Identification of One or Two α-Globin Gene Deletions by Isoelectric Focusing Electrophoresis

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ABSTRACT

Objectives: To investigate the utility of isoelectric focusing electrophoresis (IEF) for identifying patients with α-thalassemia, which results from the deletion of 1 or more of the α-globin genes.

Methods: Samples were selected based on their hemoglobin H (HbH) concentration observed using IEF. The samples were analyzed for the most common α-globin gene deletions using molecular analysis.

Results: α-Globin gene deletions corresponding to α-thalassemia trait or silent carrier were observed in all samples with the HbH less than 2% phenotype. The genotypes of the specimens with HbH greater than 5% were consistent with HbH disease, while the wild-type phenotype control samples showed a wild-type genotype.

Conclusions: Low concentrations of HbH can be detected in a patient with 1 or 2 α-gene deletions using IEF.

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of HbH. The concentration of HbH/Hb Barts and the severity of α-thalassemia depend on the number of affected α-globin genes. Approximately 95% of α-thalassemia cases result from gene deletion(s) rather than nondeletional mutations. The most common single α-gene deletions are the African –α4.2 and –α4.4. The most frequent double gene deletions are the Southeast Asian (–αSEA, –αFIL, and –αTHAI) and the Mediterranean (–αMED and –α20), which result in deletion of both α-globin genes (HBA1 and HBA2) in cis (––) (same chromosome).7,8

When evaluating cases of α-thalassemia, 4 primary genotypes are possible: silent carrier (αα/–α), α-thalassemia trait (αα/–α or –ααα), HbH (β4) disease (–α/–α), and hydrops fetalis or Hb Barts (γ4) (–γ/–γ). HbH disease (3-gene deletion) usually presents as moderately severe hemolytic anemia, while deletion of all 4 α-globin genes (Hb Barts) is incompatible with life. Hb Barts is almost always fatal and is characterized by massive tissue edema (hydrops fetalis), pleural and pericardial effusions, and severe hypochromic microcytic anemia.9 Carriers of the α-thalassemia trait have only a mild hypochromic microcytic anemia, but couples with these genotypes can be at risk for having a fetus with hydrops fetalis or a child with HbH disease.

The accepted practice for diagnosing α-thalassemia assumes that HbH is detected only by biochemical methods when there are 3 α-globin gene deletions. This is hypothesized to occur because the limit of detection for HbH using high-performance liquid chromatography (HPLC) is too low (due to elution in the void volume/coelution with bilirubin) or because there is no HbH present in these patients’ samples.4 Prenatal algorithms therefore suggest that if the α-thalassemia trait is suspected, DNA analysis of 1 or both parents should be performed.11

In this study, we report the detection of concentrations of HbH less than 2% using isoelectric focusing electrophoresis (IEF) that corresponds to silent carriers or the α-thalassemia trait. Thus, this work provides the foundation for low-cost screening methods for α-thalassemia to be adopted.

Materials and Methods

Patient Samples

Sixty-seven whole-blood samples were selected based on their HbH concentration observed using IEF at Northern California Kaiser Permanente over a 6-month period. Fifty-nine of these samples had an HbH concentration less than 2%, 3 samples had an HbH concentration less than 5%, and 5 samples, selected at random, had no detectable HbH or other variant hemoglobin. Each specimen was analyzed by IEF, HPLC, and α-thalassemia deletion testing of the HBA1 and HBA2 genes. All samples were blinded before genotype analysis so that the researcher (A.M.A., R.H.N.) performing the molecular analysis was unaware of the phenotype results. Patient medical record review was used to collect the complete blood count and ferritin results associated with each specimen as well as to assess patient demographics. This study was approved by the Kaiser Permanente institutional review board.

Isoelectric Focusing Electrophoresis

Using a pH gradient of 6.0 to 8.0, IEF was performed with a 1-mm precast agarose gel and reagents found in the RESOLVE Hb Neonatal Hemoglobin Screen Kit (PerkinElmer, Waltham, MA). A 1:10 dilution was performed on each specimen using the elution solution provided in the RESOLVE kit, and 3 to 5 μL of the resulting mixture was electrophoresed using a Multiphor II (Model 17) Horizontal Electrophoresis Unit (Pharmacia LKB Bio Technologies) under 30 W for 80 minutes, followed by 5 minutes at 35 W. Hemoglobin bands were visualized by an o-dianisidine stain using the JB-2 Staining System (PerkinElmer). Staining is a multistep process that includes fixing gel in 10% trichloroacetic acid for 10 minutes, washing the gel with continuously running deionized water for 15 minutes, allowing the gel to soak in freshly prepared staining solution for 15 minutes, washing the gel with continuously running deionized water for 30 minutes, and finally drying the gel for 30 to 45 minutes. HbA, HbF, HbS, and HbC controls were included on all gels; HbN, HbE, HbD, and HbG controls were used as needed (Analytical Control Systems, Fishers, IN). All patient results were visually inspected and compared with the HPLC results. Percent HbH was estimated using scanning densitometry.

HPLC

High-performance liquid chromatography analysis was performed using the manufacturer’s instructions for the Bio-Rad Variant II β-Thalassemia Short Program (Bio-Rad, Hercules, CA), which separates hemoglobin variants by cation-exchange chromatography using a salt gradient.

Molecular Analysis of the α-Globin Genes HBA1 and HBA2

α-Globin gene deletions were detected using a previously published Gap–polymerase chain reaction (PCR) multiplex assay designed to detect the 7 most common deletions causing α-thalassemia.12 Selective amplification of specific deletions was achieved by using primers flanking the deletion boundaries and by limiting the PCR elongation to match the amplicon size. Briefly, genomic DNA was extracted from whole blood and the α-globin gene cluster amplified by Gap-PCR. Agarose gel electrophoresis of the PCR amplicons was used to detect the deletions (–α3.7, –α4.2, –(α)20.5, –αSEA, –αMED, –αFIL, and –αTHAI) in the α-globin gene cluster.
Statistical Analysis

All statistical calculations were performed using Microsoft Excel (Microsoft, Redmond, WA).

Results

Patient Samples

Isoelectric focusing was positive for low concentrations (<2%) of HbH in 59 of 67 patients (representative gel shown in Figure 1). In contrast, HbH was undetectable by HPLC analysis of these specimens (data not shown). Each of these patients had a mean corpuscular volume (MCV) less than 80 fL. More than 90% of these patients showed some combination of abnormal RBC morphology, including polychromasia, microcytosis, hypochromia, ovalocytes, anisocytosis, and schistocytes. Approximately 50% of the patients also had decreased hemoglobin and an increased RBC count. A smaller percentage of the patients (~22%) had a decreased hematocrit. Demographics were available for 63% of the patients and revealed that all were of Southeast Asian descent. Origin countries included the Philippines, China, Cambodia, and Vietnam. Clinical findings and demographics are summarized in Table 1.

Three patients had an HbH concentration greater than 5%. For these patients, HPLC showed a small yet detectable early eluting peak corresponding to the β-tetramers (data not shown). All 3 patients were of Southeast Asian descent (Chinese, Filipino, and Vietnamese). All had HbA2 less than 2%, low total hemoglobin, low MCV, high red cell distribution width, and RBC morphology abnormalities, including polychromasia, microcytosis, hypochromia, ovalocytes, anisocytosis, and schistocytes. A low hematocrit was observed in 2 of 3 patients.

Five specimens with undetectable HbH by IEF were used as wild-type controls. These patients were of varying demographics, including African American, Hispanic, white, and Vietnamese. Three of 5 of the control patients had an MCV less than 80 fL; 1 patient had an MCV less than 80 fL, low total hemoglobin, and low hematocrit. No abnormalities in RBC morphology were reported for any of these patients.

Genotype Associated With Patient Samples

Polymerase chain reaction analysis for the 7 common deletions showed that all samples selected for their HbH concentration of less than 2% had a corresponding α-thalassemia genotype (Table 1). Fifty-eight of these samples had 2 genes deleted (α-thalassemia trait); 1 specimen had only a single gene deleted (silent carrier). The most common deletion (54 of 59 specimens) observed was the Southeast Asian deletion (– – SEA); 2 patients (2 of 59 specimens) harbored the – –FIL deletion. Each of these genotypes corresponds to the α-thalassemia trait. Three genotypes were observed only once: a homozygous 3.7-kb deletion, compound heterozygous 3.7/4.2-kb gene

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deletions, and a heterozygous 3.7-kb deletion. The latter is the only specimen that contained a single gene deletion.

Polymerase chain reaction analysis also confirmed 3-gene-deletion α-thalassemia genotypes in all 3 samples with HbH greater than 5%. Each of the following genotypes was observed in 1 of 3 samples: −α²⁴.₂, −SEA, −α³⁷, −FIL, and −α³⁷, −SEA. The 5 negative controls (wild-type phenotype) all showed a wild-type genotype.

### Discussion

α-Thalassemia, the most common genetic disorder of Hb synthesis, results from decreased expression of the α-globin genes HBA1 and HBA2.¹³ Deletion of 1 or more of these genes is the most common cause of α-thalassemia. Two gene deletions on the same chromosome (−; −, −SEA, −FIL, and −α³⁷) are common in Southeast Asian populations, making the risk of HbH disease and β-thalassemia severe in these demographics high.⁷,¹³ As such, prenatal screening of these patients is critical to avoid catastrophic events during late pregnancy. If a pregnant woman is suspected of having the α-thalassemia trait, she is encouraged to (1) have her partner’s risk of α-thalassemia evaluated and/or (2) confirm the presence of α-thalassemia using molecular techniques. If the fetus is determined to be at risk for hydrops fetalis, invasive techniques such as chorionic villus sampling or amniocentesis are used to gather the tissue required to assess the fetal genotype. Because this process can be traumatic for the family, accurate paternal diagnosis and genetic counseling are critical.

Molecular techniques are an excellent way of diagnosing α-thalassemia.¹³ However, they are often expensive with long turnaround times. This is due to 2 main reasons. First, the equipment and personnel necessary to perform such molecular assays are generally available only at reference laboratories. Second, interpretation of the results is highly complex, requiring a board-certified MD or PhD to sign out each individual case.

At Kaiser Permanente Northern California, we noticed a consistent trend of visualizing concentrations of HbH less than 2% using IEF in patients with mild anemia. Our hypothesis was that these patients were carriers of α-thalassemia trait. Using molecular analysis, we determined that the presence of such low concentrations of HbH was 100% specific for the deletion of 1 or 2 α-globin genes. Importantly, most patients identified by IEF harbored cis 2 gene deletions (n = 54 WT/SEA; n = 2 WT/FIL), which are arguably the most important genotypes to detect for prenatal purposes. In this study, a single sample positive for HbH was shown to have only a single gene deletion. While this suggests that the use of IEF can detect silent carriers, we cannot conclude that IEF has the sensitivity necessary to detect HbH in most patients with a single α gene deletion.
Historically, it has been assumed that commonly used biochemical techniques for hemoglobin variant analysis (HPLC, acid/alkaline gel electrophoresis, and capillary electrophoresis) do not detect HbH in carriers of the α-thalassemia trait. Others have tried to compensate for this and detect the α-thalassemia trait by alternative biochemical techniques. One group used an enzyme-linked immunosorbent assay to quantify β-globin as a marker for α-thalassemia. The specificity and sensitivity of this method was promising, but it required purchasing a kit that has utility only for α-thalassemia. Others have recently shown that they can detect the α-thalassemia trait using a capillary electrophoresis platform designed exclusively for newborn screening. While valuable, this study was limited to cord blood samples eluted from dried blood spots and focused on the detection of Hb Barts. We successfully detected HbH at concentrations less than 2% using IEF. We believe this is of particular value because IEF is a low-cost, rapid turnaround-time technology that can be used to identify all types of hemoglobinopathies using any source of whole blood.

While the diagnostic capabilities of IEF highlighted in our work for the detection of α-thalassemia carriers are exciting, the following question arises: why is HbH undetectable by commonly used biochemical testing in these patients? Routine methods such as HPLC and capillary electrophoresis are based on spectroscopy for the detection and quantification of hemoglobin. More specifically, these methods detect the heme moiety within hemoglobin by measuring absorbance at 415 nm (aka, the Soret peak). Four heme prosthetic groups and their side chains in the globin subunits, which can directly influence the heme molecule’s ability to absorb light at a given wavelength. In fact, the distinct environments found in both α- and β-globin chains have been shown to directly affect the extinction coefficient of heme.

For most hemoglobin tetramers, this is of no consequence since they include 2 α-globin subunits per tetramer. However, HbH is a tetramer of β-globin chains and consequently has a lower extinction coefficient relative to the heme within the β-globin subunit. For hemoglobin tetramers, this is of no consequence since they include 2 α-globin subunits per tetramer. However, HbH is a tetramer of β-globin chains and consequently has a lower extinction coefficient at 415 nm than does HbA. The IEF assay used in this study detects hemoglobin using o-dianisidine (a peroxidase stain), which stoichiometrically detects the presence of iron. We speculate that the use of this stain enhances sensitivity of the IEF technique, thus enabling detection of very low concentrations of hemoglobin variants—namely, HbH less than 2%.

In summary, we confirmed that patients with 1 or 2 α-globin gene deletions have low concentrations of HbH in their RBCs, which is detectable by IEF. Most important, our results strongly support the use of IEF as a cost-effective approach to screening and diagnosis of α-thalassemia carriers.

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