β-thalassemia is a genetic disorder resulting in decreased or completely absent production of the normal β-globin chain usually due to nucleotide base substitution, minor deletion, or insertion. The β-thalassemia mutations at codons 17(A>T), 41/42(-TCTT), 71/72(+A), and IVSI-nt1 (G>T) are commonly found in Southeast Asia. Moreover, one of the most common point mutations in the β-globin gene is known as hemoglobin (Hb) E [β26 (B8) Glu→Lys, GAG>AAG], which induces alternative splicing and thus results in decreased β-globin E chain. The compound β-thalassemia/HbE mutations cause anemia that can vary from nearly asymptomatic to severe transfusion-dependent anemia.

Due to the high cost for treatment of β-thalassemia/HbE disease, the reduction of the number of newborns with this disease is desirable. The preliminary screening of β-thalassemia is performed in a couple by using a modified one-tube osmotic fragility test (OF test) and/or red blood cell (RBC) indices while the screening of HbE is performed by using dichlorophenolindophenol (DCIP) precipitation test. The confirmation test for both β-thalassemia and HbE is performed by high-performance liquid chromatography (HPLC), low-pressure liquid chromatography (LPLC), and capillary electrophoresis (CE).

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Proportions of HbA2/E and HbF in β-thalassemia/HbE disease, measured by HPLC, are 30 to 60% and ≥15%, respectively, while those in homozygous HbE were ≥65% and <5%, respectively. However, some cases of β-thalassemia/HbE disease have a high proportion of HbA2/E (≥75%) and a low fraction of HbF (<15%). Therefore, diagnoses of both β-thalassemia/HbE disease and homozygous HbE are usually uncertain in samples that have HbA2/E levels higher than 75% and HbF levels lower than 5%.
varying from 5% to 15%. Molecular analysis is needed to confirm the diagnosis in these cases.

However, in low-resource settings where molecular analysis is not available, HbF ($\alpha_{2}^\gamma$) levels >5% measured by HPLC, in combination with MCV <55 fL, hemoglobin <10.0 g/dL, and hematocrit <30% have been recommended in screening for β-thalassemia/HbE disease. CE is capable of distinguishing HbA$_2$ from HbE, thus permitting quantification of HbA$_2$ in patients with HbE. Nevertheless, whether the HbA$_2$ fraction measured by CE can be diagnostic of β-thalassemia/HbE disease has not been demonstrated. Thus, the aim of this study was to compare the levels of HbA$_2$ in β-thalassemia/HbE and homozygous HbE patients who have HbE levels higher than 75% and HbF levels varying from 5% to 15%.

**Materials and Methods**

**Blood Samples and Thalassemia Diagnosis**

Blood specimens were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) and submitted for investigation of thalassemia to the Associated Medical Sciences Clinical Service Center laboratory at Chiang Mai University in Chiang Mai, Thailand. Hemoglobin, hematocrit, and mean corpuscular volume (MCV) of RBCs were measured using an automated blood counter (Sysmex KX-21; Sysmex Corporation, Kobe, Japan). Quantification of HbA$_2$ (for detection of β-thalassemia) and identification of hemoglobinopathies including HbE was performed using HPLC (VARIANT β-thalassemia Short Program, Bio-Rad Laboratories, Hercules, California, USA). Blood samples that had HbA$_2$/E fractions higher than 75% and HbF levels varying from 5% to 15% were analyzed by CE (CAPILLARYSTM 2, Sebia, Norcross, Georgia, USA) within 24 hours. The α-thalassemia-1 Southeast Asian (SEA) type deletion was detected by using real-time polymerase chain reaction (PCR) with SYBR Green1 high-resolution melting (HRM) analysis as previously described.

**DNA Preparation and Molecular Diagnosis for β-thalassemia and HbE**

The genomic DNA was extracted from blood samples containing HbA$_2$/E levels higher than 75% and HbF levels varying from 5% to 15% by using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The DNA was stored at ~20°C until analysis. The β-thalassemia codons 71/72 (+A), 41/42 (-TCTT), 17 (A>T), and IVS1-nt1 (G>T) mutations were analyzed by the multiplex amplification refractory mutation system (MARMS)-PCR according to the protocol described previously. The diagnosis for heterozygous and homozygous HbE was based on amplification refractory mutation system (ARMS)-PCR which has been described elsewhere.

**Statistical Analysis**

The data are presented as median and interquartile range (IQR). Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences 11.0, Chicago, IL, USA). Hematological parameters and HbA$_2$, HbE, and HbF were compared between β-thalassemia/HbE disease and homozygous HbE using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

**Results**

Based on the molecular analysis, 9 patients were diagnosed with β-thalassemia/HbE disease and 19 patients with homozygous HbE. Among the 9 β-thalassemia/HbE patients, 6 had mutations in the β-thalassemia codons 41/42, 2 had the codon 17 mutation, and 1 had the intervening sequence I (IVSI)-nt1 mutation. Moreover, 2 of the 9 β-thalassemia/HbE disease, and 1 of the 19 patients with homozygous HbE had co-inheritance of the α-thalassemia-1 SEA type deletion. The means (ranges) age of β-thalassemia/HbE disease and homozygous HbE patients were 38.2 (15 to 58) and 28.9 (2 to 71) years, respectively. Median levels of MCV, hemoglobin, and hematocrit of the β-thalassemia/HbE patients were significantly lower than those of homozygous HbE patients (Figures 1A, 1B, and 1C, respectively). Whereas 18 of 19 (95%) of homozygous HbE patients had MCV higher than 55 fL, only 1 β-thalassemia/HbE patient had MCV higher than that value and this patient co-inherited the α-thalassemia-1 SEA type deletion (Figure 1A). Fourteen of 19 (74%) homozygous HbE patients and no β-thalassemia/HbE patients had hemoglobin higher than 10.0 g/dL (Figure 1B). Moreover, 16 of the 19 (84%) homozygous HbE patients and 2 of 9 (22%) β-thalassemia/HbE patients had hematocrit higher than 0.30 L/L (Figure 1C).

Median HbA$_2$ and HbF fractions determined by HPLC in β-thalassemia/HbE patients were not significantly different from those in homozygous HbE patients.
Figure 1
Comparison in levels of MCV (A), hemoglobin (B), hematocrit (C), HbA₂ (D), HbE (E), and HbF (F) between homozygous HbE and β-thalassemia/HbE patients. Results are shown by median (horizontal line) and IQR (whisker line). The horizontal dashed line indicates the cutoff values. The values of each sample were presented by filled cycles while those of patients with α-thalassemia-1 SEA type deletion were presented by open cycles.
Discussion

CE has the ability to resolve and quantify HbA₂ and HbE. Thus, CE might facilitate the diagnosis of β-thalassemia/HbE disease in blood specimens that have HbE fractions higher than 75% and HbF levels varying from 5% to 15%. The present study showed that levels of HbA₂ measured by CE in β-thalassemia/HbE disease were significantly higher than those of homozygous HbE disease. Moreover, all β-thalassemia/HbE patients had HbA₂ levels higher than 6% while only 1 of 19 (5%) homozygous HbE patients had HbA₂ levels higher than the cutoff. These results suggest that the occurrence of α- and δ-globin chains in β-thalassemia/HbE disease was higher than that in homozygous HbE. Only 1 homozygous HbE patient, who co-inherited the α-thalassemia-1 SEA type deletion, had a HbA₂ level higher than 6%. Further, 2 β-thalassemia/HbE patients, who co-inherited α-thalassemia-1 SEA type deletion, also had high levels of HbA₂ (8.3% and 12.8%). This finding was consistent with a previous study that reported β-thalassemia/HbE or homozygous HbE patients who co-inherited the α-thalassemia-1 SEA type deletion had higher levels of HbA₂ than those who did not co-inherit the α-thalassemia-1 SEA type deletion. These results imply that the reduced α-globin chains in β-thalassemia/HbE or homozygous HbE patients who co-inherited the α-thalassemia-1 SEA type deletion preferentially bind to the δ-globin chain.

In 1 reported study, the combination of HbF level (>5%) analyzed by HPLC with MCV (<55 fL), hemoglobin (<10.0 g/dL), and hematocrit (<0.30 L/L) was used as an alternative method of screening for β-thalassemia/HbE disease. However, in our study, the levels of HbF measured by either HPLC or CE among β-thalassemia/HbE and homozygous HbE patients were not significantly different. Although HbE levels of homozygous HbE patients were significantly higher than those of β-thalassemia/HbE disease, their variations in both groups of patients were high. Thus, it was not possible to use the HbE levels to screening for β-thalassemia/HbE disease and homozygous HbE. All β-thalassemia/HbE patients had hemoglobin levels lower than 10.0 g/dL; however 5 of 19 (26%) homozygous HbE patients also had hemoglobin levels lower than this threshold. In addition, 2 of 9 (22%) β-thalassemia/HbE patients had hematocrits higher than 0.30 L/L. Therefore, it is also difficult to use hemoglobin and hematocrit to classifying β-thalassemia/HbE disease and homozygous HbE. Most of the β-thalassemia/HbE patients (89%) had MCV lower than 55 fL; however, there was a case of homozygous HbE which had MCV lower than this value. Moreover, among 2 β-thalassemia/HbE patients who co-inherited the α-thalassemia-1 SEA type deletion, 1 patient had MCV higher than 55 fL while the other patient had MCV less than 55 fL. This observation was consistent with a previous study that reported MCV levels of homozygous HbE patients with and without co-inheritance of α-thalassemia-1 SEA type deletion varied from 51–61 fL and 47–80 fL, respectively. Thus, the use of MCV only is inadequate for screening of β-thalassemia/HbE disease and homozygous HbE. Increased HbA₂ is specific for diagnosis of β-thalassemia; thus, the combination of HbA₂ level and MCV might be helpful to differentiate between β-thalassemia/HbE disease and homozygous HbE.

In conclusion, β-thalassemia/HbE disease may be differentiated from homozygous HbE in specimens that have HbA₂/E fractions higher than 75% and HbF fractions varying from 5% to 15% by using a combination of the HbA₂ level >6% measured by CE, and MCV <55 fL. This approach might be used to decrease the number of specimens referred for molecular testing, which would be especially beneficial in low-resource settings where β-thalassemia and HbE are prevalent.

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