

## Original article

## Correlation of hematologic parameters and molecular characterization of thalassemia: Phramongkutklo Hospital Experiences

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**Abstract:**

**Background:** Thalassemia is one of the most common hereditary hemolytic anemias worldwide. The prevalence of thalassemia traits in Thai population is 20-30% for  $\alpha$ -thalassemia, 3-9% for  $\beta$ -thalassemia and 20-30% for Hb E.

**Objective:** This study aimed to investigate the prevalence of thalassemia as well as the correlation between phenotypic characteristics and associated parameters, and alpha- and beta-globin genotypes at Phramongkutklo Hospital, a tertiary care center for thalassemic patients in Thailand.

**Materials and Methods:** Four-hundred blood specimens were collected and complete blood counts and hemoglobin typing were performed. All cases were tested for common  $\alpha$ -globin mutation, whereas  $\beta$ -globin analysis was tested only in cases of abnormal hemoglobin typing. **Results:** Among 400 blood specimens, the most common Hb type was  $A_2A$  pattern accounting for 229 specimens (57.3%) with  $\alpha$ -thal 1 identified in 27 specimens (6.8%) and  $\alpha$ -thal 2 identified in 37 specimens (9.3%). We found 12 specimens (3.0%) demonstrating  $A_2A$  pattern with elevated Hb  $A_2$  more than 3.5% on Hb typing. Beta thalassemia mutation analysis revealed mutations of codon 41/42 (-TCTT) (1.5%), codon 17 (A>T) (0.8%), nt-28 (A>G) (0.5%) and IVSI-I (G>T) with heterozygous alpha-thal 1 deletion with SEA type (0.3%). Our study found Hb E trait in 127 specimens (31.8%) and homozygous Hb E was detected in 20 specimens (5%). Abnormal hemoglobin was observed in 0.5%. **Conclusion:** Our study demonstrated the prevalence of thalassemia trait and thalassemia disease up to 62%. Molecular diagnosis is useful to confirm diagnosis and evaluate genotype-phenotype correlation.

**Keywords :** ● Genotype ● Hematologic parameters ● Hemoglobin E ● Alpha thalassemia  
● Beta thalassemia

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## นิพนธ์ต้นฉบับ

# การศึกษาความสัมพันธ์ระหว่างพารามิเตอร์ทางโลหิตวิทยาและลักษณะทางอณูพันธุศาสตร์ในผู้ป่วยโรคโลหิตจางธาลัสซีเมีย: ประสบการณ์จากโรงพยาบาลพระมงกุฎเกล้า

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### บทคัดย่อ

**ความเป็นมา** โรคโลหิตจางธาลัสซีเมียเป็นโรคโลหิตจางที่มีการถ่ายทอดทางพันธุกรรมที่พบได้บ่อยที่สุดในประชากรชาวไทยพบความชุกของพาหะธาลัสซีเมียชนิดแอลฟาไร้อยู่ระหว่าง 20-30 พาหะธาลัสซีเมียชนิดเบต้าไร้อยู่ระหว่าง 3-9 อย่างไรก็ตามพบความชุกของพาหะธาลัสซีเมียชนิดฮีโมโกลบินอี มากถึงร้อยละ 20-30 **วัตถุประสงค์** เป็นการศึกษาถึงความชุกของโรคโลหิตจางธาลัสซีเมียและความสัมพันธ์ระหว่างพารามิเตอร์ทางโลหิตวิทยา รวมถึงการตรวจแยกชนิดของฮีโมโกลบินโดยวิธี capillary electrophoresis (CE) และลักษณะทางอณูพันธุศาสตร์ทั้งยีนแอลฟาและเบต้าโกลบิน ในโรงพยาบาลพระมงกุฎเกล้า ซึ่งเป็นโรงพยาบาลระดับตติยภูมิสำหรับรักษาผู้ป่วยโรคโลหิตจางธาลัสซีเมีย **วิธีการ** ตัวอย่างเลือดของผู้ป่วยจำนวน 400 ราย นำมาตรวจนับเม็ดเลือดโดยสมบูรณและลักษณะของเม็ดเลือดตรวจแยกชนิดของฮีโมโกลบินและตรวจการกลายพันธุ์ของแอลฟาโกลบินยีนทุกสาย ส่วนการตรวจการกลายพันธุ์ของเบต้าโกลบินยีนจะตรวจในรายที่มีผลตรวจแยกชนิดของฮีโมโกลบินที่ผิดปกติ นำผลการตรวจที่ได้ทั้งหมดมาศึกษาวิเคราะห์ **ผลการศึกษา** ตัวอย่างเลือดของผู้ป่วยจำนวน 400 ราย พบฮีโมโกลบินในรูปแบบ  $A_2A$  มากถึง 229 ราย (ร้อยละ 57.3) การขาดหายไปของแอลฟาโกลบินชนิดที่ 1 พบในผู้ป่วย 27 ราย (ร้อยละ 6.8) และพบการขาดหายไปของแอลฟาโกลบินชนิดที่ 2 พบในผู้ป่วย 37 ราย (ร้อยละ 9.3) ผู้ป่วยจำนวน 12 ราย (ร้อยละ 3) ที่ตรวจแยกชนิดของฮีโมโกลบิน ฮีโมโกลบินเป็นรูปแบบ  $A_2A$  แต่มี ปริมาณฮีโมโกลบินชนิด  $A_2$  มากกว่าร้อยละ 3.5 ได้ศึกษาถึงการกลายพันธุ์ของเบต้าโกลบินยีน พบการกลายพันธุ์ที่ตำแหน่ง codon 41/42 (ร้อยละ 1.5) codon 17 (ร้อยละ 0.8) nt-28 (ร้อยละ 0.5) และ IVSI-I ร่วมกับ heterozygous alpha-thal 1 deletion ชนิด SEA type (ร้อยละ 0.3) ในการศึกษาครั้งนี้ยังพบพาหะธาลัสซีเมียชนิดฮีโมโกลบินอี 127 ราย (ร้อยละ 31.8) และผู้ป่วยชนิด homozygous Hb E อีกจำนวน 20 ราย (ร้อยละ 5) การตรวจแยกชนิดของฮีโมโกลบินยังพบรูปแบบที่ผิดปกติ พบได้ไม่บ่อยอีก ร้อยละ 0.5 **สรุป** การศึกษาที่พบความชุกของพาหะและผู้ป่วยโรคโลหิตจางธาลัสซีเมียมากถึงร้อยละ 62 การศึกษาถึงลักษณะทางอณูพันธุศาสตร์จะช่วยให้สามารถยืนยันการวินิจฉัยที่ถูกต้องและสามารถพยากรณ์ความรุนแรงของโรคในอนาคตได้

**คำสำคัญ :** ● ลักษณะทางอณูพันธุศาสตร์ ● พารามิเตอร์ทางโลหิตวิทยา ● ฮีโมโกลบินอี ● แอลฟาธาลัสซีเมีย ● เบต้าธาลัสซีเมีย

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## Introduction

Thalassemia is a common inherited hemolytic anemia categorized as alpha-thalassemia and beta-thalassemia. Pathophysiology of the disease is characterized by reduced or the absence of globin chain synthesis attributed to imbalance of the globin chains and subsequently resulting in chronic hemolytic anemia and decreased hemoglobin level. The causative mutations of thalassemia are often specific to each population<sup>1,2</sup>.

In Thailand, the prevalence of thalassemia traits ranges from 20 to 30% for  $\alpha$ -thalassemia and 3 to 9% for  $\beta$ -thalassemia in which the prevalence increases to 20 to 30% in hemoglobin E (Hb E)<sup>3</sup>. The prevalence of beta-thalassemia and Hb E carriers were reported as 3-9% and 13-50%, respectively<sup>3</sup>. Beta-thalassemia is caused by mutations in the beta-globin gene (*HBB*) on chromosome 11 causing reduced beta-globin chain synthesis. One common *HBB* mutation in Southeast Asia is a single point mutation of codon 26 found in Hb E which can manifest as hemoglobinopathy or thalassemic phenotype associated with various clinical findings. The common identified types of *HBB* mutations are point mutations, small deletions or insertions within the coding regions and the exon-intron junctions. At present, more than 30 different  $\beta$ -globin genes mutations have been identified in Thailand<sup>4</sup>.

Alpha-thalassemia is one of the major thalassemia types and is caused by mutations in either the  $\alpha_1$ -globin gene (*HBA1*) or  $\alpha_2$ -globin gene (*HBA2*) on chromosome 16. At present, more than 20 different  $\alpha$ -globin genes mutations associated with  $\alpha$ -thalassemia have been identified in Thailand<sup>5-13</sup>. Deletion of either one ( $\alpha$ -thalassemia 2) or both ( $\alpha$ -thalassemia 1)  $\alpha$ -globin genes is among the most common mutations; whereas, nondeletional forms of  $\alpha$ -thalassemia 2 are occasionally found. Hb Constant Spring (Hb CS) is a variant of hemoglobin arising from a termination codon mutation of the *HBA2* and is considered the most common nondeletional mutations with prevalence ranging from 1 to 8%<sup>14</sup>.

The clinical and hematological manifestations of  $\alpha$ -thalassemia are heterogeneous, ranging from a silent carrier with normal clinical findings to catastrophic diseases with transfusion-dependent phenotype such as Hb Bart's hydrops fetalis syndrome. Hemoglobin H (Hb H) disease is another common type of alpha-thalassemia caused by interaction of  $\alpha$ -thalassemia 1 and  $\alpha$ -thalassemia 2 attributed to impaired production of three of the four alpha globins. The disease is classified in two main forms including deletional and nondeletional Hb H diseases. Deletional Hb H disease is characterized by a combined deletion of both *HBA1* and *HBA2* on one arm and deletion of either *HBA1* or *HBA2* on the other arm of chromosome 16. Nondeletional Hb H disease is described as a compound heterozygous of the deletion of both *HBA1* and *HBA2* on one arm and a point mutation or small insertion/deletion involving either the *HBA1* or *HBA2* gene on the other arm of chromosome 16. The correlation of clinical phenotypes, hematological characteristics, laboratory parameters and  $\alpha$ -globin genotypes in different populations had been studied<sup>15-17</sup>.

Herein, we aimed to study the prevalence of thalassemia and investigate the correlation between phenotypic characteristics and associated parameters including hematologic parameters, hemoglobin types analyzed by capillary electrophoresis (CE) and alpha- and beta-globin genotypes at Phramongkutklao Hospital, a tertiary care and referral center for thalassemia patients in Thailand.

## Materials and Methods

### Subject selection

Four hundred subjects who had available leftover blood specimens from investigations of clinical or laboratory suspicion for thalassemia disease or thalassemia traits or failed screening for thalassemia in prenatal diagnosis at the Department of Pathology, were enrolled in this study. The study protocol was approved by the Institutional Review Board of Phramongkutklao Hospital and Phramongkutklao College of Medicine, Bangkok, Thailand following the ethical principles of the Declaration

of Helsinki of 1975 and its revision. Blood specimens leftover from hemoglobin typing and taken from subjects older than 1 year of age with a minimal volume of 500 microliters between January 2017 and December 2017 were included in this study. The specimens taken from subjects with a history of recent red cell transfusion within 120 days were excluded.

#### **Hematological parameters and hemoglobin typing**

Complete blood counts were performed using a Coulter HMX Automated Hematology Analyzer (Beckman Coulter Corporation, Miami, FL, USA). This machine is a quantitative, automated hematology analyzer and leukocyte differential counter for in vitro diagnostics used in clinical laboratories. Hemoglobin profiles including Hb E, Hb A, Hb A<sub>2</sub> and Hb F concentrations were determined using Capillary Electrophoresis (CE, Minicap System, Sebia, Norcross, France). The MINICAP HEMOGLOBIN(E) assay is generally based on the principle of capillary electrophoresis in free solution. Hemoglobin fractions were separated in silica capillaries, by their electrophoretic mobility and electroosmotic flow at a high voltage in an alkaline buffer. Hemoglobin fractions were directly detected at the specific absorbance of 415 nm and the pattern was divided in 15 zones. Each zone displayed a drop-down library of possible variants migrating within this zone.

#### **Mutation analysis**

##### **Alpha-globin genotypes**

A total of 400 EDTA peripheral blood samples were collected. Genomic DNA was extracted from peripheral blood lymphocytes using commercially available kits (magLEAD<sup>®</sup> 12gC, Precision System Science Co., Ltd.) according to manufacturer protocol. The  $\alpha$ -globin gene mutations were first characterized using multiplex gap polymerase chain reaction (gap-PCR) to detect common deletions in Chinese and Southeast Asian populations consisting of  $\alpha$ -thalassemia 1 [SEA (-<sup>SEA</sup>) and THAI (-<sup>THAI</sup>) deletion] and  $\alpha$ -thalassemia 2 [3.7-kb (- $\alpha$ <sup>3.7</sup>) and 4.2-kb (- $\alpha$ <sup>4.2</sup>) deletion] as previously described<sup>18</sup>.

Second, allele specific PCR or multiplex amplification refractory mutation system (M-ARMS) was performed to detect Hb CS and Hb Pakse (Hb PS) as described previously<sup>10,11,19,20</sup>.

##### **Beta-globin genotypes**

Twelve EDTA peripheral blood samples were investigated from subjects previously reported from hemoglobin typing with suspicion of beta-thalassemia trait (Hb A<sub>2</sub> > 3.5%), and an additional 2 EDTA peripheral blood samples presenting EF pattern as well as 2 EDTA peripheral blood samples from subjects with suspicion of abnormal band. First, the *HBB* mutations were analyzed using the multiplex amplification refractory mutation system (M-ARMS) to detect seven common *HBB* mutations in Thailand including codon 41/42 (-TCTT), codon 17 (A>T), codon 71/72 (+A), nt-28 (A>G), IVS-II-654 (C>T), IVS-I-1 (G>T) and nt-90 (C>T) as previously described<sup>21</sup>. Second, unknown *HBB* gene mutations or abnormal hemoglobin were further characterized by direct DNA sequencing of all three exons and exon-intron junctions to detect uncommon mutations according to protocols previously described elsewhere.

##### **Statistical analysis**

Baseline values of selected variables were calculated as mean, median, and standard deviation. Continuous variables were compared between two groups using the unpaired t-test for data with a parametric distribution. Statistical analysis was performed using IBM Statistical Package for the Social Science (SPSS) Software, Version 23 (IBM, NY, USA) and p-value < 0.05 was considered to be statistically significant.

## **Results**

### **Population characteristics**

Population characteristics including age, sex, Hb and red blood cell indices were analyzed from 400 leftover blood specimens as shown in Table 1. The population's age ranged between 1 and 90 years old with median age of 31 years old. The majority of the population were adults aged older than 18 years old (372 specimens,

**Table 1** Population characteristics (n = 400)

	Specimen (%)
Age	
< 18 years old	28 (7)
≥ 18 years old	372 (93)
Sex	
Male	188 (47)
Female	212 (53)
MCV (fL)	
< 80 fL	221 (55.2)
≥ 80 fL	179 (44.8)
Hemoglobin typing	
A <sub>2</sub> A	229 (57.3)
A <sub>2</sub> ABart'sH	4 (1.0)
CS A <sub>2</sub> A	5 (1.3)
CS A <sub>2</sub> ABart'sH	6 (1.5)
CSEA	5 (1.3)
EA	127 (31.8)
EE	14 (3.5)
EE/EF	6 (1.5)
EF	1 (0.3)
EFA	1 (0.3)
Rare abnormal hemoglobin	2 (0.5)

Notes: Data are presented as number (%) for categorical variables

Abbreviation: MCV, mean corpuscular volume

93%). Females were more predominant than males at a ratio of 1.12:1. Microcytic red blood cells were more commonly found (221 specimens, 55.2%) compared with normocytic red blood cells (179 specimens, 44.8%).

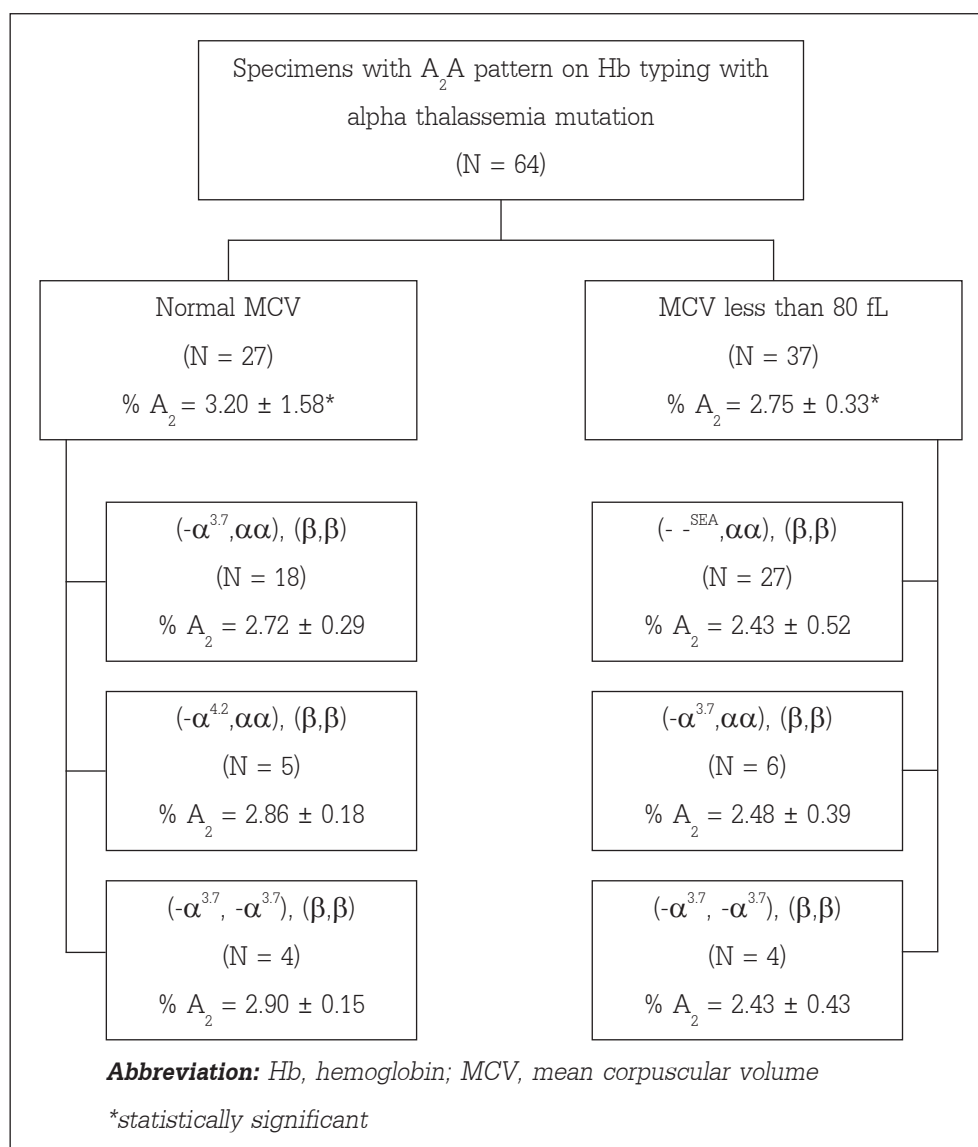
#### Alpha thalassemia mutations and hematological parameters

Among 400 leftover blood specimens, the most common Hb type was A<sub>2</sub>A pattern (A<sub>2</sub> < 3.5%) accounting for 229 blood specimens (57.3%). Of those 229 specimens, males were more predominant than females with a ratio of 1.2:1, and the majority comprised adults aged older than 18 years (216 specimens, 94.3%) and normocytic red blood cells (134 specimens, 58.5%).

Normocytic blood specimens with A<sub>2</sub>A pattern were analyzed for alpha thalassemia mutations. Alpha thalas-

semia traits were identified in 27 blood specimens including heterozygous alpha-thal 2 with -3.7 kb deletion (18 specimens), heterozygous alpha-thal 2 deletion with -4.2 kb deletion (5 specimens) and homozygous alpha-thal 2 deletion with -3.7 kb deletion (4 specimens). Notably, none of those normocytic specimens were positive for heterozygous alpha-thal 1 deletion. Microcytic blood specimens with A<sub>2</sub>A pattern (95 specimens, 41.5%) were also analyzed for alpha thalassemia mutations. Alpha thalassemia traits were identified in 37 blood specimens including heterozygous alpha-thal 1 deletion with SEA type (27 specimens), heterozygous alpha-thal 2 deletion with -3.7 kb deletion (6 specimens) and homozygous alpha-thal 2 deletion with -3.7 kb deletion (4 specimens). The mean (±SD) of hemoglobin A<sub>2</sub> was significantly higher in normocytic blood specimens with A<sub>2</sub>A pattern (3.20 ± 1.58) compared with microcytic blood specimens with A<sub>2</sub>A pattern (2.75 ± 0.33) with a p-value of 0.002 as shown in Diagram 1.

Hb H disease was observed in 10 specimens (2.5%) consisting of deletional Hb H (4 specimens, 1%) and nondeletional Hb H (6 specimens, 1.5%). All specimens with deletional Hb H had A<sub>2</sub>ABart'sH pattern on Hb typing and a combination of alpha-thal 1 deletion with SEA type and alpha-thal 2 deletion with -3.7 kb deletion on alpha thalassemia mutation analysis. The mean ± SD of Hb, hematocrit and other red blood cell indices were as follows: Hb 9.08 ± 0.92 g/dL, hematocrit 29.8 ± 2.96%, MCV 60.9 ± 3.24 fL, MCH 18.6 ± 0.92 pg and RDW 25.8 ± 2.95%. Moreover, all specimens with nondeletional Hb H had CSA<sub>2</sub>ABart'sH on Hb typing. However, from alpha thalassemia mutation analysis, 5 of those had a combination of alpha-thal 1 deletion with SEA type and nondeletional alpha-thal (constant spring), 1 of those had a combination of alpha-thal 1 deletion with SEA type and nondeletional alpha-thal (Pakse). The mean ± SD of Hb, hematocrit and other red blood cell indices were as follows: Hb 7.68 ± 1.97 g/dL, hematocrit 27.2.8 ± 8.02%, MCV 68.5 ± 11.50 fL, MCH 19.4 ± 2.47 pg and RDW 27.1 ± 9.43% which did not significantly differ compared with data from deletional Hb H.



**Diagram 1** Specimens with A<sub>2</sub>A pattern on hemoglobin typing with alpha thalassemia mutations

#### Beta thalassemia mutations and hematological parameters

Among 400 leftover blood specimens, 12 specimens (3.0%) had A<sub>2</sub>A pattern with elevated Hb A<sub>2</sub> more than 3.5% on Hb typing. The mean ± SD of Hb A<sub>2</sub> was 5.29 ± 0.96 and the mean ± SD of red blood cell indices were as follows: Hb 10.49 ± 1.29 g/dL, hematocrit 33.3 ± 4.04%, MCV 64.3 ± 5.34 fL, MCH 20.5 ± 1.79 and RDW 18.2 ± 3.48. Beta thalassemia mutation analysis was performed revealing mutations of codon 41/42 (-TCTT) on 6 specimens (1.5%), codon 17 (A>T) on 3 specimens (0.8%), nt-28 (A>G) on 2 specimens (0.5%) and IVSI-I (G>T) with heterozygous alpha-thal 1 deletion with SEA type on 1 specimen (0.3%) as shown in Table 2.

Hb E trait was observed in 127 specimens (31.8%) which could be divided in Hb E trait with Hb A<sub>2</sub>/E more than 25% (96 specimens, 24.0%) and Hb E trait with Hb A<sub>2</sub>/E less than 25% (31 specimens, 7.8%). Interestingly, most specimens with Hb A<sub>2</sub>/E more than 25% had normal Hb levels with mean ± SD of Hb and other red blood cell indices as follows: Hb 12.6 ± 1.67 g/dL, hematocrit 38.3 ± 5.23%, MCV 78.5 ± 4.78 fL, MCH 25.9 ± 1.82 pg and RDW 14.1 ± 1.55 percent. Among 31 specimens with Hb A<sub>2</sub>/E less than 25%, most specimens had mildly decreased Hb levels with mean ± SD of Hb and other red blood cell indices as follows: Hb 11.1 ± 2.49 g/dL, hematocrit 34.9 ± 7.11%, MCV 69.6 ± 7.66

**Table 2** Characteristics of specimens with A<sub>2</sub>A pattern on hemoglobin typing with hemoglobin A<sub>2</sub> more than 3.5%

Hemoglobin typing N (%)	CD41/42 (-TCTT)	CD17 (A>T)			nt-28 (A>G)		IVSI-1 (G>T)
	mutation	mutation			mutation		Mutation
	( $\alpha\alpha,\alpha\alpha$ ), ( $\beta,\beta^0$ )	( $\alpha\alpha,\alpha\alpha$ ), ( $\beta,\beta^0$ )			( $\alpha\alpha,\alpha\alpha$ ), ( $\beta,\beta^+$ )		(- <sup>SEA</sup> , $\alpha\alpha$ ), ( $\beta,\beta^+$ )
	6 (1.5)	3 (0.8)			2 (0.5)		1 (0.3)
Case		1	2	3	1	2	1
Age (years)		24	29	55	29	39	21
HbA <sub>2</sub> (%)	6.1 ± 0.67	5.6	6.2	6.4	5.8	6	4.7
Hb (g/dL)	9.9 ± 1.45	9.6	11.3	11.1	10.8	12	11.7
Hct (%)	31.7 ± 4.67	30	35.2	34.9	33.8	37.5	37.9
MCV (fL)	62.6 ± 3.98	62.9	64.1	65.4	70.4	71.7	56.0
MCH (pg)	19.5 ± 1.40	20.1	20.5	20.8	22.4	22.6	17.3
RDW (%)	17.0 ± 3.00	16.1	16.4	15.8	14.3	15	20.5

Notes: Data are presented as mean ± SD and median (range) for continuous variables and number (%) for categorical variables  
Abbreviation: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin;  
RDW, red cell distribution width

**Table 3** Association between hematological parameters and hemoglobin E

Hemoglobin typing	Hemoglobin E trait	Hemoglobin E trait	p-value
	(Hb A <sub>2</sub> /E > 25%)	(Hb A <sub>2</sub> /E < 25%)	
Specimens; N (%)	96 (24.0)	31 (7.8)	
Hb (g/dL)	12.6 ± 1.67	11.1 ± 2.49	0.013*
Hct (%)	38.3 ± 5.23	34.9 ± 7.11	0.011*
MCV (fL)	78.5 ± 4.78	69.6 ± 7.66	< 0.05*
MCH (pg)	25.9 ± 1.82	22.0 ± 3.08	< 0.05*
RDW (%)	14.1 ± 1.55	16.24 ± 3.36	0.007*

Notes: Data are presented as mean ± SD and median (range) for continuous variables and number (%) for categorical variables  
Abbreviation: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin;  
RDW, red cell distribution width

fL, MCH 22.0 ± 3.08 pg and RDW 16.24 ± 3.36 percent which significantly differed compared with specimens with Hb A<sub>2</sub>/E more than 25% as shown in Table 3. Alpha thalassemia mutations were detected in all 31 specimens consisting of heterozygous alpha-thal 2 deletion with -3.7 kb deletion in 15 specimens, heterozygous alpha-thal 1 deletion with SEA type in 7 specimens, homozygous alpha-thal 2 deletion with -3.7 kb deletion in 4 specimens, heterozygous alpha-thal 2 deletion with -4.2 kb deletion in 3 specimens and nondeletional alpha thalassemia (heterozygous hemoglobin constant spring) in 2 specimens as shown in Table 4.

In addition, homozygous Hb E was observed in 20 specimens (5%) which could be divided in EE pattern (14 specimens, 3.5%) and EE/EF pattern (6 specimens, 1.5%) from Hb typing. As of 14 specimens with EE pattern, the mean ± SD of Hb and hematocrit were 11.7 ± 2.07 g/dL and 36.3 ± 6.29%, respectively with low MCV/MCH and widening RDW as follows: MCV 67.3 ± 6.02 fL, MCH 21.6 ± 2.28 pg and RDW 15.9 ± 1.93 percent. Alpha thalassemia mutations were detected in 6 specimens consisting of heterozygous alpha-thal 2 deletion with -3.7 kb deletion in 5 specimens and heterozygous alpha-thal 1 deletion with SEA type in

**Table 4** Characteristics of hemoglobin E trait (n = 127)

Hemoglobin typing	Hemoglobin E trait (Hb A <sub>2</sub> /E >25%)				Hemoglobin E trait (Hb A <sub>2</sub> /E <25%)					
	N (%)	(αα,αα), (β,β <sup>E</sup> )	(-α <sup>3.7</sup> ,αα), (β,β <sup>E</sup> )	(- <sup>SEA</sup> -α <sup>3.7</sup> ,αα), (β,β <sup>E</sup> )	(-α <sup>3.7</sup> , -α <sup>3.7</sup> ), (β,β <sup>E</sup> )	4 (1.0)	1	2	3	2 (0.5)
Case	96 (24.0)	15 (3.8)	7 (1.8)	4 (1.0)	1	2	3	4	1	2
Age (years)		11.5 ± 2.13	13.5 ± 2.49	9.4	65	23	21	30	76	32
Hb (g/dL)	12.6 ± 1.67			30.0	9.4	8.8	13.1	12.0	7.5	12.2
Hct (%)	38.3 ± 5.23	35.6 ± 6.32	40.8 ± 7.12	30.8	30.0	30.8	38.8	36.9	23.7	37.0
MCV (fL)	78.5 ± 4.78	73 ± 7.64	84.7 ± 5.39	70.1	70.1	75.2	80.3	76.2	78.3	79.9
MCH (pg)	25.9 ± 1.82	23.5 ± 3.10	27.9 ± 2.04	21.9	21.9	21.5	27.1	24.9	24.7	26.4
RDW (%)	14.1 ± 1.55	15.2 ± 2.32	13.5 ± 1.56	17.9	17.9	17.4	13.2	13.8	18.4	13.3

Notes: Data are presented as mean ± SD and median (range) for continuous variables and number (%) for categorical variables

Abbreviation: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width

1 specimen. The mean ± SD of red blood cell indices among those specimens were as follows: MCV 64.4 ± 3.29 fL, MCH 20.7 ± 1.34 pg and RDW 18.0 ± 3.09 percent compared with red blood cell indices on 8 specimens with no alpha thalassemia mutations (MCV 66.9 ± 6.55 fL, MCH 21.5 ± 2.37 pg and RDW 16.4 ± 2.56%) without significance. Six specimens with EE/EF pattern on Hb typing with Hb F more than 5% (mean ± SD: 12.6 ± 2.69) were observed among 2 children and 4 adults. All 6 specimens had mildly decrease Hb levels. The mean ± SD of Hb and hematocrit were 11.4 ± 2.01 g/dL and 35.2 ± 5.95%, respectively with low MCV/MCH and widening RDW as follows: MCV 63.0 ± 5.3 fL, MCH 20.4 ± 1.68 pg and 20.0 ± 2.88%. Similar to specimens with EE pattern, 7 beta thalassemia common mutations were investigated on those EE/EF specimens revealing no identified mutations. However, alpha thalassemia mutations were detected in 2 specimens consisting of heterozygous alpha-thal 2 deletion with -3.7 kb deletion and homozygous alpha-thal 2 deletion with -3.7 kb deletion.

Interestingly, 2 specimens (0.5%) with Hb E/β-Thalassemia were found in this study. Specimen #1 was obtained from a 64-year-old Thai woman with underlying rheumatoid arthritis and a history of anemia requiring daily folic acid supplement. Complete blood count was obtained revealing Hb 9.8 g/dL, hematocrit 31%, MCV 68.9 fL, MCH 21.8 pg and RDW 26.4%. Hb typing showed EFA pattern with Hb A 20.3%, Hb F 33.0%, Hb E 41.7% and Hb A<sub>2</sub> 5.0%. Beta thalassemia 7 common mutation analysis was positive for nt-28 along with codon 26 (Hb E). Specimen#2 was obtained from a 46-year-old Thai man initially presented a mediastinal mass and was noted to received thalassemia required blood transfusion since 20 years of age. However, he had poor compliance and ended up receiving blood transfusion once to twice annually. Complete blood count was obtained revealing Hb 8.5 g/dL, hematocrit 27.5%, MCV 73.4 fL, MCH 22.8 pg and RDW 26.9%. Hb typing showed EF pattern with Hb F 51.6%, Hb E



44.2% and Hb A<sub>2</sub> 4.2%. Beta thalassemia 7 common mutation analysis was positive for codon 41/42 (-TCTT) along with Hb E.

Rare types of abnormal Hb were detected in 2 specimens. Specimen#3 was obtained from a 24-year-old Thai pregnant woman who was previously healthy. Complete blood count revealed Hb 13.6 g/dL, hematocrit 41.5%, MCV 81.4 fL, MCH 26.6 pg and RDW 14.2%. Hb typing was performed due to low MCH revealing A<sub>2</sub>A pattern with rare abnormal Hb (hemoglobin A 64.0%, abnormal Hb 32.7% and Hb A<sub>2</sub> 3.3%). Beta thalassemia 7 common mutation analysis was negative. However, *HBB* gene sequencing revealed heterozygous Hb Tak (*HBB*; c.440\_441dup or p.\*148Thr\*11).

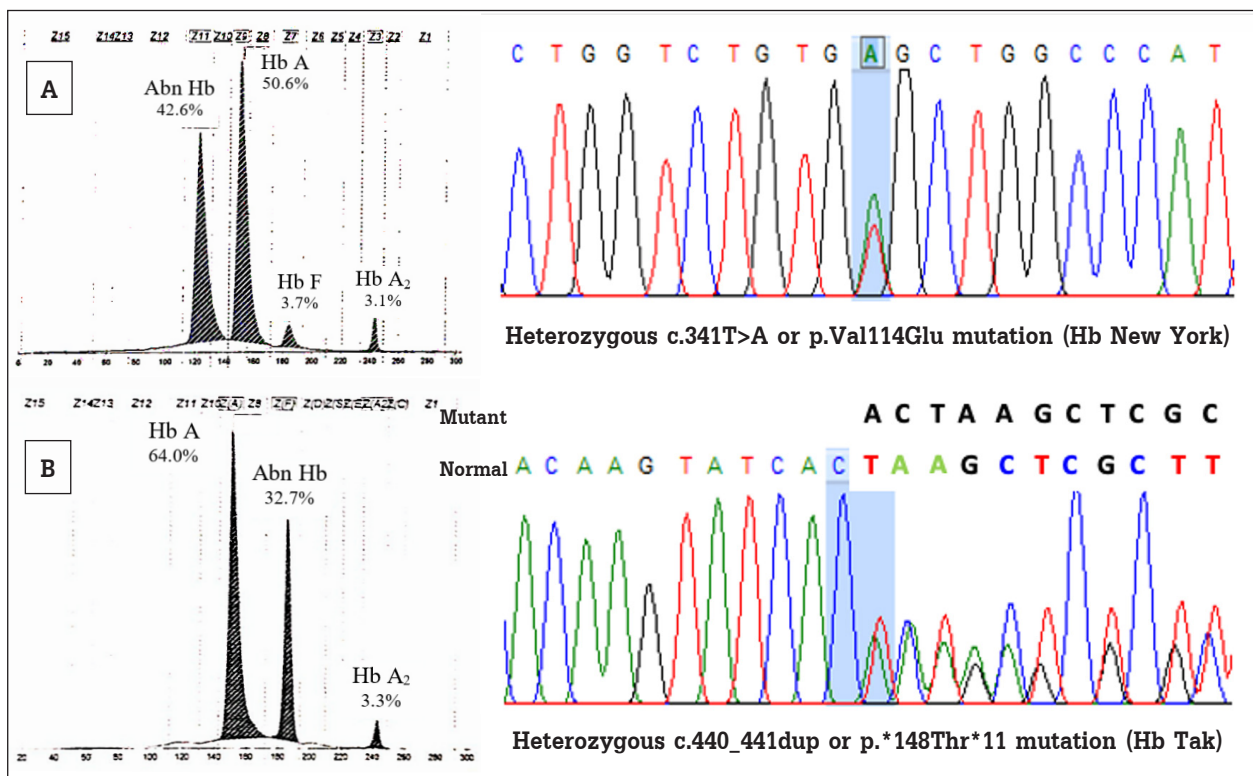
Specimen#4 was obtained from a 2-year-old Thai boy initially presenting mild anemia. Complete blood count revealed Hb 10.7 g/dL, hematocrit 29.9%, MCV 70.7 fL, MCH 25.2 pg and RDW 21.2%. Hb typing showed A<sub>2</sub>A pattern with rare abnormal Hb (abnormal Hb 42.6%, Hb A 50.6%, Hb F 3.7% and Hb A<sub>2</sub> 3.1%). Beta thalassemia 7 common mutation analysis was negative. However,

*HBB* gene sequencing revealed heterozygous Hb New York (*HBB*; c.341T>A or p.Val114Glu). The capillary electrophoresis of rare abnormal hemoglobin is shown in Figure 1.

## Discussion

The ultimate goal of this study was to determine the prevalence of thalassemia trait and disease as well as associations between hematological parameters from an automated complete blood count analyzer including Hb, hematocrit and red blood cell indices (MCV, MCH and RDW), hemoglobin types evaluated by capillary electrophoresis (CE) and types of alpha- and beta- globin gene mutations. Four hundred leftover blood specimens were obtained from subjects who were suspected of thalassemia trait or disease in which the majority of adults accounted for 93% of total subjects' specimens.

Among 400 blood specimens, 62.5% were found to have thalassemia trait or disease. The prevalence of alpha thalassemia traits was 16% and the most common alpha thalassemia mutation was heterozygous alpha-thal 1



**Figure 1** Capillary electrophoresis and direct DNA sequencing results of rare abnormal hemoglobin; (A) Hemoglobin New York (c.341T>A or p.Val114Glu) (B) Hemoglobin Tak (c.440\_441dup or p.\*148Thr\*11)

deletion with SEA type. The prevalence was higher than the normal population because we studied hemoglobin typing in suspected cases of anemia mainly from the Department of Medicine and Obstetrics exhibiting a positive screening test for thalassemia. In Southeast Asia, the SEA deletion ( $--^{SEA}$ ) is the most common  $\alpha$ -thalassemia 1 mutation caused by a deletion of approximately 19.3 kb in length removing both linked  $\alpha$ -globin genes but sparing the embryonic  $\zeta$ -globin gene. Another form of  $\alpha$ -thalassemia 1 occasionally found in this region is the THAI type deletion ( $--^{THAI}$ ) which removes a 33.5 kb-DNA segment including the embryonic  $\zeta$ -globin gene<sup>22,23</sup>. THAI type deletion was not detected in this study. However, the rarity of THAI type deletion is concordant with a prevalence ratio of 99:1 of SEA to THAI type deletion in related studies conducted in Thai populations<sup>19,23,24</sup>.

The most common  $\alpha$ -thalassemia 2 mutation in this study was 3.7 kb or rightward deletion ( $-\alpha^{3.7}$ ) found in 32 specimens, followed by 4.2 kb or leftward deletion ( $-\alpha^{4.2}$ ) found in 5 specimens. All specimens with alpha thalassemia mutations had  $A_2A$  pattern. The mean  $\pm$  SD Hb  $A_2$  in normocytic blood specimens (MCV > 80 fL) with  $A_2A$  pattern and microcytic blood specimens (MCV < 80 fL) with  $A_2A$  pattern were  $3.20 \pm 1.58\%$  and  $2.75 \pm 0.33\%$ , respectively in which this difference was significant. Alpha thalassemia traits were detected among 6.8% of those normocytic blood specimens with  $A_2A$  pattern; however, none were alpha thal-1 trait. According to the studies from Pornprasert et al.<sup>25</sup> and Sin et al.<sup>26</sup>, the cut-off MCV of 75 fL was recommended to further investigate alpha thalassemia trait. In addition, Chanhathai et al.<sup>27</sup> recommended the workup for alpha thalassemia-1 trait among obstetric patients with MCV less than 76.15 fL. The aim of using lower MCV numbers was to increase the possibility of detecting subjects with alpha thalassemia traits. According to these findings, the use of MCV with cut-off at 80 fL could provide a higher chance of detecting alpha thal-1 and alpha thal-2 traits. Iron deficiency must be

known among patient with alpha thalassemia traits with microcytic blood picture.

In our study, Hb H disease was detected in 10 specimens classified as either deletional or nondeletional Hb H disease. However, hematologic parameters including Hb, hematocrit and additional red blood cell indices did not significantly differ between those two groups which contrasted with the study by Traivaree et al.<sup>19</sup> reporting less severities of deletional Hb H disease compared with nondeletional Hb H disease due to gaining function of the remaining alpha gene. This might have resulted from the small sample size of this study.

In addition, our study found beta thalassemia traits in 12 specimens for which the codon 41/42 (-TCTT) mutation was the predominant genotype (6 specimens, 33.3%), followed by codon 17 (A>T) mutation, nt-28 (A>G) mutation and IVSI-I (G>T). This result was compatible with the study by Trivaree et al.<sup>21</sup>. However, several beta globin gene mutations were not found in this study including IVS-I-5 (G>C), IVS-II-654 (C>T) or codon 71/72 (+A). Those mutations might have been detected if we had used a larger sample size as described in Table 2. Interestingly, among 12 specimens with beta thalassemia mutations, one specimen showed an alpha-thal 1 deletion with SEA type ( $--^{SEA}$ ) but still noted Hb  $A_2$  of more than 3.5% which accounted for 8.3% of specimens with alpha thal-1 in specimens with beta thalassemia trait (12 specimens). In fact, the patient with beta-thalassemia trait should not have anemia, but all patients with beta-thalassemia trait in our study had mild anemia which might have been caused by iron deficiency.

Our study also explored the prevalence of Hb E trait found to be 31.8% at Phramongkutklo Hospital compared with related studies reporting the prevalence of Hb E trait ranging between 8 and 60%<sup>14</sup> depending on the regions in which the northeast region exhibited the highest prevalence of disease. Subjects with Hb E trait having Hb  $A_2/E$  more than 25% were not anemic.

On the other hand, those having Hb A<sub>2</sub>/E less than 25% might have had mild anemia and were noted to have significantly lower MCV/MCH and wider RDW. However, iron deficiency also causes lower Hb A<sub>2</sub>/E, lower MCV/MCH and wider RDW. AEBart's disease was not observed in this study.

According to Table 4, we also found 7 patients with compound heterozygous between the alpha-thal 1 deletion with SEA type (--SEA) and Hb E trait had normal MCV (84.7 ± 5.39 fL) and 3 patients with α-thalassemia 2 with -4.2 kb deletion (-α<sup>42</sup>) and Hb E trait had lower MCV (76.6 ± 6.40 fL) that differed from the related study<sup>21</sup> probably cause from a small sample population.

In 2 specimens with Hb E/β-thalassemia, the Specimen#1 found mutations of nt-28 along with Hb E which could be milder clinical symptoms due to β<sup>+</sup>-thalassemia. Specimen#2 was obtained from a subject who received a delayed diagnosis of anemia causing the first blood transfusion to be given to the subject at 20 years of age. Given the additional poor compliance of this subject in which blood transfusion was given only once to twice annually times, the subject unfortunately developed extramedullary hematopoiesis in response to chronic hypoxia and presented a mediastinal mass as mentioned from the study from Zhang et al<sup>28</sup>. Moreover, the study from Huan-Zhu Zhang et al. found that appropriate transfusion and treatment among patients with Hb E/β<sup>0</sup>-thalassemia could prevent extramedullary hematopoiesis<sup>29</sup>. Also, those two cases had no associated alpha thalassemia mutations.

One child in our study was found to have rare abnormal Hb. Further investigation confirmed Hb New York. Hb New York was first reported in 1971 by Blackwell, et al<sup>30</sup>. According to the study of Li, et al.<sup>31</sup> and Panyasai, et al.<sup>32</sup>, patients with Hb New York usually have normal clinical and hematological parameters. However, the subject in our study did have mild anemia with low MCV, MCH and wide RDW, which could be from iron deficiency anemia because iron deficiency anemia is commonly found in this age group.

In addition, Hb Tak was also found in one asymptomatic subject during antenatal care visit. The subject had no anemia and her hematological parameters were in normal ranges. Hemoglobin Tak was first described in Thailand in 1971 by Flatz, et al.<sup>33</sup>, and is one of the commonest Hb variant found in the Southeast Asia region, presently with an unclear prevalence. In general, patients with heterozygous Hb Tak usually are asymptomatic; however, homozygous Hb Tak patients might present symptomatic secondary polycythemia according to a report from Tanphaichitr, et al<sup>34</sup>. However, the subject in this study had no clinical or laboratory findings compatible with the findings of related studies<sup>34</sup>.

### Limitations

Several limitations were encountered this study including a small sample size with short study duration which could not entirely represent the country as a whole. In addition, beta thalassemia mutation analysis was not performed in all specimens for which specimens with normal A<sub>2</sub>A might have been undetected.

### Conclusion

This study could identify the prevalence of thalassemia trait and thalassemia disease up to 62% from leftover blood specimens from Hb typing as well as associations between results from automated complete blood count, Hb typing using CE and mutation analysis using PCR technique. The most common type of thalassemia trait was hemoglobin E trait found at 31.8% in our study.

However, the alpha thal-2 mutation (6.8%) but not alpha thal-1 was detected in specimens with normal MCV in which alpha thal-1 trait combined with beta thalassemia trait was detected in one specimen (0.3%) accounting for 8.3% when compared with all beta thalassemia traits. Further study is essentially needed to evaluate the prevalence of alpha thalassemia trait combined with beta thalassemia trait to lower the risk of thalassemia disease.

### Conflicts of interest

The authors declare they have no conflicts of interest.

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### Contribution

**PJ** contributed to laboratory investigations, genetic diagnosis and data collection (30%)

**BB** contributed to confirm rare genetic diagnosis (10%)

**CT** contributed to overall management of the program and study design (10%)

**PR** contributed to data analysis and drafting the manuscript (10%)

**AP** contributed to overall management of the study, diagnosis, data analysis and writing the manuscript (40%)

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