Laboratory Diagnosis of Hemoglobinopathies and Thalassemia

Archana M Agarwal, MD

Medical Director, Hematopathology and RBC Laboratory
ARUP Laboratories
Assistant Professor of Pathology
University of Utah Department of Pathology
Learning Objectives

• Understand the pathophysiology of hemoglobinopathies

• Recognize the most important expected test results in hemoglobinopathies and thalassemias

• Understand different testing methodologies

• To be able to direct ordering physician to appropriate tests for these disorders
Hemoglobin (Heme+Globin)

• Hemoglobin is a tetramer composed of 4 globin molecules; 2 alpha globins and 2 beta globins or beta like globins

• The alpha globin chain is composed of 141 amino acids and the beta globin chain is composed of 146 amino acids

• Each globin chain also contains one heme molecule
Ribbon Diagram of Hemoglobin
Genetics of Globin Genes

**Chromosome 16**

\[\zeta\]
\[\alpha_1\]
\[\alpha_2\]

**Chromosome 11**

\[\zeta\]
\[\alpha_1\]
\[\alpha_2\]

\[\epsilon\]
\[\gamma_G\]
\[\gamma_A\]
\[\delta\]
\[\beta\]

\[HS\ 1-6\]

\[\epsilon\]
\[\gamma_A\]
\[\gamma_G\]
\[\delta\]
\[\beta\]

\[HS\ 1-6\]
Hemoglobin-Development Switching
Normal Adult Human Hemoglobin Composition

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Structure</th>
<th>% of Normal Adult Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>$\alpha_2\beta_2$</td>
<td>$&gt;96%$</td>
</tr>
<tr>
<td>Hb A2</td>
<td>$\alpha_2\delta_2$</td>
<td>$\sim2.5%$</td>
</tr>
<tr>
<td>Hb F</td>
<td>$\alpha_2\gamma_2$</td>
<td>$&lt;1%$</td>
</tr>
</tbody>
</table>
Hemoglobinopathy (structural)

- Due to mutations in either alpha or beta globin
- **Structural** – substitution, addition or deletion of one or more AAs in the globin chain
  - i.e HbS, HbC, HbE, HbD, HbO, etc…
- Over 1000 identified
  - Majority are benign & discovered incidentally
  - Pathogenic mutations can cause
    - Change in physical properties (sickling, crystalizes)
    - Globin instability (Heinz body formation, lower expression)
    - Altered oxygen affinity
Thalassemia (quantitative)

- A quantitative decrease in the production of alpha or beta globin chain
  - Large deletions, point mutations, small insertion/deletion that leads to decreased transcription or an unstable transcript
- Beta thalassemia results from mutations in beta gene(s)
  - Pathogenesis a result of the **free alpha subunits**
  - Two classes: $\beta^0$ and $\beta^+$
- Alpha thalassemia results from large deletions in the alpha gene(s)
  - Pathogenesis a result of the **free beta subunits**
Demographics: Thalassemias

- Found most frequently in the Mediterranean, Africa, Western and Southeast Asia, India and Burma

- Distribution parallels that of *Plasmodium falciparum*
Classification & Terminology: Alpha Thalassemia

- Normal \(\alpha\alpha/\alpha\alpha\)
- Silent carrier \(-\alpha/\alpha\alpha\)
- Minor /trait \(-\alpha/-\alpha\)
- Hb H disease \(--/-\alpha\)
- Barts hydrops fetalis \(--/--\)
Clinical Presentations of Alpha Thalassemia

- **A single** deletion (α-thalassemia minor)
  - silent carrier state
  - RBC morphology and hemoglobin concentrations are usually normal
- **Two** gene deletion (α-thalassemia minor)
  - Mild microcytic anemia
- **Three** gene deletion (hemoglobin H disease)
  - Precipitated β chains—Hb H
  - Patients have moderate anemia, marked microcytosis, splenomegaly, and bone marrow erythroid hyperplasia
- **Four** gene deletion (Hydrops fetalis)
  - Not compatible with life (barring very early intervention)
  - Hemoglobin is primarily comprised of γ4 (Bart’s), which has a very high affinity for O2 and is a poor oxygen transporter
Classification & Terminology: Beta Thalassemia

- Normal \( \beta/\beta \)
- Minor / trait \( \beta/\beta^0 \), \( \beta/\beta^+ \)
- Intermedia \( \beta^0/\beta^+ \)
- Major \( \beta^0/\beta^0 \), \( \beta^+/\beta^+ \)
Clinical Significance of $\beta$ Thalassemia

- Heterozygous asymptomatic
- Homozygous $\beta^0$ is a severe disorder associated with transfusion dependent hemolytic anemia
- Homozygous $\beta^+$ is a heterogeneous disorder
  - severity depending on mutation and % of HbA
  - Increased HbA = decreased severity
Sickle Cell Anemia

• Single nucleotide base change codes for valine instead of glutamic acid at the 6th position from the N-terminus of the β-globin chain

• Affects the shape and deformability of the red blood cell

• Leads to veno-occlusive disease and hemolysis
Peripheral Smear: Sickle Cell Anemia
Hb E

- 2\textsuperscript{nd} most prevalent hemoglobin variant
  - 30,000,000 world wide
  - 80% in Southeast Asia

- Hb E trait: microcytosis (mean MCV=65fl). No anemia

- Hb E disease: MCV =55-65fl with minimal anemia

- *On HPLC has similar migration pattern as Hb A2
**Hb C**

- Mutation in β-globin gene β(6glu->lys)
- Seen predominantly in blacks: Gene prevalence in US black population is 2 to 3%
- May confer malaria resistance
- Often asymptomatic, mild anemia, splenomegaly
- Blood smear shows many target cells, rare intracellular crystals
- Hb S/C disease causes moderate to severe anemia and hemolysis
Diagnosis

• **Indications for Testing**
  – Hemolytic anemia; family history of hemoglobinopathy

• **Laboratory Testing**
  – Initial testing – CBC with peripheral smear
  – Polychromasia, spherocytes, schistocytes, sickle cells, Heinz bodies, basophilic stippling; however, the lack of any of these cells does not rule out hemolytic anemia
  – Many hemoglobinopathies can be diagnosed using electrophoretic or high performance liquid chromatography (HPLC) techniques, but some may be missed
  – Genetic testing
Importance of CBC

• Thalassemias
  – Red cell indices are critical to diagnosis
  – Hypochromic microcytic anemia
    • MCV (mean corpuscular volume or size of the cell) is key
    • RDW (red cell distribution width) changes are variable
    • Increased RBC count → one distinguishing factor between thalassemias and other microcytic anemias
Distinguishing Features Between Iron Deficiency and Thalassemia

- The RBC count in thalassemia is either normal or on higher side of normal
- MCV usually less than 70 in
- The RDW is usually in the normal range

- Low RBC count
- MCV usually more than 70
- RDW is usually more than 17
Diagnosis of Thalassemias
High-Pressure Liquid Chromatography

- Cation Exchange
- Analytical cartridge contains negatively charged silica
- Buffers contain Na+ and K+ ions
- Hemolysates contain positively charged hemoglobin
- Hemoglobin binds to negatively charged silica at injection
- Na+ and K+ concentration increased and separates hemoglobin fragments from silica
Normal Patient Chromatograms
Summary of HPLC

Advantages

• Fast
• Small amounts of sample
• Accurate quantitation of A2

Disadvantages

• Hemoglobin E cannot be separated from A2
• Hemoglobin H and Barts elute too quickly from column
Capillary Electrophoresis

[Diagram of Capillary Electrophoresis]

http://www.sebia-usa.com
Phoresis Reports

Phoresis
Standard layout for printer report

Name

Sample num. 9  Date: 1/11/2007  ID: 0700386000

Electrophoresis

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>Hb A</td>
<td>96.2</td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

Comment:
Ratio: 1.00  T.P.: 

http://www.sebia-usa.com
Alkaline and Acid Gel Electrophoresis

- Electrophoresis (pH 8.4 (alkaline) and pH 6.2 (acid) on agarose gels)

- Slow, labor-intensive, and inaccurate in the quantification of low-concentration Hb variants (e.g., Hb A₂) or in the detection of fast Hb variants (Hb H, Hb Barts)

- The precision and accuracy of Hb A₂ measurements using densitometric scanning of electrophoretic gels is poor, especially when compared with HPLC techniques
Isoelectric Focusing

- IEF is an electrophoretic technique with excellent resolution.
- IEF is an equilibrium process in which Hb migrates in a pH gradient to a position of 0 net charge.
- The Hb migration order of IEF is the same as that of *alkaline electrophoresis* with better resolution.
Molecular Analysis

- **Alpha thalassemia**
  - Multiplex ligation dependent probe amplification (MLPA) and multiplex PCR
  - Alpha globin sequencing

- **Beta thalassemia**
  - Beta globin sequencing
    - The test examines the complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5’ and 3’UTR regions.
    - Clinical sensitivity is up to 97% based on the ethnicity
  - Beta globin del/dup testing by MLPA
α–Thalassemia Diagnosis

• Hb gel/HPLC migration patterns
  – Not helpful for α–Thalassemia, unless β4 (Hb H) and γ4 (Hb Barts) are present

• Genetic analysis
  – MLPA: will identify all deletions and duplications
  – Multiplex PCR for 7 common deletions-only 7 common deletion
  – Alpha globin sequencing
    • PCR amplification followed by bidirectional sequencing of the complete protein coding sequence with exon/intron boundaries, proximal promoter region, 5’ and 3’ untranslated regions, and polyadenylation signal
    • Only useful in 5-10% of cases where alpha thal is due to point mutation
β-Thalassemia Diagnosis

- **HPLC**: Elevated HB A2 diagnostic
- **Molecular analysis**: Complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5’ and 3’UTR regions
- Clinical sensitivity is up to 97% based on the ethnicity
- Beta globin del/dup in some cases (about 5%) where beta thalassemia is due to large deletions
Sickle Cell Disease Diagnosis

• Sickledex test (Screening test)
  – Deoxygenated Hb-S is insoluble in a concentrated phosphate buffer solution and forms a turbid suspension
  – Normal Hemoglobin A and other hemoglobins remain in solution
  – It does not differentiate between Sickle Cell Disease (S/S) and Sickle Cell Trait (A/S)
Sickle Cell Disease Diagnosis

Electrophoresis

HPLC

Suspected hemoglobinopathies and thalassemia

Order: HPLC/Capillary electrophoresis

- Increased Hb A2
  - Likely Beta thal
    - Beta globin sequencing
      - Normal
      - Beta globin del/dup

- Normal/HbH
  - Alpha thal
    - Alpha globin del/dup or 7 common deletion
      - Normal
      - Alpha globin sequencing

- Variant hemoglobin
  - Either Alpha or Beta globin sequencing
References and Acknowledgement


• Color Atlas of Hemoglobin Disorders: A compendium Based on Proficiency Testing (2003), updated in 2010

• Acknowledgement:
  – Josef T. Prchal, M.D, Professor of Medicine, Genetics and Pathology. University of Utah and ARUP Laboratories
  – Dottie Hussie, M.T, ARUP Laboratories
P.A.C.E.®/FL Password: HT62816

Go to www.aruplab.com/hemoglobinopathies and click on the P.A.C.E.®/FL Credit Redemption Link

Credit redemption for this webinar will be available through July 12, 2016

This webinar can be viewed after August 1, 2016 at www.arup.utah.edu where CME/SAM, P.A.C.E.® and Florida continuing education credit will be available.