Molecular Diagnosis of Thalassemias and Hemoglobinopathies

An ACLPS Critical Review

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ABSTRACT

Objectives: To describe the use of molecular diagnostic techniques for patients with hemoglobin disorders.

Methods: A clinical scenario is presented in which molecular diagnosis is important for genetic counseling. Globin disorders, techniques for their diagnosis, and the role of molecular genetic testing in managing patients with these disorders are described in detail.

Results: Hemoglobin disorders, including thalassemias and hemoglobinopathies, are among the commonest genetic diseases, and the clinical laboratory is essential for the diagnosis of patients with these abnormalities. Most disorders can be diagnosed with protein-based techniques such as electrophoresis and chromatography. Since severe syndromes can result due to inheritance of combinations of globin genetic disorders, genetic counseling is important to prevent adverse outcomes. Protein-based methods cannot always detect potentially serious thalassemia disorders; in particular, α-thalassemia may be masked in the presence of β-thalassemia. Deletional forms of β-thalassemia are also sometimes difficult to diagnose definitively with standard methods.

Conclusions: Molecular genetic testing serves an important role in identifying individuals carrying thalassemia traits that can cause adverse outcomes in offspring. Furthermore, prenatal genetic testing can identify fetuses with severe globin phenotypes.

Clinical Scenario

You are consulted by a genetic counselor who wants to know what laboratory testing you would recommend for her clients. She is advising a couple, each of whom is of Cambodian ancestry. They had a baby who has some form of α-thalassemia based on newborn screening, which detected the presence of hemoglobin Bart’s by high-performance liquid chromatography and isoelectric focusing. While neither parent is symptomatic, both are microcytic, and hemoglobin analysis demonstrated that the father is a carrier of hemoglobin E, while the mother has β-thalassemia trait (thalassemia minor).
Questions

1. What are the likely α-globin (HBA) and β-globin (HBB) genotypes for each parent?
2. What outcomes are possible in offspring from this couple?
3. What additional testing would you recommend for this couple and their child?

Hemoglobin disorders are the most frequent genetic diseases in the world, particularly in those parts of the world where malaria has been endemic. These disorders include hemoglobinopathies, which are caused by structural changes in the globin protein chains of hemoglobin, and thalassemias, which are disorders of globin expression. These disorders can result in anemia, shortened RBC life span/hemolysis, and other systemic pathology depending on the specific mutation(s) present. Diagnostic techniques include protein electrophoresis and chromatography, special RBC preparations and stains, and nucleic acid testing. In this review, I describe the diagnostic approach in laboratory testing for globin disorders with a focus on the application of nucleic acid testing.

Thalassemia

The thalassemias are diseases caused by decreased expression of one of the two globin chains of the hemoglobin molecule, α (HBA) and β (HBB). Decreased expression can result from deletion of the structural gene(s); mutations that result in decreased RNA synthesis, processing, or stability; or mutations resulting in decreased protein synthesis or stability. The decrease in expression of one of the globin chains results in accumulation of excess polypeptides encoded by the unaffected gene. This chain imbalance causes abnormal RBC maturation, resulting in microcytosis as the characteristic laboratory abnormality. Milder forms of thalassemia do not cause anemia; more severe forms can cause microcytic anemia, hemolysis, iron loading, and transfusion dependence in the most severe forms. Thalassemia is a significant public health burden in affected regions, and thus prenatal screening and genetic counseling are important in preventing the most severe forms of thalassemia.

α-Thalassemia

α-Thalassemia is caused by deletion in approximately 95% of cases, with the remaining minority due to point mutations. The HBA genes consist of two highly similar structural genes that encode identical protein chains at the far telomeric end of the short arm of chromosome 16. The most common deletions involve loss of 3.7 kb of sequence resulting in a HBA2-HBA1 fusion gene. Several different breakpoints are associated with this mutation, variously referred to as α-thalassemia or trans deletion α-thalassemia, which is likely due to unequal crossing over during meiosis. The chain imbalance caused by these deletions is relatively mild, so affected individuals may not be microcytic. Since individuals with these deletions by definition will transmit at least one HBA gene to their offspring, there is no risk of having children with a life-threatening form of α-thalassemia (hemoglobin Bart’s hydrops fetalis), which occurs due to loss of all four HBA genes. As a result, genetic testing is not necessarily indicated for the purposes of genetic counseling or prenatal diagnosis, although the definite risk of a significant α-thalassemia syndrome in offspring can only be determined by genetic testing of the prospective parents. Diagnosis of α-thalassemia is often one of exclusion: patients are microcytic with no evidence of increased HbF (hemoglobin F) and decreased HbA2 (hemoglobin A2) levels.
of iron deficiency, but no abnormal findings are present by hemoglobin electrophoresis or chromatography. There may be insufficient excess β-globin to form hemoglobin H (HbH) inclusions, but it is possible to detect the small amount of HbH present in these individuals using special gel electrophoresis and staining techniques.3

In contrast, deletion of both HBA genes on the same chromosome, referred to as α0-thalassemia or cis deletion α-thalassemia, results in a greater degree of chain imbalance, since there is a 50% decrease in HBA expression. This deletion is very common in individuals of Southeast Asian ancestry, and there are various α0-thalassemia mutations found in other populations, including Filipino, Thai, and Mediterranean. The main significance of α0-thalassemia is that couples each heterozygous for these deletions are at 25% risk of having offspring with hemoglobin Bart’s hydrops fetalis. This syndrome manifests as severe anemia and hydrops in affected fetuses, who either die in utero or are stillborn. Mothers carrying these fetuses are at increased risk for preeclampsia and other obstetrical complications.5,6 For this reason, genetic counseling and prenatal diagnosis are critical for preventing this condition.

Compound heterozygosity for α*-thalassemia and α+*-thalassemia (deletion of three of four HBA genes) is referred to as HbH disease. The significant globin chain imbalance associated with this genotype results in significant microcytosis (mean corpuscular volume [MCV] 55-60 fL), chronic hemolysis, and splenomegaly, but these individuals generally have a normal life span without significant complications. These individuals are at risk for transmission of hemoglobin Bart’s hydrops fetalis, so the same genetic counseling and prenatal diagnosis considerations apply as for those with α*-thalassemia.

A minority of α-thalassemia is due to point mutations affecting the HBA genes. As of this writing, 111 sequence variants are associated with α-thalassemia in the HbVar database (http://globin.cse.psu.edu/cgi-bin/hbvar/query_vars3, query category “Thalassemias,” a minority of α-thalassemia can resemble HbH disease phenotypically.

### β-Thalassemia

In contrast to α-thalassemia, 95% of β-thalassemias are due to point mutations that cause abnormal RNA transcription, processing or stability, or nonsense mutations resulting in production of abnormal proteins or nonsense-mediated RNA decay. Currently, 279 sequence variants are associated with β-thalassemia in the HbVar database (http://globin.cse.psu.edu/cgi-bin/hbvar/query_vars3, query category “Thalassemias,” query chain beta, last accessed November 23, 2016). β-Thalassemia can be further subdivided into β0-thalassemia, where no HBB protein is produced from the mutated allele, or β+*-thalassemia, where reduced quantities of protein are produced. Heterozygotes for either type of allele have microcytosis, clinically referred to as β-thalassemia minor. Compound heterozygotes for two β+*-thalassemia alleles or one β+ and one β0 allele have a more severe phenotype termed β-thalassemia intermedia, which includes anemia, hemolysis, iron loading, and occasional requirement for transfusion. Individuals with two β0-thalassemia alleles have the most severe form of the disease termed β-thalassemia major, resulting in transfusion-dependent anemia, severe transfusional iron loading requiring chelation therapy, and shortened life expectancy.

Laboratory diagnosis of nondeletional β-thalassemia generally relies on the detection of increased levels of hemoglobin A2, which comprises HBA and the minor adult β-like globin, HBD (δ). Many individuals with the various types of β-thalassemia also exhibit an increase in fetal hemoglobin (hemoglobin F, comprising HBA and HBG [γ-globin chains], although this is not a universal finding and depends on sequences located on chromosome 11 as well as other locations in the genome.5-8

A minority of β-thalassemia is due to deletion. Deletions encompassing the HBB gene, both the HBB and HBD genes, and the entire HBB complex have all been described.4 There are also rare mutations in which the regulatory region of the HBB locus, termed the locus control region (LCR), is deleted, resulting in no expression of the linked β-like globin genes, even though they are present and structurally normal.10,11 When the HBD gene (or the LCR) is deleted, there is no diagnostic increase in hemoglobin A2, and a diagnosis of β-thalassemia is difficult to make. Most HBB-HBD deletions are associated with significant increases in hemoglobin F, providing a clue as to the diagnosis, but if hemoglobin F is not elevated, it is difficult to distinguish deletional β-thalassemia from α-thalassemia. Molecular techniques are the only way to make this distinction.

### Hemoglobinopathies

Structural variants of the globin genes are termed hemoglobinopathies. Some variants are associated with clinical diseases such as sickle cell anemia and related sickling disorders, hemolysis due to unstable hemoglobins, hemoglobins with altered oxygen affinity, and hemoglobins in which iron cannot be maintained in the ferrous (Fe2+) state. However, most structural variants are clinically silent
and are only discovered incidentally, often during the measurement of hemoglobin A\textsubscript{c} in diabetics by high-performance liquid chromatography (HPLC) or capillary zone electrophoresis. In addition, when the amino acid change of a variant results in no difference in charge, the variant may not be detected by any chromatographic technique.

**Protein-Based Diagnostic Modalities**

**Hemoglobin Electrophoresis**

Separation of hemoglobins by various electrophoretic techniques is a mainstay of diagnosis of hemoglobin disorders; these techniques and their advantages and limitations are listed in **Table 1**. The most common electrophoretic method currently in use is isoelectric focusing, which has largely replaced (other than on proficiency testing surveys and board examinations) cellulose acetate alkaline electrophoresis. This method easily separates the most common normal hemoglobins, including hemoglobin A, hemoglobin F, and hemoglobin A\textsubscript{2} as well as the common variants hemoglobins S and C. The elevated hemoglobin A\textsubscript{2} associated with \(\beta\)-thalassemia and excess HBB chains associated with HbH disease are also visualized by isoelectric focusing. A number of other relatively common variants are difficult to resolve from hemoglobin S (eg, hemoglobin D or hemoglobin G) or hemoglobin C (eg, hemoglobin E or hemoglobin O\textsuperscript{Ku}) by isoelectric focusing, so another electrophoretic technique

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose acetate electrophoresis</td>
<td>Low cost, extensive laboratory experience</td>
<td>Low resolution, not generally used in modern laboratories, may miss (\beta)-thalassemia in newborn period</td>
</tr>
<tr>
<td>alkaline pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate agar electrophoresis</td>
<td>Can distinguish uncommon variants from hemoglobin S or hemoglobin C, low cost</td>
<td>Low resolution, high-performance liquid chromatography can usually substitute</td>
</tr>
<tr>
<td>acidic pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supravital staining with brilliant cresyl blue</td>
<td>When positive, definitive diagnosis of (\alpha)-thalassemia, semiquantitative results allow presumptive genotyping</td>
<td>May be negative in milder forms of (\alpha)-thalassemia, labor intensive</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>Good resolution, widely used, relatively low cost</td>
<td>Cannot definitively identify some variants, not sensitive for (\alpha)-thalassemia, not quantitative, may miss (\beta)-thalassemia in newborn period</td>
</tr>
<tr>
<td>High-performance liquid chromatography</td>
<td>Quantitative, good resolution, rapid, widely adopted</td>
<td>Cannot definitively identify some variants, not sensitive for (\alpha)-thalassemia, may miss (\beta)-thalassemia in newborn period</td>
</tr>
<tr>
<td>Capillary zone electrophoresis</td>
<td>High resolution, can resolve hemoglobin A\textsubscript{2} from hemoglobin E, quantitative</td>
<td>Cannot definitively identify some variants, not sensitive for (\alpha)-thalassemia, may miss (\beta)-thalassemia in newborn period</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Useful for rapidly characterizing hemoglobin variants</td>
<td>No role in diagnosis of thalassemia</td>
</tr>
<tr>
<td>DNA blot analysis</td>
<td>Can detect large deletions, specific breakpoints do not need characterization</td>
<td>Low resolution, requires specific probes, labor intensive, largely replaced by newer techniques, cannot detect point mutations (except for those affecting restriction sites)</td>
</tr>
<tr>
<td>Gap–polymerase chain reaction</td>
<td>Rapid, can be multiplexed, good for common deletions, especially in (\alpha)-thalassemia</td>
<td>Cannot detect point mutations, will miss deletions lacking specific primers</td>
</tr>
<tr>
<td>Multiplex ligase-dependent probe amplification</td>
<td>Can cover large chromosomal regions for deletion analysis, quantitative, exact breakpoints do not need characterization</td>
<td>Low resolution, cannot detect point mutations or small deletions</td>
</tr>
<tr>
<td>Comparative genomic hybridization</td>
<td>Can cover large chromosomal regions for deletion analysis, high resolution, exact breakpoints do not need characterization</td>
<td>Cannot reliably detect single (\alpha)-globin deletions due to cross-hybridization</td>
</tr>
<tr>
<td>Sanger sequencing</td>
<td>Best current method for characterizing globin variants and point mutations causing thalassemia</td>
<td>Not useful for detecting deletions</td>
</tr>
<tr>
<td>Allele-specific methodologies (allelic-specific polymerase chain reaction, reverse dot-blot, arrays, etc)</td>
<td>Useful in genetically homogeneous populations, high throughput, economical</td>
<td>Less useful in ethnically diverse populations</td>
</tr>
<tr>
<td>Next-generation sequencing</td>
<td>Has potential to characterize mutations and deletions throughout all globin genes in parallel</td>
<td>Not yet developed for this application, may be subject to problems in repetitive sequences, particularly (\alpha)-globin genes</td>
</tr>
</tbody>
</table>
or HPLC is required to definitively distinguish among these possibilities. Electrophoresis using citrate agar at pH 6.2 allows hemoglobin S or hemoglobin C to be distinguished from other variants that migrate similarly by isoelectric focusing. Even greater resolution is now possible using capillary zone electrophoresis, which can, for example, separate hemoglobin E from hemoglobin A\textsubscript{2}, something not possible with the techniques mentioned above or with HPLC.\textsuperscript{12} Capillary zone electrophoresis has the potential to replace older electrophoresis methods in many laboratories in the near future.

**HPLC**

A number of HPLC methods are available from various manufacturers that separate hemoglobin species, based on cation exchange chromatography. These methods are approved by the US Food and Drug Administration for quantification of hemoglobin F and hemoglobin A\textsubscript{2} as well as hemoglobin A\textsubscript{2\textsubscript{c}} (glycated hemoglobin) for monitoring therapy in patients with diabetes.\textsuperscript{13} HPLC allows separation of hemoglobin S and hemoglobin C from hemoglobins that have similar migration on electrophoresis. It also allows quantification of the variant hemoglobins. HPLC cannot separate hemoglobin E from hemoglobin A\textsubscript{2}. Although this is not generally of diagnostic significance, the ability to separate these two hemoglobins can be useful in distinguishing homozygosity for hemoglobin E from compound heterozygosity for hemoglobin E and \(\beta^2\)-thalassemia.\textsuperscript{14} Separation of hemoglobins E and A\textsubscript{2} can be accomplished by capillary zone electrophoresis. Other chromatographic techniques are available for separating hemoglobin species. Notably, boronate affinity chromatography is useful for separating glycated from nonglycated hemoglobin species for monitoring diabetic patients, even those with hemoglobin variants,\textsuperscript{15} but this technique has no utility for diagnosing thalassemias or hemoglobinopathies.

**Use of Electrophoresis and Chromatography for Diagnosing Hemoglobin Disorders**

Diagnosis of \(\beta\)-thalassemia is usually accomplished by demonstrating increased hemoglobin A\textsubscript{2} by electrophoresis and/or chromatography, although in unusual circumstances such as coexistent \(\delta\)-thalassemia or severe iron deficiency, the level of hemoglobin A\textsubscript{2} may not be increased. The diagnostic increase in hemoglobin A\textsubscript{2} will also not be observed in \(\beta\)-thalassemia due to deletion of the HBB and HBD genes, although hemoglobin F is often elevated,\textsuperscript{9} as seen in \(\delta\beta\)-thalassemia and hereditary persistence of fetal hemoglobin (HPFH). Protein-based techniques may also not detect the presence of \(\beta\)-thalassemia in the newborn period, since hemoglobin A\textsubscript{2} does not reach adult levels until about 6 months of age. However, thalassemia major due to homozygosity/compound heterozygosity for two \(\beta^{0}\)-thalassemia alleles would be suspected due to a lack of hemoglobin A at birth.

Hemoglobin electrophoresis and HPLC generally do not contribute to the diagnosis of the milder forms of \(\alpha\)-thalassemia, in which one or two (\textit{cis} or \textit{trans}) HBA genes are deleted. The excess HBB chains in these disorders are not abundant enough to visualize by standard techniques, partially due to the instability of HbH. HbH does form inclusions in rare RBCs, which can be visualized by supravital staining with brilliant cresyl blue,\textsuperscript{16} but this technique is not available in many laboratories. Thus, \(\alpha\)-thalassemia is often a diagnosis of exclusion, where a patient with microcytosis, normal iron studies, and normal hemoglobin electrophoresis/HPLC is presumed to have some form of \(\alpha\)-thalassemia, although methods have been described to detect the small amounts of HbH that are present in milder forms of \(\alpha\)-thalassemia.\textsuperscript{3} For these patients, molecular diagnosis may be the only means of definitive diagnosis, and this can be critical for genetic counseling. In the setting of HbH disease, where three HBA genes are deleted or where there is a combination of HBA deletions and point mutations, there is enough chain imbalance to visualize HbH by electrophoresis or HPLC.

Presumptive identification of a variant hemoglobin can be accomplished by gel electrophoresis, isoelectric focusing, HPLC, and/or capillary zone electrophoresis. Comparison with samples containing a previously documented variant can be analyzed in parallel for identification. Prior to the availability of DNA sequencing, hemoglobin variants were characterized by mass spectrometry of proteolytic global peptides.\textsuperscript{17,18} Although DNA sequencing is the most common means of identifying variant hemoglobins, newer techniques of mass spectrometry offer the prospect of rapidly identifying hemoglobin variants based on the mass of the intact molecule or defined globin fragments.

**Molecular Diagnostic Techniques for Globin Disorders**

**\(\alpha\)-Thalassemia**

Since most \(\alpha\)-thalassemias are due to deletion of one or more HBA genes, and since these deletions are

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**Sabath / Hemoglobin Disorder Molecular Diagnosis**

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common in the affected populations, molecular diagnostic techniques are focused on demonstrating these common deletions. DNA blot analysis has been used in the past, but polymerase chain reaction (PCR) methods using gap-PCR are most commonly used currently.\(^{10}\) PCR primers that flank the common breakpoints can demonstrate deletions by generating PCR products of specific sizes. The specific primers used in a given laboratory depend on the common deletions in the population being served. Several comprehensive sets of PCR primers to detect most HBA deletions have been described.\(^{19}\)

An alternative technique for characterizing deletions is multiplex ligation-dependent probe amplification (MLPA).\(^{20}\) This is a technique that can quantify gene copy numbers and thus can detect gene deletions based on reduction in gene dosage. In MLPA, a series of oligonucleotide probe pairs that anneal to DNA in adjacent sites is designed to cover the chromosomal area of interest at convenient intervals. When the oligonucleotides anneal to their target sequences, DNA ligase will covalently link the probes that anneal at adjacent sites, and the resulting ligated sequences are amplified by PCR. The quantity of PCR product is proportional to the target copy number, so deleted regions are identified by decreased signal for a given probe pair. Unlike with PCR, where the exact chromosomal breakpoint is required to design PCR primers, MLPA is capable of detecting unusual deletions that have not been previously described or are not covered by whatever primer sets a given laboratory uses based on its patient population. This technique has been used successfully to characterize known and novel deletions of the HBA locus that cause α-thalassemia, although MLPA had difficulty in consistently detecting trans HBA deletions.\(^{21}\)

In our laboratory, we have experimented with using array-comparative genomic hybridization (array-CGH) to characterize globin gene deletions. By creating a custom oligonucleotide array that densely tiles the HBA locus, we were able to detect heterozygotes for the common Filipino and Southeast Asian cis HBA deletions based on a 50% reduction in signal relative intensity in the deleted regions. The array-CGH method can also detect heterozygotes with the single-gene (trans) –α\(^{3.7}\) and –α\(^{4.2}\) deletions based on a reproducible pattern of array-CGH results, although the results are difficult to interpret a priori due to cross-hybridization between HBA1 and HBA2 sequences.\(^{22}\) Others have used a similar approach.\(^{23,25}\)

For nondeletional α-thalassemia, (Sanger) DNA sequencing is the most comprehensive method to detect all mutations to avoid having to design mutation-specific assays. Allele-specific PCR has a role in areas where certain mutations are common, such as hemoglobin Constant Spring in Southeast Asia.\(^{26}\) A reverse dot-blot strategy has also been described that can detect common deletions and point mutations.\(^{27}\) Sanger sequencing of HBA genes is somewhat complex methodologically since the two HBA genes on chromosome 16 are nearly identical for a stretch of over 1 kbp that includes the 5' and 3' flanking regions, coding sequences, and introns. To selectively amplify the HBA1 and HBA2 genes, one needs to use sequences at least 1,000 base pairs 5' to the structural genes to design specific PCR primers for each HBA gene. These 5' primers can be combined with a common 3' primer. In addition, the HBA sequences are relatively guanine-cytosine rich, so standard PCR conditions need to be optimized to efficiently amplify these sequences.\(^{19}\) Once successfully amplified, the PCR products can be sequenced directly using standard Sanger methods. Sequences obtained can be compared with those in the HbVar database to determine clinical significance (http://globin.bx.psu.edu/hbvar/menu.html).

**β-Thalassemia and HPFH**

Since most β-thalassemias are due to point mutations, Sanger sequencing is the most practical current method to detect all possible mutations in an individual.\(^{28}\) DNA sequencing of the HBB gene is less technically complex than that for the HBA genes, since PCR amplification is easier due to there being only a single HBB gene on each chromosome 11 and since the sequence is less guanine-cytosine rich than the HBA genes. A number of different PCR and sequencing strategies have been described.\(^{29}\)

Since some populations have a relatively small number of mutations causing β-thalassemia, a number of methods targeting a limited set of mutations have been developed that are less expensive and allow higher throughput than Sanger sequencing. Many of these more ethnically homogeneous populations are in resource-limited regions of the world, so more cost-effective methods are badly needed. Reverse dot-blot methods, in which biotinylated patient DNA is hybridized to membranes on which mutation-specific oligonucleotides are immobilized and is then detected by enzyme-linked streptavidin, can be more economical than direct sequencing and can screen for multiple mutations.\(^{30,31}\) Rather than using membranes, oligonucleotides can also be applied to an array substrate and used in a similar fashion.\(^{32}\) Another strategy is to use allele-specific PCR to detect specific mutations, as described for common Southeast Asian and Indian mutations.\(^{33}\) An approach casting a wider net to detect mutations is heteroduplex analysis using denaturing HPLC, which can distinguish various mutations based on the chromatographic characteristics of PCR products containing various HBB mutations, either as homoduplexes in the homozygous state or as heteroduplexes in
When to Use Molecular Testing for Patients With Hemoglobin Disorders?

Patients suspected of having hemoglobin disorders should initially be screened with a CBC to detect the presence of anemia with or without microcytosis. Regardless of MCV, patients should have hemoglobin analysis by isoelectric focusing, capillary zone electrophoresis, and/or HPLC to detect hemoglobin variants and to quantify hemoglobin F and hemoglobin A\textsubscript{2}. Microcytic patients should in addition have measurement of iron supply parameters (serum iron, iron binding capacity, ferritin, etc) and detection of HbH inclusions by brilliant cresyl blue staining if available.

Depending on the results and whether there are genetic counseling issues to consider, molecular testing for globin abnormalities may be indicated. An algorithm for deciding whom to test and what molecular tests to perform is illustrated in Figure 2, and common presentations and suggested diagnostic approaches are listed in Table 2. Patients with ambiguous hemoglobin testing results or unusual phenotypes are candidates for DNA testing. Couples seeking genetic counseling should be offered DNA testing when both prospective parents are suspected of having cis \(\alpha\)-globin deletions or both have \(\beta\)-thalassemia traits. In addition, it should be kept in mind that individuals with \(\beta\)-thalassemia trait or hemoglobin E trait may have coincident \(\alpha\)-thalassemia trait that is masked by the microcytosis associated with these \(\beta\)-globin disorders, and thus \(\alpha\)-globin DNA testing should be performed to evaluate risk to offspring of hemoglobin Bart’s hydrops fetalis. Prenatal genetic testing should be offered to couples at risk for producing offspring with clinically severe hemoglobin disorders.

Conclusions

Hemoglobinopathies and thalassemias remain a worldwide cause of morbidity and mortality.\(^1\) Although historically limited to regions with endemic malaria, individuals with hemoglobin disorders now live throughout the world, and laboratories in regions previously unaffected by malaria, such as North America, northern Europe, Japan, and Australia, are now called upon to diagnose hemoglobinopathies and thalassemias. In this review, I have summarized the current standard diagnostic methods as well as more novel molecular techniques that have recently become available. One challenge in this area is that much of the disease burden of hemoglobinopathies and thalassemias is in parts of the world with limited resources for health care. Some of the methods that focus on mutations that are frequent in a given region may allow relatively inexpensive diagnoses, but additional work remains to provide molecular diagnostic techniques for these populations.

There are also different considerations for pediatric diagnostic laboratories, where the focus is generally on making diagnoses for children with abnormal newborn screens or unexplained anemias vs adult diagnostic laboratories, where genetic counseling is often the focus in addition to making new diagnoses. Molecular diagnostic techniques are less commonly needed in the pediatric setting, whereas these techniques are frequently critical in the setting of genetic counseling and prenatal diagnosis.

The next advance in molecular diagnostics for hemoglobin disorders will be next-generation sequencing. There will be some technical challenges in implementing next-generation sequencing, especially with respect to the \(HBA\) genes, which, because of the nearly identical sequence between the \(HBA1\) and \(HBA2\) genes, will make it challenging to determine whether a given mutation belongs to one or the other of these genes. Aside from this challenge however, various strategies now in place for next-generation sequencing, including hybrid capture and whole-genome

heterozygotes.\(^{35-37}\) Alternatively, similar results can be obtained using single-stranded conformational polymorphism analysis.\(^{38}\) An analogous method for detecting \(\beta\)-thalassemia mutations that screens PCR products for base substitutions is high-resolution melting curve analysis.\(^{39}\) As another way to detect multiple mutations simultaneously, a method using melting curve analysis was developed to detect 24 \(\beta\)-thalassemia mutations common in the southern Chinese population using a multiplexing strategy that allows numerous mutations to be detected in a single tube.\(^{40}\) A completely alternative method involves resolving DNA fragments by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.\(^{41,42}\)

Deletions of the \(HBB\) locus that cause \(\delta\beta\)-thalassemia or HPFH, as well as rarer deletions, can be detected by gap-PCR if the deletion breakpoints are known.\(^{43}\) Strategies using MLPA have also been described\(^{20,44}\) using similar strategies as those for the \(HBA\) locus. More recently, our laboratory and others have demonstrated the utility of array-CGH for characterizing deletions of the \(HBB\) locus.\(^{22-25}\) Because these arrays use probes spaced at short intervals throughout the locus, it is possible to finely map deletion breakpoints, design PCR primers to amplify the breakpoint region, and determine the sequences flanking the breakpoints. We used this technique to confirm the breakpoints for several known mutations causing \(\delta\beta\)-thalassemia and HPFH as well as previously unmapped deletions.\(^{22,23}\)
approaches, should be applicable. It is my hope that the cost of these technologies will eventually decrease enough to make them available to resource-limited settings where diagnosis of hemoglobin disorders will be most valuable.

Case Summary

Since this couple had a child with α-thalassemia, one or both must be carriers for this condition. Since both parents have a β-globin phenotype that would mask an underlying α-thalassemia, molecular testing is required to make a diagnosis and to determine risk to future offspring. Being of Southeast Asian ancestry, the most likely genotype is the common Southeast Asian cis α-globin deletion (α0-thalassemia). α-Globin genotyping by gap-PCR would be the most cost-effective way to determine this couple’s α-globin genotypes and risk to future offspring. Any offspring who coinherit one of the parent’s β-globin disorders and a cis α-globin deletion would not be expected to

Table 2
Common Presentations With Genetic Counseling Implications, Risks for Offspring, and Suggested Workups

<table>
<thead>
<tr>
<th>Prospective Parent With</th>
<th>Consider in Partner</th>
<th>Risk to Offspring</th>
<th>Workup for Partner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin S trait</td>
<td>Hemoglobins S, C, E, D; Qζ trait; β-thalassemia trait</td>
<td>Clinically significant sickling disorder</td>
<td>Hemoglobin analysis (HPLC and/or IEF), HbA2 quantification</td>
</tr>
<tr>
<td>Hemoglobin C, E, D or Qζ trait</td>
<td>Hemoglobin S trait</td>
<td>Clinically significant sickling disorder</td>
<td>Hemoglobin analysis</td>
</tr>
<tr>
<td>α-thalassemia trait</td>
<td>α-s or α-thalassemia trait</td>
<td>Hemoglobin H disease, hemoglobin Bart’s hydrops fetalis</td>
<td>Hemoglobin analysis, BCB inclusion body study; α-globin deletion analysis if inconclusive</td>
</tr>
<tr>
<td>β-thalassemia trait (cannot exclude coincident α-thalassemia trait)</td>
<td>α and/or β-thalassemia S trait</td>
<td>β-Thalassemia intermedia or β-thalassemia major, hemoglobin Bart’s hydrops fetalis, clinically significant sickling disorder (with S trait)</td>
<td>Hemoglobin analysis, BCB inclusion body study; α-globin deletion analysis for both parents if inconclusive</td>
</tr>
<tr>
<td>Hemoglobin E trait (cannot exclude coincident α-thalassemia trait)</td>
<td>α and/or β-thalassemia S trait</td>
<td>Hemoglobin Bart’s hydrops fetalis, E/β0-thalassemia, clinically significant sickling disorder (with S trait)</td>
<td>Hemoglobin analysis, BCB inclusion body study; α-globin deletion analysis for both parents if inconclusive</td>
</tr>
</tbody>
</table>

BCB, brilliant cresyl blue; HbA2, hemoglobin A2; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing.
have a syndrome any more severe than either thalassemia trait alone. Since the father has hemoglobin E trait and the mother has some type of β-thalassemia trait, it is important to determine the specific β-thalassemia mutation that the mother carries, since if it is a β⁰-thalassemia mutation, there is a 25% chance that future offspring would be compound heterozygotes for hemoglobin E/β⁺-thalassemia. This would result in a thalassemia intermedia phenotype, with iron loading with possible transfusion dependence. The mother’s genotype would generally be determined by β-globin gene sequencing. Finally, depending on the α- and β-globin genotypes of the parents, it is even possible that future offspring could have the combination of hemoglobin E/β⁺-thalassemia/HbH disease, which can result in a severe transfusion-dependent thalassemia. Thus, the recommendation would be for both parents to have α-globin DNA analysis with additional β-globin DNA sequencing for the mother. Since newborn screening by hemoglobin analysis would not determine the child’s α-globin genotype, α-globin DNA analysis could be performed on the child to determine likely outcomes. Alternatively, the child could be evaluated at age 8 to 9 months to determine the thalassemia phenotype, from which one could possibly infer the α-globin genotype. Prenatal genetic testing would allow the parents to determine possible adverse outcomes in future pregnancies.

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References


