Molecular Diagnostic Challenges of the Thalassemias

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I. INTRODUCTION AND DEFINITIONS

Adult hemoglobins are tetramers of two alpha and two beta globin chains held together by four heme groups, one on each chain. The main function of hemoglobin is to transport oxygen from the lungs to peripheral tissues, where it exchanges oxygen to carbon dioxide for delivery to the lungs. Globin chains are differentially expressed at different stages of development from distinct genes and as a result, give rise to various hemoglobins in embryonic, fetal and adult life. In early embryogenesis, expressions of the epsilon (ε) and zeta (ζ) globin chains result predominantly in hemoglobin (Hb) Gower 1 (ζ2ε2), whereas in the late embryo/early fetus, co-expression of the alpha (α) and gamma (γ) chains produce Hb Gower 2 (α2ε2) and Hb Portland (ζ2γ2), respectively. In most of the fetal stage, expression of α and γ chains result in Hb F (α2γ2), which has a significantly high oxygen affinity to ensure adequate oxygen transport across the placenta during fetal development. In the late fetal stage, at around 30 weeks of gestation, expression of the γ chains gradually declines and expression of the β chains start to rise, resulting in predominant synthesis of β chains and very little synthesis of γ chains by 12 months after birth. On the other hand, the α chains remain highly expressed throughout fetal and adult life. Hemoglobin A (Hb A), the predominant type of hemoglobin in the adult is made up of 2 α chain and 2 β chains (α2β2). Although the δ and γ globin chains are also expressed in adulthood, albeit at very low levels, their resulting respective hemoglobins, Hb F and Hb A2 are typically found at around 1% and 3.5%, respectively, thus constituting a small percentage of overall adult hemoglobin. Circulating levels of Hb A, Hb A2, and Hb F are constant throughout life; however, the levels of Hb A may be decreased and those of Hb A2 and Hb F could increase, thus pointing to particular clinical situations.

The thalassemias are an autosomal recessive group of inherited disorders that result in the reduced synthesis of one or more of the globin chains of a specific hemoglobin. Unlike the β-globin genes, which are biallelic, the α and γ genes are duplicated in humans, such that there are 4 γ and 4 α genes in a diploid cell. Unlike hemoglobin variants, which produce structurally abnormal hemoglobins as in sickle cell anemia (Hb S), the structural integrity of hemoglobins is maintained in the thalassemias; however, the synthesis of a specific chain is reduced. As a result, the reduced synthesis of α or β globin chains alters the balance of the chains in Hb A. Thus, lack of β-chain expression, called β0-thalassemia, results in the formation of α4 rather than α2β2 tetramers due to the inability of the α chains to pair with their complementary β chains. Similarly, in α-thalassemia, β4 tetramers form, resulting in Hb H.

The molecular basis of the thalassemias has been exhaustively elucidated and represents one of the first diseases to be characterized at the molecular level. Mutations in thalassemia alter the function of the genes encoding a globin chain and these may carry point mutations, small insertions, deletions, or, in some cases, partial or large deletions encompassing one or two globin genes. For example, in β0-thalassemia, mutations result in the absence of β-chain synthesis and these may be caused by β-globin gene deletions or more commonly by subtle mutations such as nonsense, frameshift, or RNA-splicing mutations. In β+ thalassemia (where some β chain synthesis occurs), mutations in the β-globin genes may be located in promoter regions (resulting in defective RNA polymerase binding sites), at exon-intron boundaries (disrupting splice site recognition), within introns (generating cryptic splice signals) or farther
A. Postnatal Samples

The most common type of sample submitted for DNA analysis is peripheral blood, which is collected in a 2–3 ml small purple- or lavender-top tube that contains EDTA as the anticoagulant. Green-top tubes containing heparin as the anticoagulant should be avoided since heparin interferes with the activity of some restriction endonucleases and is a known inhibitor of Taq DNA polymerase, the enzyme used for polymerase chain reaction (PCR) amplification.

Other non-blood-based samples are mouthwashes or brush cheek swabs, both of which are aimed at sampling buccal epithelial cells. These types of samples are particularly useful for newborns, where blood sampling is difficult, or in situations where a patient has been freshly transfused. These samples are usually stable at room temperature for a few days and are still adequate for DNA extraction.

B. Prenatal Samples

For samples obtained by amniocentesis: two separate 15–20–ml amniotic fluid samples will be needed—one for direct DNA assays using amplification methods such as PCR and a second one for establishing a backup culture of amniocytes. It is critical that amniotic fluid samples be free of maternal blood or fibroblasts contamination. These are best avoided by careful inspection for the presence of blood in the aspirated amniotic fluid and by discarding the first sample withdrawal, which will contain maternal skin fibroblasts. Although an amniocyte's culture will be essentially free from maternal blood contamination due to the failure of white blood cells to attach to the culture flask, fibroblasts from maternal skin may seriously contaminate fetal amniocyte cultures. It is important to ensure that the first draw of amniotic fluid is discarded and that the physician sends to the laboratory only the second or subsequent draws.

For samples obtained by chorionic villi sampling (CVS): a 1–2–mm sample of tissue is adequate for PCR-based assays and a similar amount for establishing a backup culture. The importance of “cleaning” the villi from potentially contaminating maternal tissue is primordial, and a villi sample submitted for prenatal diagnosis should indicate whether the sample has been cleaned under a microscope by an expert technician. A maternal blood sample should also be submitted, along with the fetal sample for maternal cell contamination studies in either amniocentesis or CVS sampling. Maternal cell contamination is relatively more of a critical issue for CVS samples than for amniocentesis, because the source of maternal DNA contaminant from CVS samples is tissue-based as opposed to blood in amniocentesis, provided the first amniocentesis draw has been discarded due to contaminating maternal skin fibroblasts.

DNA extraction from peripheral white blood cells can be performed manually or by automated instruments such as the Qiagen DNA extractor. Manual methods that use white blood cells obtained from 6–7 ml of blood generate a larger amount of genomic DNA than automated methods that require only 200 μl of whole blood. Depending on applications, the automated method generates enough DNA to perform a few PCR-based assays. However, the DNA yield from the manual method is much larger and is ample for multiple Southern blot assays. The manual method is based on the selective lysis of red blood cells and centrifugation of the white blood cells, which are then lysed and digested with Proteinase K to liberate the DNA from DNA-binding proteins. The mixture is then either ethanol-precipitated or used as such in restriction endonucleases digests or for PCR amplification. Tissue samples and adherent cells recovered from amniocytes and villi cultures are processed with the manual DNA extraction method.

II. SAMPLE COLLECTION AND PREPARATION

A. Postnatal Samples

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III. ANALYTICAL TECHNIQUES

There are multiple techniques that are aimed at the detection of mutations in human DNA. Although the gold standard is DNA sequencing, this approach remains too cumbersome and expensive at the present time to be routinely performed in a molecular diagnostics laboratory. However, high throughput and decreasing costs of DNA sequencing may one day shift all molecular diagnostics
toward DNA sequencing. Until then, DNA sequencing is performed on selected samples when other screening assays turn out to be non-informative and the suspicion of an underlying mutation is high enough based on hematological data or family history. Generally, amplification-based assays that rely on the PCR are preferred for their simplicity and rapid turnaround time, while others that are based on Southern blotting require elaborate techniques but can be more valuable than PCR in particular systems, such as in the screening for large deletions.

A. β-thalassemia

For the β-thalassemia syndromes, our laboratory uses three molecular-based approaches to uncover mutations in β-thalassemia:

1. PCR followed by an allele-specific reverse dot blot (RDB) hybridization system to screen for 42 deleterious β-globin point mutations, including common structural hemoglobin variants such as Hb S, Hb C, and Hb E (Cai et al., 1994; Maggio et al., 1993). The assay is based on the PCR amplification of genomic DNA using 5' end-labeled biotinylated primers that amplify different segments of the β-globin gene where the mutations of interest lie. The resulting biotinylated PCR products are then hybridized to nylon-based macroarray membranes with covalently bound normal and mutant allele-specific oligonucleotide (ASO) probes for each mutation. After brief washings followed by conjugation of the membrane with anti-avidin coupled to horseradish peroxidase (HRP), the hybridized PCR products are visualized through a color reaction using tetramethylbenzidine (TMB) as a substrate for HRP (Fig 36.1A).

2. A follow-up approach to PCR and RDB is the detection of common deletions in the β-globin gene. This assay is performed by PCR amplification of three frequently encountered deletional breakpoints: the Asian Indian 619-bp deletion, the Black 1.4-Kb deletion, and the Filipino 118 kb deletion (known as 45 kb deletion). The products of the amplification reactions are then identified by polyacrylamide gel electrophoresis (Fig 36.1B).

3. DNA sequencing of the β-globin gene is used when both assays described above are negative. In this assay, DNA sequencing encompasses the entire β-globin gene of genomic DNA showing T to C substitution at intron 2, nucleotide 837. This mutation generates a cryptic splice site that alters normal splicing of β-globin mRNA.

**FIGURE 36.1** Molecular diagnosis of β-thalassemia. (A) Reverse dot blot hybridization of a panel of 42 mutations in the β-globin gene represented with normal and mutant allele-specific probes. Some adjacent mutations share the same normal probe. Four representative strips, each from a different individual, show a distinctive genotype consisting of either negative (hybridization to normal probes only), heterozygous (hybridization to a mutant probe and its corresponding normal probe), compound heterozygous (hybridization to two mutant probes and their corresponding normal probes), and homozygous mutant (hybridization to a mutant probe and not to its corresponding normal probe). (B) Detection of three different β-globin gene deletions by PCR and gel electrophoresis to identify the breakpoint fragment associated with each deletion in heterozygous and homozygous individuals. The 619-bp, 1.4-Kb, and 118-Kb deletions are found in Asian Indians, African-Americans, and Filipinos, respectively. (C) DNA sequencing of the β-globin gene showing heterozygosity for a T to C substitution at intron 2, nucleotide 837. This mutation generates a cryptic splice site that alters normal splicing of β-globin mRNA.
from the promoter region to the polyA site. We use a four-dye dideoxy nucleotides terminator fluorescent technology with four overlapping sets of sequencing reactions in each 5' and 3' directions to cover the entire gene in both orientations. Capillary electrophoresis is then used to fractionate the DNA sequencing products, which are detected by laser scanning. Base calling is performed by software analysis and the data analyzed by comparison to a reference DNA sequence and a database of known β-thalassemia mutations. A report is generated outlining the polymorphisms and mutations detected in each sample. Fig 36.1C shows DNA sequencing patterns obtained from the identification of a mutation at nucleotide 837 in intron 2 of the β-globin gene in an individual heterozygous for this mutation.

B. α-thalassemia

Most of the mutations that strike α-globin gene function are deletions of one to four α-globin genes per diploid genome. They occur as a result of misalignment of the α2 and α1 globin gene regions followed by crossing over of the misaligned chromosomes. This is a process that usually does not occur de novo but has occurred multiple times in the population and thus, chromosomes representing one α-globin gene deletion and α-gene triplication segregate readily in the population. In addition, α-globin point mutations appear sporadically worldwide but may be prevalent in selected inbred populations.

There are two major types of single α-globin deletions that occur worldwide. These are termed rightward and leftward deletions due to the relative positions of their chromosomal crossing-over regions. Rightward and leftward deletions are denoted as α3.7 and α4.2 because they result, respectively, in the deletion of 3.7 kb and 4.2 kb of DNA as a result of the chromosomal cross-over (Higgs et al., 1984). Three other common types of α-globin gene deletions, termed the Southeast Asian (SEA), Filipino (FIL), and Thai, each remove two α-globin genes from the same chromosome (referred to as cis deletion). Interaction of the SEA, FIL, or Thai deletions with either the α3.7 and α4.2 deletions or even with a point mutation usually in the α2 globin gene, results in Hb H disease. Thus, different molecular based assays are used to detect deletional and non-deletional forms of α-thalassemia.

For the deletional forms, we have used for over 15 years, genomic Southern blot assays with DNA double-digested with Bgl II and Asp718 (Lebo et al., 1990). As hybridization probes, we combine digoxigenin labeled α and ζ globin cDNA probes and follow up with chemiluminescence detection using anti-digoxigenin coupled with alkaline phosphatase (Fig 36.2A). As an adjunct to the Southern blot, we use PCR to amplify the deletional breakpoint for each type of α-globin deletion (Chong et al., 2000; Liu et al., 2000) (Fig 36.2B). We found that occasionally, PCR will miss detecting some forms of the α3.7 deletion, presumably due to DNA polymorphisms under a PCR primer (allelic dropout) and thus Southern blotting remains our method of choice.

For α-globin point mutations, a PCR/RDB system with specific normal and mutant probes is currently our preferred method for the detection of three α-globin point mutations, Hb Constant Spring, Hb Quong Sze, and Hb Pakse, each of which produces a structurally abnormal α-chain variant that may interact with deletional types of α-thalassemia (Fig 36.2C), especially in Southeast Asian populations. Other α-globin point mutations may be uncovered by DNA sequencing (Fig 36.3).

C. δβ-thalassemia and HPFH

Elevated levels of fetal hemoglobin in adults may be caused by the presence of either δβ-thalassemia or HPFH). These conditions result from a variety of deletions in the β-globin gene cluster. Therefore, short of cloning the various breakpoints to devise a PCR screening strategy, restriction endonuclease mapping by Southern blot hybridization remains the best alternative to screening for these types of deletional breakpoints. Due to the fact that restriction endonuclease mapping may also generate unanticipated DNA fragments caused by single nucleotide polymorphisms within the restriction endonuclease recognition site, it is imperative that multiple restriction enzymes be used in this screening assay such that a restriction fragment indicative of a deletional breakpoint could be detected with at least 2–3 different restriction enzymes. Using this strategy, our laboratory screens for potential δβ-thalassemia/HPFH deletions by using patient and negative control genomic DNAs cleaved individually with Bam HI, Bcl I, Bgl II, Eco R I, Sac I, or Xba I. The blots are then hybridized with a specific probe, washed, exposed, and the probe stripped off to rehybridize with another probe. Using this approach, we hybridize the same Southern blot sequentially with δ-, δβ-, or γ-globin gene specific probes. A representative Southern blot with a deletion breakpoint in the δ-globin gene is shown in Fig 36.4.

D. Prenatal Diagnosis

It is important to note that the real value of DNA testing for the thalassemias is in prenatal diagnosis (PND). Postnatally, many types of thalassemias can be diagnosed with different hematological parameters coupled with hemoglobin electrophoresis and family history.

The most critical issue in any type of PND is maternal cell contamination (MCC), especially when a fetus is found to be heterozygous for a particular mutation and could be
FIGURE 36.2 Detection of α-globin deletions and point mutations. (A) Chemiluminescence lumigraphs of Southern blots from genomic DNA doubly digested with Bgl II/Asp 718 and hybridized to a mixture of α and ζ-globin probes or with the LO probe, which is located in the upstream region of the ζ-globin gene. The LO probe will detect the Filipino and Thai deletions, while the α and ζ-globin probes will detect the −α3.7, −α4.2 and Southeast Asian (sea) deletions. Bands representative of the α2, α1, and ζ-globin genes are indicated. α-thalassemia patients with various genotypes and deletions are indicated and noticeable by their corresponding breakpoint fragment, appearing in either heterozygous or homozygous states. Each lane corresponds to a different individual. (B) PCR amplification of specific α-globin gene deletional breakpoints and detection with a Cyber Safe DNA gel stain following fractionation by electrophoresis on a 1.5% agarose gel. The gel shows an incremental 500-bp size marker (M), a normal individual (lane 1), and individuals (lanes 2–8) heterozygous for a different deletion, as denoted by their corresponding genotypes next to the gel. The normal α2 and α1 PCR amplicons are 1.8kb and 0.92kb, respectively. Each amplification reaction includes two amplicons, which serve as internal controls (IC) for PCR amplification. (C) Reverse dot blot hybridization coupled with HRP-peroxidase detection in the α2-globin gene of three common point mutations causing α-globin structural variants, Hb Constant Spring (codon 142, TAA (Stop) → CAA (Gln)), Hb Pakse (codon 142, TAA (Stop) → TAG (Tyr)) and Hb Quong Sze (codon 125, CTG (Leu) → CCG (Pro)). Each mutation is represented by a normal (N) and a mutant (M) allele-specific oligonucleotide probe. The Hb Constant Spring and Hb Pakse mutations share the same normal probe as they both occur at the α2-globin termination codon. An internal control probe (Cntrl) is included on the strip as evidence of PCR amplification. Each probe array shown here is from a different patient, whose α-globin genotype is indicated next to the strip.

FIGURE 36.3 (A) DNA sequencing of the α2-globin gene of a normal individual and of an α-thalassemia individual heterozygous for an insertion/duplication at nucleotide 118 of intron 2, resulting in ambiguous DNA sequence readout, a typical pattern generated by heterozygosity for small insertions and deletions. The arrows point to the site of the insertion and the beginning of sequence ambiguity. (B) DNA sequence of a normal individual and of an individual who carries the SEA deletion on one chromosome and the single nucleotide deletion (−C) at codon 78 (AAC) of the α1-globin gene on the other chromosome. Due to the presence of the SEA deletion, the DNA sequence appears similar to that of a homozygous (−C) deletion. The site of codon 78 is boxed on the DNA sequence and the C deletion is evident in the α-thalassemia patient.
at risk for homozygosity of the same mutation. Although MCC studies can be performed selectively on heterozygous fetuses, the best practice is to perform it on all prenatal samples. The assay is based on detecting genetically informative variable number of tandem repeats (VNTRs) between maternal and fetal DNA (Batanian et al., 1998) and in our laboratory, the assay consists of screening maternal and fetal DNA with 16 pentanucleotide markers that have over 90% heterozygosity in the general population. The assay is based first on finding informative markers between mother and fetus and then determining whether the fetus carries an additional maternal “allele” indicative of maternal cell contamination (Fig 36.5). The next issue is to determine whether any amount of maternal cell contamination could actually interfere with the molecular diagnosis of the disorder being tested for. Each laboratory should assess this crucial information during their test development in order to determine the level of contamination that could for example, result in the misdiagnosis of a homozygous fetal sample into a heterozygous as a result of contaminating maternal DNA. As an example, in our laboratory, the RDB system for β-thalassemia will tolerate up to 5% maternal DNA contamination before showing evidence that homozygosity for a particular mutation will appear as heterozygous.

**FIGURE 36.4** Lumigraph of a Southern blot hybridized with the β-globin intron 2 DNA probe. Lanes correspond to DNA from a normal individual (N) or from a patient (P) heterozygous for β-thalassemia, each digested with a different restriction enzyme. Comparison of normal and patient DNA reveals an additional band with each restriction digest. The extra band is indicative of a deletional breakpoint band in the β-globin gene. The arrows point to the sizes (in kilobases) of the λHind III DNA marker.

**FIGURE 36.5** Detection of maternal DNA contamination in a fetal chorionic villi sample. The electrophoregrams show comparisons of allelic profiles of three markers on chromosomes 12, 13, and 18 between maternal and fetal DNA. Maternal (M) and paternal (P) alleles are identified by comparison of maternal and fetal DNA profiles. A small amount of maternal contamination (C) is evident with each marker.
A common question that arises in the PND of thalassemia relates to the severity of the upcoming disease in an affected fetus. For example, the severe genotypes of \( \beta \)-thalassemia are well known based on the known deleterious nature of each mutation; however, their interactions with \( \alpha \)-thalassemia are not clinically well characterized. Thus, genetic counseling about the termination of pregnancy of a homozygous \( \beta \)-thalassemia fetus with one or two \( \alpha \)-globin gene deletions is a slippery slope despite the fact that one could predict a milder course of the anemia.

### IV. RECOMMENDATIONS/LIMITATIONS OF AVAILABLE METHODS

Every technique used in molecular diagnostics has limitations and it is critical not only to recognize these limitations but also to inquire about them in a clinical context. It is also important that molecular techniques be aimed at directly interrogating the site of a mutation as opposed to determining the effect of a putative mutation on an amplification, such as in meting profile identification, denaturing gradient gel electrophoresis, and single-strand conformation polymorphisms. These research methods are best used for mutation screening purposes, but eventually a direct method needs to be used to directly identify the site and nature of a mutation.

In screening for \( \beta \)-thalassemia point mutations, arrays of specific mutations are usually not complete for all the mutations in the most-at-risk populations, such as those present in the Mediterranean region, Southeast Asia, or Africa. Follow-up assays such as DNA sequencing may be able to rule out or uncover additional mutations in the \( \beta \)- or \( \alpha \)-globin genes.

Furthermore, PCR-based assays need to ascertain that the PCR primers will not result in allelic dropouts, which would then amplify one instead of two alleles. Such a situation could arise if a mismatch occurs between a PCR primer and genomic DNA as a result of a DNA polymorphism near or at the 3’ end of the primer, resulting in a failure of DNA amplification. Such situations are best solved either by assessing the PCR primers or by using neighboring sets of PCR primers to amplify the same target sequence.

Allele-specific oligonucleotide probe-based assays, which are common for detecting point mutations in many genetic diseases, are also susceptible to single nucleotide polymorphism (SNP) variation, which could result in a failure of probe hybridization, resulting in false negatives. Although it is quite difficult to ensure that each probe in a complex array of mutations will not be subject to a SNP failure of hybridization, the validity of the results are best interpreted in light of clinical information and/or by following up with a different assay such as DNA sequencing, if necessary.

Southern blot-based assays for the screening of deletional breakpoints in \( \delta \beta \)-thalassemia or HPFH are not as sensitive to SNP variation as allele-specific probe assays, since typically multiple restriction enzymes are used in the assay. However, restriction endonuclease mapping by Southern blots relies on the juxtaposition of a common restriction endonuclease enzyme site brought by the deletion in proximity to another restriction site located in the non-deleted segment of the area of interest, thus generating the breakpoint fragment. This approach, while extensively characterized for \( \alpha \)-globin gene deletion screening, may not lead to the detection of deletional breakpoint fragments if only 2–3 restriction enzymes are used, such as for the deletional types of \( \delta \beta \)-thalassemias and HPFH.

### V. SPECIAL CONSIDERATIONS IN QUALITY MANAGEMENT

In clinical testing, laboratory assays should be validated at least twice per year by participation in proficiency testing programs either through a professional organization (such as the College of American Pathologists, CAP) or via inter-laboratory testing programs. For the thalassemias, genetic testing is limited in the United States to a handful of commercial and academic laboratories. Thus, physicians requesting thalassemia testing ought to ensure that the laboratory performing these types of tests is certified by appropriate regulatory agencies actively engaged in proficiency testing and has a deep expertise and knowledge to interpret complicated thalassemia genotypes. For home-brewed assays, laboratories should regularly maintain their tests by ensuring, for example, the reliability of their assay platforms (e.g., \( \beta \)-thalassemia RDB strips), which contain the allele-specific probes, and by periodic testing for the integrity of biotin on PCR primers. Benchmarks for turnaround time ought to be continuously monitored, since PND is a critical component of thalassemia molecular testing.

### VI. REPORTING, INTERPRETATION, AND CLINICAL INDICATION

Reports of \( \alpha \)-, \( \beta \)-, or \( \delta \beta \)-thalassemia molecular genetic tests can be challenging and complex, due to their molecular heterogeneities, their potential interactions together, and with common structural variants such as Hb S, Hb C, and Hb E. Typically, reports should indicate the limitations of the test, the clinical significance of any detected mutation, and whether in prenatal diagnostic settings, interfering maternal DNA could obscure the fetal diagnosis. Moreover, the report should point out whether the failure to detect a mutation in a postnatal individual is consistent with the hematological findings that originally warranted molecular genetic testing for thalassemia.
A. β-thalassemia

i. Carrier Testing

The combination of a low MCV (approximately 65–72) with elevated levels of Hb A2 (>3.5% by HPLC) are strongly indicative of heterozygous β-thalassemia. If β-thalassemia molecular genetic testing of such individuals should fail to reveal the presence of a β-globin gene mutation from a specific mutational panel, then DNA sequencing is most appropriately recommended and will certainly uncover a point mutation, unless the hematological data are suspicious and not suggestive of β-thalassemia. In some cases, as in the Filipino deletion, a particularly elevated Hb A2 (>6%) suggests the presence of a β-globin gene deletion (Craig et al., 1992). In this case, neither a mutational panel nor DNA sequencing are likely to detect it due to the absence of the β-globin gene and thus failure of its amplification. On the other hand, a deletion that encompasses part of the β-globin gene such as the 619-bp deletion can be detected by DNA sequencing. In general, β-thalassemia due to large deletions (i.e. more than just a few base pairs), are best assayed by PCR screening of common deletional breakpoints or alternatively by restriction endonuclease mapping of the β-globin locus.

In another vein, iron deficiency is probably the major confounder of thalassemia when considering hematological indices, such as MCV and Hb A2 levels. In our experience, we have noted that severe iron deficiency with marginally elevated Hb A2 levels can result in near-normal Hb A2 levels that may suggest, according to hematological criteria, iron deficiency, α-thalassemia, or both. Therefore, in the presence of iron deficiency, which also results in a decreased MCV, α-thalassemia and β-thalassemia molecular testing is strongly recommended. One way of circumventing molecular genetic testing of α or β-thalassemia when suspecting coexisting iron deficiency is to treat the individual with iron supplementation and then monitor MCV and Hb A2 levels. If the MCV normalizes, then no thalassemia testing is needed, but if the MCV remains lower than the normal range, then thalassemia testing would be indicated.

ii. Homozygous β-thalassemia (Cooley’s Anemia)

Postnatal (after 1 year of age) DNA testing is usually not necessary since hematological findings (low Hb, low MCV, and elevated Hb F), parental testing (low MCV and elevated Hb A2 in both parents), physical examination and transfusion history can confirm a β-thalassemia diagnosis. However, between birth and three months of age, molecular genetic testing is recommended since hematological indices may be obscured by the declining expression of Hb F and the absence of physical symptoms. Nevertheless, it has become common practice to identify the underlying mutation(s) in an affected individual such that phenotype-genotype correlation may predict prognosis, severity of the disease and management. Actually, in a minor proportion of β-thalassemia individuals, a mild phenotype may be expected. For example, patients who are homozygous for a β-globin promoter mutation or for the mild IVS1 position 6 mutation. Although compound heterozygosity for two different mutations in the β-globin gene represents an unequivocal diagnosis of Cooley’s anemia, homozygosity for a particular mutation cannot technically rule out the presence of a deletion on one allele and the detected mutation on the other allele, namely because these methods are not quantitative with respect to zygosity (presence of one or two alleles). Parental testing is strongly recommended to ensure that both parents carry the same mutation as the one detected in the affected individual. In certain situations, the detection of homozygosity for a particular mutation along with heterozygosity for a known DNA polymorphisms in the β-globin gene (such as in codon 2, and IVSII, 74) may be indicative of true homozygosity, because heterozygosity for such polymorphisms alludes to the presence of two alleles.

B. α-thalassemia

The most common condition that tends to confound the diagnosis of the carrier state of α-thalassemia is iron deficiency. Thus, a low MCV and a negative result for common α-thalassemia deletions could hint to the presence of an uncommon α-globin deletion or to a point mutation, usually in the α2 globin gene or even to iron deficiency.

Although MCV values largely correlate with inactivation of α-globin gene copy number, there is significant overlap between the low end of normal MCV values and a single α-globin gene inactivation. Similarly, MCV values of two α-globin gene inactivation overlap with heterozygous β-thalassemia. Overlap in MCV values resulting from three α-gene inactivation (Hb H disease) can occur with two α-globin gene inactivation; however, Hb H is readily differentiated by the presence of a fast-running hemoglobin by electrophoresis or HPLC. The correlation of MCV with α-globin gene copy number identified by molecular genetic testing helps to indicate whether additional mutations that escape screening (e.g., point mutations) may further warrant extensive analysis such as DNA sequencing of the α2 (or occasionally α1) globin alleles. In the following sections, inactivation of 1 to 4 α-globin genes are discussed separately.

i. Inactivation of One α Globin Gene

The most common form of one α-globin gene inactivation is caused by the rightward deletion denoted as (−α 3.7), which, in our laboratory, was detected in the heterozygous state in 15% of 3378 individuals screened for α-thalassemia,
whereas the much less common leftward deletion (−αT) was detected at only 0.8%. The non-deletional forms of α-thalassemia (denoted αT) are much less common worldwide except in the Saudi Arabian population, where the poly A mutation in the α2 gene accounts for approximately 50% of α-thalassemia (Pressley et al., 1980), most likely due to the high rate of inbreeding in this population. Nevertheless, a non-deletional form of α-thalassemia should always be suspected in the presence of a persistently low MCV after ruling out iron deficiency, β-thalassemia and the common deletion forms of α-thalassemia (−α3.7 and −α4.2). The best approach to uncover a non-deletional form of α-thalassemia is by DNA sequencing of the α2 globin gene.

ii. Inactivation of Two α Globin Genes

The inactivation of two alpha globin genes may occur on the same chromosome (in cis) as in the Southeast Asian and Filipino deletions (denoted as −−SE/αα and −−PH/αα) or on opposite chromosomes (in trans) as in homozygosity for the rightward deletion (denoted as −α3.7/−α3.7). In the absence of iron deficiency, the MCV values resulting from homozygosity for a two-gene deletion in cis (−−/αα) or homozygosity for a one-gene deletion in trans (−α/−α) greatly overlap but differ significantly from normal or heterozygosity for a one-gene deletion, thus making MCV values an important index for the presence of these types of deletions. A result denoting homozygosity for a one-gene deletion, thus making MCV greatly overlap but differ significantly from normal or heterozygosity for a one-gene deletion, thus making MCV values an important index for the presence of these types of deletions. A result denoting homozygosity for a one-gene deletion (−α/−α) should always be suspicious of the presence of an additional two-gene deletion that is not detected by the method being used. In such a case, Hb H disease (3 α-globin gene deletion) could result and this combination may be assessed by low MCV (55–60) and the presence of a fast-running hemoglobin on electrophoresis or HPLC. On the other hand, the detection of a two-gene deletion in cis, may also underline a point mutation (such as Hb Constant Spring) in the α2-globin gene, resulting in Hb H disease (−α/−α). Again, Hb electrophoresis is strongly advised to test for the presence of a fast-running Hb.

iii. Inactivation of Three α Globin Genes

This genotype results in Hb H disease and arises from the deletion of three α-globin genes (−−/−α) or the association of a two-α-globin gene deletion with a point mutation in the α2-globin gene, such as in Hb H disease due to Hb Constant Spring or Hb Pakse, both of which are prevalent in Southeast Asian populations. These point mutations, which occur at the termination codon of the α2-globin gene, can be readily detected by molecular methods such as PCR coupled with allele-specific oligonucleotide hybridization assays. When using PCR, caution should be exercised as we have encountered a case of Hb H that was readily diagnosed by Southern blot, but where the PCR showed only a two-gene deletion due to allelic dropout of the α3.7 deletion due to a DNA polymorphism under the PCR primer. Correlation of such a case with the hematology (such as fast-running hemoglobin) could easily pinpoint the failure of the rightward deletion to amplify.

iv. Inactivation of Four α Globin Genes

Deletion of four α-globin genes (denoted as −−/−−) results in hydrops fetalis, a condition that results in death at birth. Such a condition is usually screened for at the prenatal stage and thus represents the most common form of prenatal diagnosis in α-thalassemia among Southeast Asian couples, who carry each a two-α-globin gene deletion and thus have a 25% chance of conceiving a fetus with no α-globin genes). Such a diagnosis is clear and unambiguous by Southern blot or PCR methods provided internal control reactions are present in either type of detection method and parental tests have confirmed heterozygosity for α-globin deletion in each parent. As pointed out previously, prenatal diagnosis of a heterozygous fetus should always be accompanied with maternal DNA contamination studies (especially in PCR-based assays). It is also important that in Southeast Asian couples, both the common Southeast Asian deletion and the Filipino deletions be screened for since fetuses (and their parents) may carry one copy of either type of deletion.

In uncommon types of situations, but one that we have encountered in our laboratory, it is possible to diagnose homozygous α-thalassemia with the absence of all four α-globin genes in an adult individual. Such patients have been previously reported (Beaudry et al., 1986; Liang et al., 1985). Clinically, these would be patients that have been rescued from neonatal death by in utero transfusion and maintained on a strict regular transfusion regimen. A few of such individuals have been reported sporadically (Liang et al., 1985).

C. δβ-thalassemia and HPFH

The presence of elevated Hb F in an individual with no other type of hemoglobinopathy suggests the presence of heterozygous δβ-thalassemia or HPFH, which are caused most commonly by large deletions in the β-globin gene cluster. The distinction between δβ-thalassemia and HPFH is not always straightforward (Calzolari et al., 1999) and often rests on the levels of Hb F in an individual, hematological indexes indicating the presence of anemia, and the overall clinical picture.

A minor, clinically insignificant and confounding effect are the small elevations of Hb F caused by specific point mutations in the promoter of a γ-globin gene that result in an increase in its transcriptional activity. However, association of δβ-thalassemia with β-thalassemia or with a deleterious hemoglobinopathy such as Hb S is clinically relevant. Indeed, individuals with Hb SS with a higher than usual Hb F are often suspected to be compound heterozygous for Hb S and
deletional type of $\delta$-thalassemia, thus warranting a screen for deletions in the $\beta$-globin gene cluster. In patients suspected to have Hb S/$\delta$-thalassemia, Hb S/HPFH, or $\beta$$/$$\delta$-thalassemia, definitive diagnosis may be best reached by uncovering the deleterious $\beta$-thalassemia mutation and the nature of the associated deletion on the opposite chromosome. Another way to uncover, for example, Hb S/$\delta$-thalassemia is by testing the obligate heterozygous parents and demonstrating that one parent has Hb AS and the other has an elevated Hb F (diagnostic of $\delta$-thalassemia or HPFH). Further refinement of $\delta$-thalassemia versus HPFH may originate from the presence or absence of anemia in the parent with elevated Hb F.

D. Interaction of $\alpha$-thalassemia with Hb S

In many instances, the levels of Hb S, when accurately determined such as by HPLC, may also be indicative of $\alpha$-thalassemia. In our experience, Hb AS individuals with Hb S levels hovering around 45% do not have $\alpha$-thalassemia. However Hb S levels around 25% to 35% strongly suggest one ($-\alpha/\alpha\alpha$) or two $\alpha$-globin gene ($-\alpha/-\alpha\alpha$) deletions, respectively. Co-existing $\alpha$-thalassemia in Hb SS individuals may be best assessed with low MCV levels, especially with coinheritance of a $-\alpha/\alpha\alpha$ genotype. Screening for coexisting $\alpha$-thalassemia in Hb SS is particularly useful for predicting prognosis since such individuals tend to have a milder anemia and reduced hemolysis than those with the presence of all four $\alpha$-globin genes. However, long-term survival of patients with Hb SS appears to be related not with $\alpha$-globin deletions, but rather with levels of Hb F (Ballas et al., 1997; Serjeant et al., 2008).

VII. CASE STUDIES

A. A 35-year-old woman of Asian ancestry in her first trimester of pregnancy was contemplating amniocentesis for maternal age. Her physician ordered a complete blood count (CBC) and found that she had a low MCV of 67 fl. Suspecting iron deficiency, he ordered iron studies, which came back normal. She was referred to a hematologist, who suspected thalassemia and requested Hb quantitation by column chromatography. Hb A was 93.5%, Hb F 1.7%, and Hb A2 4.8%. Thus, based on the elevated levels of Hb A2, she was diagnosed with heterozygous $\beta$-thalassemia. A molecular test confirmed the diagnosis and revealed heterozygosity for the 4-basepair deletion at codons 41–42 of the $\beta$-globin gene.

The patient was referred to a genetic counselor, who advised testing the father of the fetus for $\beta$-thalassemia. Even though the husband was unavailable for genetic testing, prenatal diagnosis was recommended since the mother was having an amniocentesis for maternal age and amniocytes had been established by the Cytogenetics Laboratory. $\beta$-thalassemia mutation screening was performed on cultured amniocytes for a panel of 42 mutations in the $\beta$-globin gene. The molecular diagnostics report stated:

“The fetus is heterozygous for the 41–42 frameshift mutation in the $\beta$-globin gene. In the absence of paternal testing, we cannot rule out that the fetus may carry an additional $\beta$-globin gene mutation not included in our mutation panel. DNA sequencing of the fetus is recommended to assess the mutational status of the paternal chromosome.”

Following insurance approval, a DNA sequencing test of the entire $\beta$-globin gene was ordered and revealed that the fetus is “compound heterozygous for the frameshift mutation at codons 41–42 and for the insertion of a C nucleotide at codons 27–28. The fetus is predicted to be affected with homozygous $\beta$-thalassemia.” Following discussion of the results with the genetic counselor, the mother decided to terminate the pregnancy.

B. An African-American couple was referred for prenatal diagnosis because of family history of sickle cell anemia. CBC, iron studies, and hemoglobin quantitation by HPLC were ordered on the parents. The mother had no iron deficiency, and Hb AS. The father had an MCV of 68, no iron deficiency, and a Hb A2 of 7.8%. Molecular testing for a panel of $\beta$-globin gene mutations confirmed Hb AS in the mother but was negative for the father. A follow-up DNA sequencing test of the $\beta$-globin gene was also negative. Noting the markedly elevated Hb A2 levels in the father, a screening test for four $\beta$-globin gene deletions was performed on the father and revealed that he was heterozygous for the 1.4-kb deletion, which is found in the African-American population. The fetus was at 25% risk for S/$\beta^0$-thalassemia. Following discussion with a genetic counselor, the couple elected not to pursue prenatal diagnosis and carry the fetus to term.

C. A pregnant woman with known Hb H disease was found to have a $3\alpha$-globin gene deletion ($-/-\alpha^{37}$). Her husband did not carry any of the common $\alpha$-globin gene deletion but was heterozygous for the $\beta^0$-thalassemia nonsense mutation at codon 17 of the $\beta$-globin gene. The couple was referred to a genetic counselor, who considered the worst case scenario for the fetus, namely the combination of heterozygous $\beta$-thalassemia with a one- or two-$\alpha$-globin gene deletion. Discussion of the case with an expert hematologist brought up the likelihood that reduction of $\alpha$-globin chain synthesis along with reduction of $\beta$-chain synthesis would balance each other and not cause any clinically significant anemia that would justify pursuing prenatal diagnosis.
VIII. FUTURE DIRECTIONS

The detection of deleterious mutations in the thalassemia syndromes has provided great insights into mechanisms of gene expression and has been at the forefront of molecular biological techniques since the advent of recombinant DNA technology in the late 1970s and of PCR in the mid-1980s. Even though in many cases, a diagnosis of α- or β-thalassemia may not necessitate molecular methods, it has become standard practice to delineate mutations in carrier and affected individuals such that prenatal diagnosis and extended family testing coupled with genetic counseling can be achieved. Even though the methodology for the molecular diagnosis of the thalassemias has been greatly simplified to allow accurate and rapid turnaround time, the techniques remain manual and labor-intensive. There are very few laboratories that offer comprehensive molecular thalassemia testing in a clinical environment and at a reasonable cost that could be covered by medical insurances. With the appearance and routine use of microarrays in clinical settings, new efforts ought to be aimed at devising a microarray chip that not only detects all the point mutations in α- and β-thalassemia, but also spans specific probes for the deletional breakpoints in α, β, δβ-thalassemia, and HPFH. Thus, with a single hybridization, one would be able to screen for any mutation that has been reported to date without the need for DNA sequencing, gene mapping, or multiplex PCR. Such a thalassemia chip would be initially difficult to devise, namely because of the probes optimization, but is certainly not a far-fetched aim, and one within the realm of modern molecular methods. Such a chip, while amenable to automation, would allow its widespread use among worldwide clinical laboratories and establish a standard in care and in cost.

On the clinical side, it has been our experience over the years that there is still a poor understanding among general practitioners of the implications of particular thalassemia mutations, especially those that may be clinically significant only when associated with other mutations, such as δβ-thalassemia. It is imperative for the laboratory that carries out the molecular testing not only to perform the mutation screening and interpretation, but also to provide explanations and extensions of the ramifications of a thalassemia mutation in a clinical setting. Thus, general instruction of the thalassemia disorders ought to be incorporated in undergraduate medical education and in genetic counseling programs, such that future physicians and genetic counselors obtain a deep understanding and appreciation for the complex yet fascinating group of thalassemia disorders. Finally, in a continuous effort to eradicate the life-threatening forms of the thalassemia disorders and with the implementation of the thalassemia chip, it would be possible to screen for any form of thalassemia and identify fetuses with a poor prognosis such that reproductive counseling can be offered to the parents. Historically, the molecular diagnostics of the thalassemia disorders have led the way to the implementation of similar approaches in other genetic disorders and thus, it would not be implausible that such a genomic approach of having an entire disease mutation chip could once again lead to similar approaches in genetic, infectious or acquired disorders.

IX. SUMMARY

Clinical prognosis and genetic counseling of the thalassemia syndromes depends largely on the delineation of their molecular heterogeneity, which remains a diagnostic challenge considering that α-thalassemia, β-thalassemia, and δβ-thalassemia/HPFH may interact together in any combination and with common structural hemoglobin variants. Although molecular diagnostic tools greatly help to clarify suspicion or confirmation of a thalassemia diagnosis, a stratified strategy for molecular testing ought to be undertaken to unravel complex thalassemia genotypes with an aim to predict prognosis, treatment modalities, molecular testing of family members and genetic counseling. Molecular testing of the thalassemia syndromes in laboratories specialized in offering a comprehensive molecular diagnostic package for these disorders along with clinical interpretation should be attractive to physicians and experts who seek to unravel complex thalassemia genotypes.

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