LABORATORY DIAGNOSIS OF HEMOGLOBINOPATHIES

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Introduction

Hemoglobinopathies include qualitative and quantitative disorders of globin synthesis [abnormal hemoglobins (Hbs) and thalassemias, respectively] and comprise the most common inherited disorders of man worldwide. They occur in particularly high frequency in many of the developing countries as a result of selection by endemic malaria, where they can pose a significant public health problem [1]. Additionally, as a consequence of population movements and immigration in the past several decades, Hb disorders are encountered with increasing frequency in industrialized Western countries [1,2].

The number of hemoglobin variants discovered to date totals 1018. Fortunately, the vast majority of these variants does not cause any clinical or hematological problems, and therefore are of interest to geneticists and biochemists (http://globin.cse.psu.edu). Most of the hemoglobin variants are missense mutations in the globin genes (a, b, g, or d) resulting from single nucleotide substitutions. Other uncommon mechanisms include deletion or insertion of one or more nucleotides altering the reading frame and fusion of globin genes with deletion of intergenic DNA sequences (γb fusion in Hb Kenya and δb fusion in Hb Lepore), mutations of the termination codon leading to the production of elongated globin chains [3].

Hemoglobin variants that significantly alter the structure, stability, synthesis, or function of the molecule have hematologic and/or clinical consequences. These can be classified in certain categories (Table 1).

Table 1. Clinically significant hb variants

- Altered physical/chemical properties:
  - a) Hb S (deoxyhemoglobin S polymerization): sickle syndromes
  - b) Hb C (crystallization): hemolytic anemia; microcytosis
- Unstable Hb Variants:
  - a) Congenital Heinz body hemolytic anemia (N=135)
- Variants with altered Oxygen affinity
  - a) High affinity variants: erythrocytosis (N=92)
  - b) Low affinity variants: anemia, cyanosis
- M-Hemoglobins
  - a) Methemoglobinemia, cyanosis (N=9)
- Variants causing a thalassemic phenotype (N=50)
  - a) β-thalassemia
    - i. Hb Lepore (δb) fusion (N=3)
    - ii. Aberrant RNA processing (Hb E, Hb Knossos, Hb Malay)
    - iii. Hyperunstable globins (Hb Geneva, Hb Westdale, etc.)
  - b) α-thalassemia
    - i. Chain termination mutants (Hb Constant Spring)
    - ii. Hyperunstable variants (Hb Quong Sze)

Modified and updated from Bunn & Forget: Hemoglobin: Molecular, Genetic, and Clinical Aspects. WB Saunders, 1986.

Hb S and Hb C are two examples of mutations on the surface of the hemoglobin molecule that alter both the charge and the physical/chemical properties of the molecule with polymer formation in the case of deoxyhemoglobin S and crystallization in Hb C with profound effects on the function, morphology, rheology, and life span of the red cells. Several mechanisms account for the pathogenesis of unstable hemoglobin variants. The common mechanism involves the precipitation of

the unstable hemoglobin molecule within the red cell with attachment to the inner layer of the red cell membrane ("Heinz body" formation); Red cells containing membrane attached Heinz bodies have impaired deformability and filterability leading to their premature destruction (congenital Heinz body hemolytic anemia). Mutations in certain residues alter the oxygen affinity of the hemoglobin molecule; a stabilization of the R (relaxed, oxy) state will result in high 02 affinity variants and erythrocytosis. Conversely, a stabilization of the T (tense, deoxy) configuration will result in a variant with low 02 affinity with enhanced unloading of 0_2 to the tissues with resultant cyanosis and anemia in certain cases (due to the suppression of the 0_2 sensing pathway). Mutations of the heme binding site, particularly those affecting the conserved proximal (F8) and distal (E7) histidine residues, lead to the oxidation of the iron atom in heme from ferrous (Fe++) to ferric (Fe+++) state with resultant methemoglobinemia (M-hemoglobins) and cyanosis. A group of mutations alter both the structure and the synthetic rate of the globin chain leading to a "thalassemic" phenotype. These include fusion hemoglobins (e.g. Hb Lepore, where the 5' d-globin sequences are fused to 3' b-globin sequences with deletion of the intergenic DNA; this puts the δb-fusion gene under the transcriptional control of the inefficient d-globin promoter with low expression of the fusion globin and hence the thalassemic phenotype), mutations that cause both a missense mutation and create an aberrant splice site (such as Hb E, Hb Knossos and Hb Malay), and "hyperunstable" globins where the nascent globin chains are highly unstable, undergo rapid proteolytic degradation, and result in a reduction in the affected globin.

Except for the commonly occurring variants (Hb S, C, E, and D-Los Angeles), very few abnormal hemoglobins have been observed in the homozygous state. Variant hemoglobins are usually found in the heterozygous state. While g-chain variants are expressed in fetal life and their level gradually decreases as the g to b (fetal to adult) globin switch progresses during the post-natal period, b and a-chain variants are expressed throughout life. d-globin variants are expressed at very low levels and can be detected only after the switch to adult globin synthesis is complete. Since a-globin chains are present in all of the hemoglobins expressed after the embryonic stage (Hb F-$\alpha_2\gamma_2$; Hb A-$\alpha_2\beta_2$, and HbA2-$\alpha_2\delta_2$), a-chain variants are associated with the production of variant Hb F ($\alpha^x{}_2\gamma_2$) and HbA2 ($\alpha^x{}_2\delta_2$) as well. In heterozygous states, b-chain variants constitute 40-50 percent of the hemoglobin in red cells; it should, however, be kept in mind that certain factors affect the amount of variant b-chains in carriers. These include the stability of the variant, the surface charge of the variant b-chain, and the presence of concomitant a or b-thalassemia. The more unstable the variant, the lower the quantity. Surface charge of the variant also plays a role in determining the quantity in red cells; this is because the formation of the αb-dimers (α1β1 and α2β2 contacts) is the critical first step in hemoglobin tetramer formation, and this step is primarily driven by electrostatic interactions between a and b-chains. The a-globin chains have a relatively positive surface charge and therefore interact more readily with relatively negatively charged b-globin variants to form αb-dimers. This is reflected in the higher percentage of negatively charged b-globin variants such as Hb N-Baltimore (b95Lys®Glu), which is found in ~50 percent in heterozygotes compared to b-globin variants with a positive surface charge, Hb S (b6Glu→Val) or Hb C (b6Glu®Lys) whose quantity in the heterozygote is 40-45 percent. In the presence of a-thalassemia, negatively charged b-globin variants compete more favorably for the available a-chains; this phenomenon is reflected in even lower percentages of Hb S and Hb C in heterozygous carriers of these variants in the presence of common deletional forms of a-thalassemia (Hb S of 30-35% in individuals with heterozygous $^+$-thalassemia, -a/αa; and 25-30% in homozygous a^+-thalassemia, -a/-a) [4,5]. Conversely, the amount of a b-globin variant will increase if there is a b-thalassemia allele in trans; the percentage of the variant will be inversely proportional to the output of the b-thalassemia allele; thus, the higher the variant the lower the output of the b^+-thalassemia allele. In the case of a β^0-thalassemia allele in trans, the variant will amount to ≥90 percent of the Hb in red cells with Hb A_2 and Hb F constituting the remainder. The quantity of a-globin variants is also variable, depending on the a-globin gene involved, and the presence of concomitant a- or b-thalassemia. Since there are normally four a-globin loci (αa/αa) and the 5'a-globin genes (α_2) are expressed at a higher level, some of the variation in the level of a-globin variants will depend on which a-globin gene carries the mutation; α_2 globin mutations are usually present at 20-25 percent of the total hemoglobin, whereas α_1-globin variants are expressed at a lower level (15-20%). Concomitant a-thalassemia results

Table 2. Diagnostic aproach to the detection of hemoglobinopathies

CBC, Reticulocyte count, peripheral smear, Heinz body prep
IEF on Agarose
HPLC:
a) Cation exchange HPLC (Hb quantitation)
b) Reversed phase HPLC (globin chain separation)
Special tests:
a) Isopropanol, heat stability (unstable Hbs)
b) Oxygen affinity (p50)
DNA-based methods:
a) PCR amplification and sequencing of globin genes
b) PCR detection of deletions

in a higher level of expression of a-globin variants. Observations on the different levels of expression of the common a-globin variant, Hb G-Philadelphia (a68Asn®Lys), is a case in point [6]. While this variant is found at ~25 percent in Northern Italians, its percentages in Americans of African descent can be either 33 or ~50 percent. This is clearly related to the different genotypes found in these two distinct populations; In Northern Italy and Sardinia, the genotype is $a^{G}a/\alpha a$, with an expression level of 25 percent, whereas in Americans of African descent, the G-Philadelphia mutation is commonly found on a hybrid $\alpha_2\alpha_1$ gene associated with the common 3.7 kb a^{+}-thalassemia deletion ($-a^{G}/\alpha a$) with ~33 percent expression. When there is an a^{+}-thalassemia deletion in trans ($-a^{G}/-a$ genotype), as expected, the level of Hb G-Philadelphia will be ~50 percent. Coinheritance of a-chain variants with b-thalassemia results in the reduction of the a-chain variant.

It is important to keep in mind that the vast majority of the over 1000 known Hb variants do not cause any clinical and hematological problems [7]. Nevertheless, many laboratories are referred specimens where clinically significant Hb variants such as Hb S [b6(A3)Glu→Val, GAG®GTG] or thalassemia mutations are found in compound heterozygosity with an "unknown" Hb variant; thus, the characterization of the "unknown" variant and delineation of its role, or lack thereof in the clinical and/or hematological phenotype in question, becomes an important function of the reference laboratory [7]. A stepwise approach and algorithm to the laboratory diagnosis of hemoglobinopathies is summarized in Table 2 [8].

The evaluation of the hematological picture, complemented by concise clinical information, is an important starting point. The ascertainment of the presence and type of anemia or erythrocytosis, red blood cell (RBC) indices [with particular emphasis on mean corpuscular volume (MCV)] and the review of peripheral blood smears are simple initial steps that can provide useful information and guide the next step in laboratory evaluation. This is followed by the application of protein-based screening methods; in the Hemoglobinopathy Laboratory at the Medical College of Georgia, electrophoresis on cellulose acetate and citrate agar have now been replaced with isoelectric focusing (IEF) on agarose gels, pH 6.0–8.0. The next step is usually cation exchange high performance liquid chromatography (HPLC) [using a weak cation exchange column, SynChropak CM-300 (SynChrom Inc., Linden, IN, USA)] [7,9]. With these methods, a positive diagnosis of commonly occurring b chain variants such as Hb S, Hb C [b6(A3)Glu®Lys, GAG®AAG], Hb E [β26(B8)Glu®Lys, GAG®AAG], Hb D-Los Angeles [β121(GH4) Glu®Gln, GAA®CAA] and Hb O-Arab [β121(GH4)Glu®Lys, GAA®AAA], as well as the a chain variant Hb G-Philadelphia [a68(E17) AsnLys, AAC®AAG or AAA] can be established. Quantitative data obtained from cation exchange HPLC will allow the initial diagnosis of b-thal trait as well as other b-thal syndromes and hereditary persistence of fetal Hb (HPFH). The percentage of the uncommon or unusual variant on cation exchange HPLC will many times yield clues as to whether the variant in question is a mutant a- or b-globin (commonly, a chain variants are 15–25% of the total Hb, whereas b chain variants comprise 30–45%). However, there are many exceptions to this general rule: these include unstable Hb variants and/or the presence of concomitant a- or b-thal. The next step in these cases is the application of reversed phase HPLC to separate the globin chains using a Vydac C4 column (The Separations Group, Hesperia, CA, USA). This technique is helpful not only in localizing the abnormality to a, or b (and g) chains, but also in quantifying Gg and Ag chains of Hb F in cases with elevated fetal Hb, and thus aid in the diagnosis of different types of HPFH and δb-thal [4–7,9].

The next step in the diagnostic approach is the definitive identification of the unknown or uncommon variant. In the Hemoglobinopathy Laboratory at the Medical College of Georgia, this is achieved by the sequencing of the polymerase chain reaction (PCR) amplified globin gene in question [11]. The

sequencing reaction can be performed on genomic DNA extracted from peripheral blood [white blood cells (WBC)] [11]; however, in many cases we have increasingly applied the method of sequencing the cDNAs of the respective globin gene, obtained from the reverse transcription of total RBC mRNA, that contains an abundance of the globin message. The advantage of this method over the sequencing of genomic DNA is that all of the globin cDNAs can easily be sequenced with one set of primers with a single sequencing reaction [12]. In the case of the b-thalassemias, the identification of the mutation obviously involves sequencing of the genomic DNA, particularly the important regulatory regions such as the promoter, exon/intron boundaries, introns and the 3' untranslated region (3'UTR) up to the polyadenylation signal [13].

Another application of the molecular diagnostic methods involves the detection of deletions in the globin genes by PCR amplification and the detection of the amplicon sizes on agarose gels (Gap PCR). These include the detection of common deletional a+-thalassemias ($-\alpha^{3.7}$, $-a^{4.2}$) and α^0-thalassemias ($--^{SEA}$, $--^{MED}$, $--^{20.5}$, $--^{FIL}$, $--^{THAI}$) [14,15]. With this approach, some of the relatively common forms of deletional b-thalassemias (such as the 619 bp Indian deletion and the 1394 bp deletion of African-Americans) can be detected. This approach also allows the molecular characterization of hybrid Hbs, such as the δb hybrid Hb Lepore [15].

The application of special tests can be very helpful and provide important guidance in the work-up of certain hemoglobinopathies. These include the isopropanol and heat stability tests for the confirmation of the presence of an unstable Hb variant in cases of Heinz body positive hemolytic anemia, and the determination of the oxygen affinity (p50) of the hemolysate in suspected cases of erythrocytosis due to the presence of a high oxygen affinity Hb variant [17].

Summary - A step-wise approach guided by clinical features and hematological data has proven to be practical and useful in the diagnostic work-up of hemoglobinopathies. Although several DNA-based techniques, such as reverse dot blot for the identification of common thalassemia mutations were developed and utilized in the late 1980s and early 1990s, the widespread availability of PCR and the development of automated sequencing techniques, have largely replaced these methods in the identification of mutations underlying hemoglobinopathies. Incorporation of novel technologies such as capillary electrophoresis, mass spectrometry/proteomics, and chip-based methods for the identification of mutations, may expedite and simplify the diagnosis of hemoglobinopathies if they are proven to be cost-effective.

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