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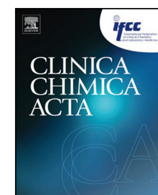

Diagnostic utility of isoelectric focusing and high performance liquid chromatography in neonatal cord blood screening for thalassemia and non-sickling hemoglobinopathies

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A B S T R A C T

Background: Thalassemia syndromes are highly prevalent in Southeast Asia. In Thailand, high performance liquid chromatography (HPLC) is the most common technique routinely performed in diagnosis of thalassemia and hemoglobinopathies, while isoelectric focusing (IEF) is rarely employed. We compared the diagnostic utility of IEF and HPLC in neonatal screening for thalassemia and non-sickling hemoglobinopathies.
Methods: Two-hundred and forty-one cord blood samples were analyzed using IEF and HPLC, β-thalassemia short program. The results were correlated with red cell indices and molecular analyses. Hemoglobin (Hb) Bart's was quantified only on IEF.
Results: Of 241 newborns, IEF and HPLC yielded 85.4% and 76.4% sensitivity to identify α-thalassemia syndrome, respectively. HbBart's $\geq$ 2% yielded 100% sensitivity to identify 2 α-globin gene deletions and/or mutations, while MCV $\leq$ 95 fl and MCH $\leq$ 30 pg yielded 100% sensitivity to identify 2 α-globin gene deletions. DNA analysis revealed HbE mutation in all 61 subjects with $HbA_2 > 1\%$ on both IEF and HPLC.
Conclusion: IEF is an effective method in neonatal screening for thalassemia and non-sickling hemoglobinopathies. The HbBart's level, MCV and MCH are helpful for identifying α-thalassemia. The presence of HbA_2 higher than 1% in cord blood indicates HbE carriers in Southeast Asian newborns.

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1. Introduction

Thalassemia syndromes are the most common monogenic disease in the world and pose an emerging global health burden. The majority of patients and more than 300,000 births each year of severe thalassemia diseases live in developing countries such as Africa, Asia and Mediterranean countries [1]. Thalassemia and non-sickling hemoglobinopathies, especially hemoglobin E (HbE) and hemoglobin Constant Spring (HbCS), are primarily prevalent in India, China and Southeast Asia [1,2]. However, a rapid increase in Asian immigration to North America and Europe results in the significant growth in thalassemia patients in the United States (US), Canada and many countries in Europe. Hemoglobin H disease (HbH and HbHCS) and β-thalassemia/HbE (β-thal/HbE) recently become a growing public health problem in the Pacific coast of the US, especially in California [2,3]. Therefore, neonatal screening for thalassemia and non-sickling hemoglobinopathies is currently advocated in the newborn screening program [4]. Early diagnosis of thalassemia and clinically significant hemoglobin variants will provide early clinical management as well as family education and genetic counseling.

Several techniques, such as gel electrophoresis, isoelectric focusing (IEF), capillary electrophoresis and high performance liquid chromatography (HPLC), were investigated to determine their diagnostic performance in cord blood screening for thalassemia and clinically significant hemoglobinopathies [5–9]. In Thailand, HPLC, β-thalassemia short program on Variant Hemoglobin Testing System, is the most common technique performed in clinical practice, while IEF is rarely employed. However, HPLC, β-thalassemia short program, cannot quantify HbBart's as well as HbH. Additionally, the acetylated HbF, which is frequently found in cord blood, may interfere with the HbBart's and HbH peaks on HPLC [10]. These factors could impact on diagnostic performance of HPLC in detection of α-thalassemia, which relies on the presence of HbBart's in cord blood. Until now, there have been no studies directly comparing diagnostic performance between these 2 methods in newborn screening in population, which thalassemia and non-sickling hemoglobinopathies are highly prevalent. This study primarily aimed to compare diagnostic utility of IEF and HPLC in neonatal cord blood screening for thalassemia and non-sickling hemoglobinopathies as well as compound mutations in Thailand.

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2. Materials and methods

2.1. Subjects

Neonatal cord blood specimens from the National Cord Blood Bank, Thai Red Cross, were sent to analyze consecutively at the Hematology Laboratory Unit, King Chulalongkorn Memorial Hospital, Bangkok, from January 2011 to July 2012. An individual cord blood specimen was collected into a bag containing 30 ml of CPDA-1 anticoagulant. The total volume of each bag was varied and depended on obtainable cord blood volume, which was approximately 80 to 120 ml. The 3 ml of each cord blood was separated for hemoglobin analysis and molecular testing.

2.2. Samples and analytical methods

Complete blood count was measured by an automated cell counter (Haematologie). Hemoglobin analysis was performed using both HPLC, Variant II β-thalassemia short program (Bio-Rad Laboratories), and IEF method to determine the quantity of each hemoglobin type. After electrophoresis, each band of hemoglobin variant on the gel was scanned and quantified by scanning densitometry using IsoScan Imaging System for Hemoglobin Testing (PerkinElmer Life and Analytical Sciences). The accuracy of scanning densitometry for quantification of HbA_2, HbBart's and HbH as well as HbCS in our laboratory was validated with molecular study and previously published [11–14]. HbBart's on HPLC was subjectively determined by the presence of a sharp spike at the retention time of zero on the chromatogram. HbBart's levels were quantified only on the IEF method.

The α-globin genes were analyzed based on multiplex gap polymerase chain reaction method for detection of the majority of deletional α-thalassemia determinants as previously described [11]. The multiple primers were used to amplify specific DNA fragments that were indicative of $--^{SEA}$ and $--^{THAI}$ for α^0-thalassemia and $-\alpha^{3.7}$ and $-\alpha^{4.2}$ for α^+-thalassemia. HbCS and Hb Pakse (HbPS) were detected using a single-tube multiplex amplification refractory mutation system method to identify HbCS [Termination codon (TAA ➔ CAA)] and HbPS [Termination codon (TAA ➔ TAT)] as previously described [14]. The β-thalassemia mutations were identified using the reverse dot blot method, which covers 15 β-thalassemia mutations and accounts for more than 95% of β-thalassemia in Thailand [15]. Subjects showed abnormal unidentifiable bands underwent direct DNA sequencing of entire β-globin gene and/or α-globin gene using the ABI BigDye system (Applied Biosystems).

2.3. Statistical analysis

Red cell indices as well as the quantity of HbBart's and HbA_2/E were demonstrated as means and standard deviations. The comparison of means was analyzed by independent sample *t*-test or one-way ANOVA test and Post Hoc analysis for multiple comparisons as appropriate. The p-value < 0.05 was considered statistically significant. Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of each selected cut-off to identify α-thalassemia, β-thalassemia and non-sickling hemoglobinopathies was calculated each time on the total number of individuals. All statistic parameters were computed using the SPSS version 16 for Window.

3. Results

Out of 241 neonatal cord blood analyses, there were 16 genotypes: 111 normal, 37 heterozygous α^+-thalassemia, 3 homozygous α^+-thalassemia, 14 heterozygous α^0-thalassemia (1 $--^{THAI}$), 9 heterozygous HbCS, 2 compound heterozygosity for α^+-thalassemia and HbCS, 1 HbH, 1 HbHCS, 2 heterozygous β-thalassemia, 38 heterozygous HbE, 10 double heterozygosity for HbE and α^+-thalassemia, 6 double heterozygosity for HbE and HbCS, 1 HbE with homozygous α^+-thalassemia, 2 double heterozygosity for HbE and α^0-thalassemia, 3 heterozygous HbE with compound heterozygosity for α^+-thalassemia and HbCS (1 HbPS) and 1 homozygous HbE. Their hematological parameters were summarized (Table 1).

3.1. Screening for α-thalassemia

The presence of HbBart's on either IEF or HPLC was used as a criterion for identifying α-thalassemia. There were 118 cases with HbBart's identified on IEF, whereas there were 84 cases demonstrating HbBart's on HPLC. Out of 89 α-thalassemia syndromes detected on genetic analyses, IEF yielded sensitivity and specificity of 85.4% and 72.4%, respectively, while HPLC yielded sensitivity and specificity of 76.4% and 89.5%, respectively. There were 12 α-thalassemia carriers with undetectable HbBart's on both IEF and HPLC (11 heterozygous α^+-thalassemia and 1 heterozygous HbCS), 9 undetectable on HPLC alone (8 heterozygous α^+-thalassemia and 1 heterozygous HbCS) and 1 undetectable on IEF alone (heterozygous α^+-thalassemia). All samples carrying at least 2 α-globin gene defects showed detectable HbBart's on both techniques.

HbBart's levels measured on IEF were correlated with a number of α-globin gene abnormalities and statistically significant different in all subgroups (Table 2). No hematological parameters were able to clearly separate a single α-globin gene defect from normal genotype with acceptable diagnostic values (data not shown). However, a single α-globin gene defect confers a little clinical impact. Therefore, we focused on screening for 2 α-globin gene mutations. The sensitivity, specificity, PPV and NPV of each selected cut-off point in screening for 2 α-globin gene defects, 2 α-globin gene deletions and heterozygous α^0-thalassemia were summarized (Table 3). Hb Bart's level ≥ 2% yielded

Table 1
Hematological and hemoglobin analysis data in correlation with thalassemia genotypes.

Genotypes (N)	Hb (g/dl)	MCV (fl)	MCH (pg)	HbBart's on IEF (%)	HbA2 on IEF (%)	HbA2 on HPLC (%)
Normal (111)	11.3 ± 1.5	106.2 ± 4.1	35.2 ± 1.6	0.4 ± 0.6	0	0.2 ± 0.2
αα/-α (37)	11.2 ± 1.6	99.5 ± 3.5	32.5 ± 1.4	1.2 ± 0.9	0	0.1 ± 0.1
$\alpha\alpha/\alpha^{CS}\alpha$ (9)	10.0 ± 0.8	101.8 ± 5.6	33.2 ± 1.6	1.8 ± 0.5	0	0.1 ± 0.1
αα/– (14)	9.9 ± 1.0	85.9 ± 4.3	27.2 ± 1.3	5.5 ± 1.9	0	0.1 ± 0.2
-α/-α (3)	9.9, 10.8, 8.9	87, 85, 80	26.9, 27.4, 26.7	3.6, 2.4, 2.2	0	0, 0.3, 0.5
$-\alpha/\alpha^{CS}\alpha$ (2)	10.5, 13.5	98, 97	29.5, 29.8	7.7, 7.2	0	0
-α/– (1)	9.8	79	22.9	13.8	0	0
$–/\alpha^{CS}\alpha$ (1)	7.9	93	27.7	16.0	0	0
$\beta/\beta^{0(+)}$ (2)	10.5, 11.0	99, 106	32.0, 34.6	0, 1.2	0	0, 0.4
β/β^E (38)	11.3 ± 1.7	104.1 ± 4.2	34.3 ± 1.7	0.2 ± 0.4	6.9 ± 1.9	5.0 ± 1.4
β/β^E, -α/αα (10)	11.4 ± 1.3	98.9 ± 3.9	32.1 ± 1.1	0.9 ± 0.9	5.1 ± 1.4	4.4 ± 1.1
β/β^E, $\alpha\alpha/\alpha^{CS}\alpha$ (6)	10.6 ± 1.5	100.2 ± 3.1	32.0 ± 1.0	1.3 ± 0.7	8.5 ± 3.6	6.4 ± 2.6
β/β^E, --/αα (2)	13.8, 9.4	95, 85	29.4, 26.8	7.5, 5.1	6.6, 5.4	4.4, 4.2
β/β^E, -α/-α (1)	10.8	86	27.1	2.8	6.2	4.3
β/β^E, $-\alpha/\alpha^{CS}\alpha$ (3)	11.2, 8.2, 10	100, 93, 94	30.9, 28.4, 30.1	6.4, 4.2, 2.0	6.2, 5.9, 4.9	4.6, 4.9, 2.7
β^E/β^E (1)	9.7	104	32.8	0	11.2	10.7

All parameters were demonstrated as mean ± SD.

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