Usefulness of brilliant cresyl blue staining as an auxiliary method of screening for α-thalassemia

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Hemoglobin (Hb) electrophoresis is widely used in thalassemia screening. Most Hb variants express a specific abnormal band on the cellulose acetate membrane. The technique is useful in the diagnosis of the type of thalassemia but is not sensitive enough to detect α-thalassemia minor because the quantity of the HbH is too small to be expressed on the supporting medium. We used simple staining of blood smears rather than the sophisticated molecular method to detect HbH inclusions. To evaluate the effectiveness of this method, we used brilliant cresyl blue (BCB) staining of red blood cells in 509 patients with microcytosis and erythrocytosis caused by various conditions. The results indicate that BCB staining is useful in the identification of subjects who possess α-thalassemia traits. Coexisting conditions such as β-thalassemia and iron-deficiency anemia did not affect the detection of the HbH inclusions with the use of BCB staining. We conclude that BCB staining is helpful and reliable as an auxiliary method of detecting HbH inclusions in the diagnosis of α-thalassemia traits, especially in places where medical resources are limited. (J Lab Clin Med 2005;145:94–7)

Abbreviations: BCB = brilliant cresyl blue; CBC = complete blood counts; EDTA = ethylenediaminetetraacetic acid; Hb = hemoglobin; HbA = hemoglobin A; HbA2 = hemoglobin A2; HbE = hemoglobin E; HbF = hemoglobin F; HbG = hemoglobin G; HbH = hemoglobin H; IDA = iron-deficiency anemia; MCV = mean corpuscular volume; RBC = red blood cell; RDW = red cell–distribution width; RDW-SD = SD of RDW

α-thalassemia is a common genetic disease that is usually caused by the deletion of 1 or more α-globin genes. α-Thalassemia trait that occurs on the deletion of 1 or 2 of 4 α-globin genes is characterized by a lower ratio of α- to β-globin synthesis and results in thalassemia minor by hematologic examination.1–3 However, it is not unusual for us to encounter “thalassemia-like” RBC indexes with normal hemoglobin fractions on electrophoresis in our medical practice. Traditional screening protocols for thalassemia involve the differentiation of various disorders that impair hemoglobin synthesis. These disorders are characterized by a reduction in the hemoglobin content of erythrocytes and are manifested in laboratory data as a reduction in RBC indexes.1,2,4 Reduced indexes may be caused by defects in the synthesis of heme, usually resulting from iron deficiency5; in the utilization of iron, as in lead poisoning or in globin synthesis, as in α- and β-thalassemia.

For patients with α-thalassemia, disturbed globin production results in a relative excess of β-like polypeptide chains. The excess β-chain can polymerize to form soluble β4 tetramers – HbH.1–3,6 Diagnosis of α-thalassemia with the use of molecular analysis is a common practice in places where molecular confirmation is available and affordable. However, in places where medical resources are limited and molecular analysis is not available or is expensive, a simple, easy, and cheap method of screening and diagnosing

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α-thalassemia is important. After supravit staining of RBCs with the redox dye BCB, HbH, being unstable, precipitates and aggregates to form intraerythrocytic inclusions. HbH inclusions occur in approximately 1 of 10,000 RBCs in individuals with α-thalassemia minor, the result of 2 gene deletions on the same chromosome, α-thalassemia-1, which is common in Southeast Asians. The number of cells with HbH inclusions is influenced by the α-thalassemia genotype, and the number of cells with HbH inclusions increases in syndromes with a greater β-globin excess. Because this method of HbH detection is easy to conduct, cheap, and easy to interpret by an experienced and trained laboratory staff, it can be used as a screening procedure for α-thalassemia, especially in areas where the molecular diagnosis of α-thalassemia is not possible. In this study, we evaluated the reliability of BCB inclusion-body determinations for the detection of α-thalassemia in 509 patients with microcytosis resulting from various conditions.

**METHODS**

A total of 764 venous blood samples taken from patients with microcytic anemia were submitted to the hematologic section of our clinical laboratory for thalassemia screening. Our research was carried out in accordance with the principles of the Declaration of Helsinki, informed consent was obtained, and the institutional review board of Chang Gung Memorial Hospital approved the study.

MCV was less than 70 fL in all cases, and the mean age of the patients was greater than 12 years. Hb electrophoresis, CBC, serum ferritin and iron concentrations, and total iron-binding capacity were determined. BCB staining was performed on all blood samples. Of the 764 patients, only 509 for whom complete data were available were included in the study. Patients were assigned to 1 of several groups on the basis of the results of Hb electrophoresis, serum ferritin and iron concentrations, and total iron-binding capacity. Individuals found to have the α-thalassemia trait had mild microcytic hypochromic anemia with MCVs of less than 70 fL, low RDW-SD, normal hemoglobin fractions on electrophoresis (HbA > 96.5%, HbA2 < 3.5%, HbF 0%), and no evidence of iron deficiency. Patients with HbH disease are usually more anemic, with abnormal HbH fractions on electrophoresis. We also used 63 venous-blood samples with normal CBC findings as controls.

We performed Hb electrophoresis using a cellulose acetate membrane at a pH of 8.2 to 8.6. Hemolysate was prepared with the use of commercial hemolysin (hemolysate reagent containing 0.005 mol/L EDTA [catalog no. 5127; Helena Laboratory, Beaumont, Texas]). Electrophoresis was carried out on cellulose acetate palate (Titan III-H; Helena Laboratory) with the use of a superheme buffer (Tris–EDTA–boric acid, pH 8.2–8.6) at 350 V for 20 minutes. After staining with Ponceau S, we visualized hemoglobin fractions H, A, F, and A2 as different bands and quantified them with the use of an Appraise densitometer (Beckman Instruments, Fullerton, Calif).

CBC, including MCV and RDW, was determined with the use of a Sysmex NE-8000 automated cell counter (TOA Co, Kobe, Japan) with standard calibration. The RDW-SD is taken at the 20% relation height level, with the histogram peak considered 100%. The RBC histograms of normal blood samples cross this 20% level twice. The distance between the 2 crossed points defined as RDW-SD is reported in femtoliters.

We prepared a 1% BCB-staining solution by dissolving 1.0 g of BCB (Sigma-Aldrich, St Louis, Mo) in 100 mL of 0.85% normal saline solution. K3-EDTA blood and 1% BCB-staining solution were added to a 12 × 75-mm plastic Kohn tube at a ratio of 1:1. We next sealed the mixture with paraffin and incubated it in a 37°C water bath for 3 hours, then prepared a BCB smear and observed 10,000 RBCs for HbH inclusions. In the case of a negative result, 50,000 RBCs were observed. Inclusions in a typical preparation are shown in Fig 1.

**RESULTS**

We observed numerous cells containing HbH inclusions in the BCB-stained smears of all 25 patients with HbH disease (of the total of 509 patients included in this study; Table I). Our most important observation: All 180 patients with the α-thalassemia trait had RBCs containing HbH inclusions. The number of HbH inclusions varied from 0.1 to 30 per 1000 RBCs. Among 304 patients without the α-thalassemia trait, 25 (8.2%) were found to have inclusion-containing cells, including 23 patients with IDA and 2 with β-thalassemia complicated by IDA. The BCB-stained smears of all patients with δβ-thalassemia, hereditary persistence of fetal Hb, and other hemoglobinopathies, as well as 63 normal controls, consistently showed negative findings. The sensitivity of BCB staining in the detection of the
α-thalassemia trait was 100%, and the specificity was 92.8%.

**DISCUSSION**

The synthesis of an α-globin chain is controlled by 4 genes (2 inherited from each parent) located on chromosome 16. Under normal circumstance, the production of α-chains is in balance with the production of equal quantities of non-α-chains. The suppression of normal α-globin–chain synthesis results from the deletion of 1 or more of the α-globin genes, leading to an increased proportion of β-chain and tetramers of the β-chain (HbH).1–3 The phenotypes of individuals with deletional α-thalassemia are diverse, dictated by the number of genes deleted and the type of deletion.1,2,10

Individuals with α-thalassemia traits have mild microcytic hypochromic anemia, which is usually caused by a deletion of (−α/αα, −α−α, or −α/αα) α-genes. Patients with HbH disease (−/−α−α) have moderately severe hemolytic anemia of varying clinical course. In cases of hydrops fetalis with Hb Bart’s (−/−−−), severe intrauterine anemia develops, resulting in death at or around the time of birth.11–13

α-Thalassemia traits are common in Taiwan.14–16 It is estimated that homozygous α-thalassemia-1 causes about 60% of the hydrops fetalis encountered in Taiwan.13 Because the disease in question involves autosomal-recessive inheritance, diagnosis of the thalassemia trait is important not only for genetic counseling but also for the prevention of serious obstetric complications in mothers bearing fetuses with homozygous disease. The incidence of α-thalassemia in Taiwan has been underestimated. Although homozygous forms result in severe anemia or death, the heterozygous forms (thalassemia trait) frequently go unrecognized. A correlation exists between the number of α-globin genes that are deleted and the extent of microcytosis.

However, there is considerable overlap in indexes among the various genotypes, and an absolute genotype assignment based solely on erythrocyte indexes is not possible.2,4 It is especially difficult to differentiate between 2-gene deletions that occur on the same chromosome (cis, αα/−−, α-thalassemia-1) and those that occur on different chromosomes (trans, α/−α−α, α-thalassemia-2).2,4,11 On the other hand, HbH is almost always detected on electrophoresis when 3 of the 4 α-globin genes are deleted. When only 1 or 2 α-globin genes are affected, the quantity of HbH is almost always undetectable on cellulose acetate membrane and normal Hb fractions appear.2,3,9 However, the distinction between the cis and trans forms of gene deletion is important because cis 2-gene carriers have a higher risk of having a 3-gene–deletion child than trans 2-gene carriers do. Genetic counseling of the affected family and DNA study to confirm the type of gene deletion of the affected individual are necessary. Molecular diagnosis of Hb variants and DNA probe study can provide firm evidence of Hb variants, and DNA-probe study can provide firm evidence of α-thalassemia.17–19 The definitive study for the detection of couples at risk for hemoglobin Bart’s hydrops fetalis is α-gene DNA analysis.20–22 However, the complexity of this technique limits its applicability as a screening tool, especially in areas where molecular analysis is not available or affordable. DNA analysis is available in only a few specialized laboratories and is not a popular technique in most laboratories. Given the limitations described above, many clinicians and laboratories make the diagnosis of α-thalassemia traits on the basis of exclusion when RBC indexes are low, iron deficiency is absent, and hemoglobin electrophoresis findings are normal.23 Thus the supplemental screening method of BCB staining becomes necessary. In addition, BCB staining is a popular technique in laboratories without the means to conduct molecular analysis. It is believed to be more sensitive than the electrophoresis technique for the detection of α-thalassemia trait and does not carry the risk of false-positive results.6–9 A correlation between the number of RBCs with HbH inclusion bodies and α-thalassemia genotypes has been documented.24

As shown in this study, BCB staining is highly sensitive and specific in the diagnosis of the α-thalassemia trait. As many as 23 patients with IDA and 2 in whom electrophoresis findings were suggestive of β-thalassemia were also shown to have HbH inclusion bodies on BCB staining. But it is noteworthy that there are no false-positive findings for normal individuals and the other Hb variants were elicited. Therefore the presence of HbH inclusion bodies in patients with IDA and β-thalassemia may explained on the basis of the coexistence of α-thalassemia with IDA and β-thalassemia.

### Table I. Results of the HbH inclusion-body staining in various patient groups

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Case no.</th>
<th>HbH inclusion bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbH disease</td>
<td>25</td>
<td>++ + 25 (100%)</td>
</tr>
<tr>
<td>α-Thalassemia trait</td>
<td>180</td>
<td>+ 180 (100%)</td>
</tr>
<tr>
<td>β-thalassemia</td>
<td>146</td>
<td>+ 2 (1.4%)</td>
</tr>
<tr>
<td>IDA</td>
<td>130</td>
<td>+ 23 (17.7%)</td>
</tr>
<tr>
<td>ββ-thalassemia</td>
<td>17</td>
<td>− 0</td>
</tr>
<tr>
<td>Hereditary persistent fetal hemoglobin</td>
<td>3</td>
<td>− 0</td>
</tr>
<tr>
<td>Other hemoglobinopathies‡</td>
<td>8</td>
<td>− 0</td>
</tr>
<tr>
<td>Normal controls</td>
<td>63</td>
<td>− 0</td>
</tr>
</tbody>
</table>

‡Including HbG Taichung-3, Hb-J Meinung-3, Hb Kaohsiung-1, and Hb-E.

*Including 11 cases with IDA.

†Including HbG Taichung-3, Hb-J Meinung-3, Hb Kaohsiung-1, and Hb-E.
It seems that the presence of iron deficiency does not make the detection of HbH inclusions more difficult, and HbH inclusions can be easily demonstrated in sideropenic patients without the need to wait for the complete correction of iron deficiency. Further study to confirm the coexistence of the conditions in these patients is necessary.

Genetic studies are usually time-consuming, complex, and expensive. Adoption of BCB staining as a screening tool for the detection of minute quantities of HbH is an alternative method. This staining is easy to perform, inexpensive, and highly sensitive and specific. However, its accuracy in the diagnosis of the α-thalassemia trait depends on several factors: The performance of BCB dye may vary widely, depending on the manufacturer or even the lot used. The appropriate timing of the analysis is also important; better results are obtained with the use of fresh blood. However, as in this study, good staining results may be obtained as long as 1 week after blood sampling. Finally, the number of HbH inclusion bodies is proportional to the severity of the genetic alternation.

The results of this study indicate that BCB staining for inclusion bodies is a useful tool with which to identify subjects who have α-thalassemic traits and therefore run the risk of giving birth to hydropic offspring. Coexistent conditions such as β-thalassemia and iron deficiency may not affect the detection of the HbH inclusion bodies with the use of this method. Antenatal screening is advised to reduce the frequency of occurrence of Hb Bart’s hydrops fetalis. We recommend that BCB staining be included in the screening protocol as an auxiliary method of detecting α-thalassemia trait in patients with microcytic blood diseases.

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REFERENCES