	20 (0.606)	30 (0.909)			
Allele	C	46 (0.697)	29 (0.440)	0.003	2.93 (1.43-6.00)	C
	T	20 (0.303)	37 (0.560)			
rs2071348 (A/C), *HBBP1*						
Genotype	AA	11 (0.333)	3 (0.091)	0.016	5.00 (1.24-20.08)	AA
	AC+CC	22 (0.667)	30 (0.909)			
Allele	A	43 (0.652)	28 (0.424)	0.009	2.54 (1.26-5.13)	A
	C	23 (0.348)	38 (0.576)			
rs766432 (C/A), *BCL11A*						
Genotype	AA	20 (0.606)	17 (0.515)	0.458	1.45 (0.54-3.84)	AA
	AC+CC	13 (0.394)	16 (0.485)			
Allele	A	53 (0.803)	49 (0.742)	0.406	1.41 (0.62-3.21)	A
	C	13 (0.197)	17 (0.258)			
rs9376074 (T/C), *HBS1L*						
Genotype	TT	13 (0.394)	10 (0.303)	0.438	1.50 (0.54-4.14)	TT
	TC+CC	20 (0.606)	23 (0.697)			
Allele	T	42 (0.636)	37 (0.561)	0.374	1.37 (0.68-2.76)	T
	C	24 (0.364)	29 (0.439)			

[a]Risk genotypes/alleles were set as reference genotypes/alleles and the recessive model was used to analyze the case-control association study. [b]At age ≤30 years (case group) vs. at age >30 years (control group) were analyzed. SNP; single nucleotide polymorphism.

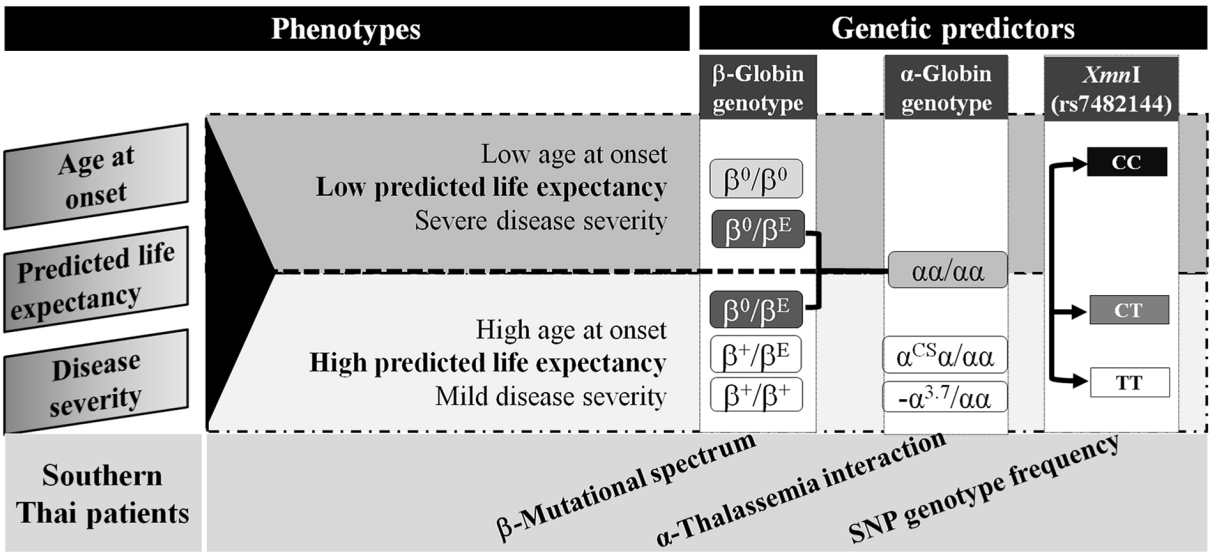

Figure 4. Genetic prediction pipeline for phenotype of β-thalassemia. β^+, reduced β-globin chain synthesis; β^0, absent β-globin chain synthesis; $\alpha^{CS}\alpha$, Hb Constant Spring allele; SNP, single nucleotide polymorphism; $-\alpha^{3.7}$, 3.7 kb rightward deletion.

frequency of disease severity among β^0/β^E southern Thai patients with mild, moderate, and severe disease phenotypes was distributed in a different pattern than in previous studies because of the different genetic backgrounds (17,24) of the

studied patients. This study revealed that the β-thalassemia mutations are very heterogeneous, with a wider distribution in southern Thailand than in other parts of Thailand. The 3 most common β-thalassemia mutations in this study were 61.4%, which differed from the central (72.4%), northern (83.1%), and northeastern (80.3%) regions of Thailand (12). A total of 17 β-thalassemia mutations, including Hb E, were identified in 181 southern Thai β-thalassemia patients in the present study. Comparing these results to previously published reports in the southern Thai population, the 4 most common mutations, codons 41/42 (-TTCT), IVS I-5 (G>C), codon 17 (A>T), and codon 19 (A>G) accounted for 67.7% of mutations in the present study and revealed slightly different patterns and frequencies due to the differences in the collected sample backgrounds, such as ethnicity (11), thalassemia status (trait or disease) (48), different provinces (12-14) of southern Thailand and migration (47) (Fig. S1). Although this study recruited patients from several provinces of southern Thailand, a similar pattern of the most common β-thalassemia mutations was observed. The origin of patients may explain the difference in distribution; for example, codons 41/42 (-TTCT) are very common in individuals of Chinese origin (45,46), whereas IVS I-5 (G>C) is very common in the Malay (49) and Asian Indian (50) populations. Interestingly, the present study demonstrated comparable frequencies of codons 41/42; -TTCT (23.9%) and IVS I-5; G>C (23.4%) because of the higher sample size of Thai-Muslim patients. The spectrum and frequency of β-thalassemia mutations in the southern Thai population were different from those in other regions of Thailand (3,51). Hb Malay was found at the highest frequency (11.7%) in the southern region of Thailand compared with other parts of Thailand (12,13). The frequency of Hb Malay in our study was 6.3%, ranking as the fourth most common β-thalassemia mutation in southern Thailand. Heterozygous β-thalassemia (IVS II-837; T>G) was first described in Asian Indians with unclear β^+ or β^0 thalassemia showing a typical asymptomatic carrier, and the incidence of this mutation was found in the Gaud Saraswat (44), Brahmins in Goa, and Karnataka (52) states of southern India. Phenotypes of the homozygous state of IVS II-837 (T>G) were transfusion-dependent (52). Interestingly, compound heterozygotes of IVS II-837 (T>G) and Hb E were found for the first time in the present study and showed a moderately affected phenotype with regular blood transfusion. According to the disease phenotype from this study and previous reports, IVS II-837 (T>G) could be categorized as β^+-thalassemia (severe form) (44,52).

Coinheritance of α-thalassemia in β-thalassemia patients is one of the ameliorating factors due to more balanced globin chain synthesis (35,36). Heterozygous α-thalassemia 2 and Hb CS were found only in 7 mild and 6 moderate cases in the present study. The α-thalassemia 1 allele was not detected in our β-thalassemia patients. A possible reason is that the coinheritance of α-thalassemia 1 leads to mild β-thalassemia; thus, these patients were not found in a hospital-based sample collection (35). Furthermore, several genetic markers in the *HBB* cluster (10,15,17,51), *BCL11A* (15,17,19), and *HBS1L-MYB* (15,17) have been associated with fetal hemoglobin and disease severity in several populations. In addition, mutations in human Krüppel-like factor 1 (*KLF1*) were found to be associated with increased fetal hemoglobin (Hb F) and hemoglobin A_2 (Hb A_2) (53,54). *KLF1* mutations have been studied in patients with β-thalassemia/Hb E, and a higher Hb F level was observed in the cases with *KLF1* mutations (38,51).

According to hospital-based sample collection, the present study failed to enroll sufficient mild cases (n=18) for SNP analysis in disease severity because the mild case has a lower frequency of going to the hospital. However, *Xmn*I and rs2071348 were associated with disease severity in β^0-thalassemia/Hb E (with low power). The T allele frequency of *Xmn*I in mild cases (0.611) was significantly higher than that in severe cases (0.329). No associations were found in rs766432 and rs9376074 because of the low sample size in mild cases (Table SIV). An increased sample size could improve the statistical power in all SNPs due to the similar trend of the allele frequencies (17).

Currently, the life expectancy between thalassemia major and thalassemia intermedia is comparable due to the use of safe blood transfusions, effective iron chelation, and improved management of cardiovascular complications (23,55). Proper patient management should be initiated during the age at onset for improved quality of life and increased life expectancy. Therefore, the prediction of the age at onset is important not only for patient management in newborns but also for genetic counseling in prenatal diagnosis (PND). Our study showed the association between SNPs and the age at onset and the predicted life expectancy of southern Thai β^0-thalassemia/Hb E patients. Interestingly, the *Xmn*I polymorphism and rs2071348 were associated with the age at onset and the predicted life expectancy. The *Xmn*I polymorphism is the strongest marker for predicting the age at onset and the predicted life expectancy in southern Thai patients with β^0-thalassemia/Hb E. This polymorphism is well identified in association with fetal hemoglobin levels and disease phenotypes in different groups of populations (10,15,17-19,51). Due to the improved and individualized management of the patients, an improved life expectancy and quality of life were observed in β-thalassemia patients. In addition, the life expectancy of thalassemia major patients was similar to that of thalassemia intermedia patients (23,55,56). Therefore, the genetic prediction of age at onset and life expectancy is suggested for better patient management after newborn screening. Concerning precision medicine, the β-thalassemia mutations and *Xmn*I (rs7482144) polymorphism could be simultaneously genotyped to improve genetic counseling in PND. However, this suggested guideline should be validated on a national scale and with considerably larger sample sizes in the future.

In summary, genetic heterogeneity and a broad spectrum of β-globin gene mutations were observed in southern Thai β-thalassemia patients. This study provides an updated spectrum of β-thalassemia mutations. Hb Malay, IVS I-5 (G>C), 105-bp deletion, and 3.5-kb deletion were primarily found in the southern Thai population, accounting for 34.1% of all mutations. The type of β-globin gene mutation and co-inheritance of α-thalassemia are strong predictors of disease severity. The *Xmn*I polymorphism and rs2071348 were associated with the age at onset and predicted life expectancy. However, SNPs on *BCL11A* and intergenic *HBS1L-MYB* are required to confirm the genetic association in a larger sample size. This study demonstrates that the *Xmn*I polymorphism is the best genetic predictor for age at onset and life expectancy. Therefore,

genetic prediction for age at onset and life expectancy may be beneficial and practical during PND or newborn screening for better genetic counseling and optimal management.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MN designed and performed the experiments, analyzed the data, and wrote the manuscript. PR performed PCR-HRM. TB, AC, KS, NS, KL, and OT provided clinical data and performed the physical examination and helped in obtaining blood specimens. SS and SF provided DNA controls and helped to design the experiments. All authors have read and approved the final manuscript. MN, TB, AC, KS, NS, KL, and OT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was conducted according to the Declaration of Helsinki guidelines and approved by the Human Research Ethics Committee of Walailak University (Nakhon Si Thammarat, Thailand; approval no. 12/030).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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