Integration of Molecular Techniques Into the Evaluation of Hemoglobin Disorders and Erythrocytosis

James D. Hoyer, MD
December 6, 2013
DISCLOSURES:

Relevant Financial Relationship(s)
None

Off Label Usage
None
Learning Objectives

Hemoglobin Disorders

• Illustrate the appropriate use of molecular techniques in the evaluation of complex hemoglobin disorders
  • 2 Cases

Erythrocytosis

• Utilize an algorithmic approach in the evaluation of causes of secondary erythrocytosis
  • 2 cases
Hemoglobin Disorders
• α thalassemia
• β thalassemia
• α chain variants
• β chain variants
• HPFH or δβ thalassemia
• α thalassemia + α chain variants
• α thalassemia + β chain variants
• β thalassemia + α chain variants
• β thalassemia + β chain variants
• α thalassemia + β thalassemia
• α chain variants + β chain variants
• β thalassemia + HPFH
• β thalassemia + δβ thalassemia
Protein Methods

• Cellulose acetate electrophoresis, Isoelectric focusing, HPLC, capillary electrophoresis

• All exploit the charge difference that can occur with amino acid substitution

• Good at identifying Hb variants, do not give information about thalassemias
Molecular Techniques

- DNA (Sanger) sequencing – point mutations, small deletions (Hb variants, beta thalassemia)
- MLPA – large deletions (alpha thalassemia, HPFH, $\delta\beta$ thalassemia)
  - Southern Blot
  - PCR based assays (gap PCR)
MLPA

Mutliplex Ligation Dependent Probe Amplification

• Series of probe sets that hybridize to your gene of interest

• Method of choice for identifying large deletions (alpha thalassemia, HPFH, δβ thalassemia), but can also identify duplications
MLPA Probe Design

Region of interest

Exonic Sequence

5’ Exon
Specific Probe

3’ Exon
Specific Probe

Ligation Site

Universal Primer

Stuffer Sequence

Universal Primer
Deletion of Exon 3

Ratio to control marker
Case 1

- 16-month-old girl of Hispanic heritage, diagnosed with beta thalassemia trait
- Iron studies are normal (Ferritin = 67 mcg/L)

<table>
<thead>
<tr>
<th>CBC</th>
<th>Hgb</th>
<th>7.4 g/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC</td>
<td>$4.47 \times 10^{12}$/L</td>
</tr>
<tr>
<td></td>
<td>MCV</td>
<td>56.0 fL</td>
</tr>
<tr>
<td></td>
<td>RDW</td>
<td>26.1%</td>
</tr>
<tr>
<td></td>
<td>Reticulocytes</td>
<td>3.4%</td>
</tr>
</tbody>
</table>
Case 1
Capillary Electrophoresis
Case 1

HPLC
Case 1

• DNA sequencing of the beta globin gene is appropriate to assess for the severity of the underlying beta thalassemia mutation

• MLPA testing of the alpha globin gene cluster may also provide information
Case 1

DNA Sequencing Exon 1, β Globin Gene

IVS-I-I, G → A, β°
Anti 3.7 Duplication, MLPA
Alpha Thalassemia

Rightward Crossover

\[ \psi \alpha_1 \quad \alpha_2 \quad \alpha_1 \]

\[ X \quad Y \quad Z \quad X \quad Y \quad Z \]

\[ \alpha_1 \alpha_1 \text{anti}3.7 \]

\[ -\alpha^{3.7} \]
Case 1

Diagnosis

• Beta thalassemia trait associated with a triplicated alpha gene
Case 1

Key Points

- The anemia is more severe than expected because the presence of a triplicated alpha gene accentuates the globin chain imbalance that is the hallmark of all thalassemic disorders
Case 1

Key Points

• DNA sequencing of the beta globin gene is appropriate to assess for the severity of the underlying beta thalassemia mutation

• MLPA testing of the alpha globin gene cluster may also provide information
Case 2

- 4-year-old Cambodian boy; he is clinically well
- No organomegaly; iron studies are normal (Ferritin = 75 mcg/L)

**CBC**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hgb</strong></td>
<td>11.1 g/dL</td>
<td></td>
</tr>
<tr>
<td><strong>RBC</strong></td>
<td>$7.05 \times 10^{12}$/L</td>
<td></td>
</tr>
<tr>
<td><strong>MCV</strong></td>
<td>47.7 fL</td>
<td></td>
</tr>
<tr>
<td><strong>RDW</strong></td>
<td>23.3%</td>
<td></td>
</tr>
</tbody>
</table>
Capillary Electrophoresis

HPLC

Mix
Case 2

- The Hb variant present is easily identified as Hb E.
- However, there is uncertainty whether this represents Homozygous Hb E or Hb E in combination with a beta thalassemia mutation.
- The Hb F level is intermediate between these two possibilities.
- The distinction is important because Homozygous Hb E is a benign condition whereas Hb E/β^° thalassemia is a moderate to severe thalassemic syndrome.
Case 2

DNA Sequencing, β globin gene

Exon 1

Hb E, β 26 GAG → AAG, Glu to Lys

Exon 2

Codon 71/72 +A, TTTAGT → TTTAAGT, β°
Case 2

• The molecular results confirm Hb E/β° thalassemia, which is a moderate to severe β thalassemic disorder

• It is unusual to have a Hgb level of 11.1 g/dL

• Further testing for alpha thalassemia by MLPA method demonstrated a -3.7 kb deletion (“rightward”) on each chromosome 16
Case 2

Diagnosis

• Hb E/β° thalassemia with concurrent alpha thalassemia trait
Case 2

Key Points

• The presence of co-existent alpha thalassemia in this case has lessened the clinical severity of the disease

• This is due to the decreased imbalance between alpha and beta chains
Erythrocytosis
Causes of Erythrocytosis

- Lifelong (hereditary)
- Developed later (acquired)
Acquired Erythrocytosis

- Polycythemia vera
  - JAK2 V617F
  - Other JAK2 mutations including exon 12
- Cardiac/pulmonary dysfunction
  - Congenital heart disease
  - Hypoventilation – sleep apnea/chronic lung disease
  - Smoking/chronic CO exposure
- Renal dysfunction
  - Renal artery stenosis, ESRD
  - Hydronephrosis, renal cysts

- Tumor-associated
  - Renal CA, hemangioblastoma, hepatoma
  - Paraganglioma, pheochromocytoma, somatostatinoma
- Androgen/Epo/transfusion abuse
- High altitude
Hereditary Mutations

• High oxygen affinity hemoglobin variants
• EPO receptor mutations
• Mutations in the oxygen sensing pathway
  • HIF$\alpha$ mutations
  • VHL mutations
  • PHD2 mutations
• Methemoglobin Reductase/Hb M
• Bisphosphoglycerate mutase deficiency
High Oxygen Affinity Hb Variants

• Typically beta chain variants cause clinical symptoms

• May be electrophoretically silent (neutral charge substitution)

• Must have high index of suspicion

• Oxygen dissociation curve is always left shifted
Epo and Receptor

A. EpoR and JAK2 monomers and tyrosine (Y) residues

B. EpoR dimerization, JAK2 activation and effector signaling

C. Truncation of EpoR due to mutations

Gene transcription for cell proliferation, anti-apoptosis, etc.
O$_2$ Sensing Pathway

Hypoxia-inducible Factor
Prolyl Hydroxylase Domain
von Hippel Lindau

Alpha subunit half-life
↓ normal O$_2$
↑ hypoxia
Peripheral blood JAK2 V617F and serum Epo

**JAK2 V617F positive**
- P Vera
- JAK2 exon 12

**Epo low**

**JAK2 V617F negative**
- Epo normal/high

**EPOR**
- Lab error

**p50**
- Lifelong erythrocytosis
- Acquired erythrocytosis

- High O₂ affinity Hb
- 2,3-BPG deficiency
- Methemoglobinemia

- PHD2
- HIF2α
- VHL

NL
Case 3

- 19-year-old American man – African heritage
- Erythrocytosis
- Headaches, chest pain “all his life”
- 3 cigarettes/day
- PE unremarkable
- Father and other family members with headaches and ruddy faces
- Cardiac W/U: R bundle branch block
## Case 3

<table>
<thead>
<tr>
<th>CBC</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb</td>
<td>20.6 g/dL</td>
</tr>
<tr>
<td>Hct</td>
<td>61.6%</td>
</tr>
<tr>
<td>RBC</td>
<td>$8.97 \times 10^{12}$/L</td>
</tr>
<tr>
<td>MCV</td>
<td>68.7 fL</td>
</tr>
<tr>
<td>WBC</td>
<td>$5.30 \times 10^{9}$/L</td>
</tr>
<tr>
<td>PLT</td>
<td>$252 \times 10^{9}$/L</td>
</tr>
</tbody>
</table>
Case 3

**JAK2 V617F:** Negative

**Serum Epo:** 21.4 mIU/mL  
(NL 3.7-31.5)
Peripheral blood JAK2 V617F and serum Epo

- JAK2 V617F positive
  - P Vera
  - EPOR
  - Lab error
  - High O₂ affinity Hb
  - 2,3-BPG deficiency
  - Methemoglobinemia

- JAK2 V617F negative
  - Epo low
  - JAK2 exon 12
  - p50
  - Lifelong erythrocytosis
  - Acquired erythrocytosis
  - PHD2
  - HIF2α
  - VHL

- Epo normal/high
  - NL
Oxygen Dissociation Curve

\[ p_{50} = 12 \text{ mm Hg} \]
Case 4
Capillary Electrophoresis
Case 4

HPLC

Hb Lepore
# Case 4

<table>
<thead>
<tr>
<th>CBC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hgb</strong></td>
<td>20.6 g/dL</td>
</tr>
<tr>
<td><strong>Hct</strong></td>
<td>61.6%</td>
</tr>
<tr>
<td><strong>RBC</strong></td>
<td>$8.97 \times 10^{12}$/L</td>
</tr>
<tr>
<td><strong>MCV</strong></td>
<td>68.7 fL</td>
</tr>
<tr>
<td><strong>WBC</strong></td>
<td>$5.30 \times 10^9$/L</td>
</tr>
<tr>
<td><strong>PLT</strong></td>
<td>$252 \times 10^9$/L</td>
</tr>
</tbody>
</table>
• The presence of Hb Lepore explains the microcytosis but not the prominent erythrocytosis

• The abnormal p50 indicates that further workup is needed to exclude a silent high $O_2$ affinity Hb variant

• DNA sequencing and mass spectrometry identified Hb Johnstown ($\beta^{109}$ Val to Leu)
Case 4

• 12-year-old American girl – Spanish heritage
• Noted on 10 mo well baby exam
• Hgb 15-20 g/dL  (NL 12.2-14.8)
• Asymptomatic “feels great”
• No known family history
• No cardiac or pulmonary disorders
### Case 4

<table>
<thead>
<tr>
<th>CBC</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb</td>
<td>19.4 g/dL</td>
</tr>
<tr>
<td>Hct</td>
<td>58.2%</td>
</tr>
<tr>
<td>RBC</td>
<td>$6.50 \times 10^{12}$/L</td>
</tr>
<tr>
<td>MCV</td>
<td>89.5 fL</td>
</tr>
<tr>
<td>WBC</td>
<td>$4.20 \times 10^9$/L</td>
</tr>
<tr>
<td>PLT</td>
<td>$310 \times 10^9$/L</td>
</tr>
</tbody>
</table>
Case 4

JAK2 V617F: Negative

Serum Epo: 14.4 mIU/mL  (NL 9-28)
Peripheral blood $\textit{JAK2} \text{ V617F}$ and serum $\textit{Epo}$

- $\textit{JAK2} \text{ V617F}$ positive
  - P Vera
  - EPOR: Lab error
  - High $O_2$ affinity Hb
    - 2,3-BPG deficiency
    - Methemoglobinemia

- Epo low
  - $\textit{JAK2} \text{ exon 12}$
  - p50

- $\textit{JAK2} \text{ V617F}$ negative
  - Epo normal/high
  - Lifelong erythrocytosis
  - Acquired erythrocytosis
    - PHD2
    - HIF2$\alpha$
    - VHL

NL
Oxygen Dissociation Curve

Oxyhemoglobin (%)

p O2

p50 = 27 mm Hg
Case 4

**HPLC**

**CE**

[Graphs and charts showing HPLC and CE results]
Case 4

PHD2: Normal

HIF2α: P534R, 1601 C → G

VHL: Normal
Summary

• The evaluation of hemoglobin disorders can become very complex

• The key is to use the appropriate technology for the question being asked

• Molecular methods do not have to be used if the answer is easily obtained with routine methods

• Correlation with the patient's clinical history, physical findings and laboratory data is essential
Summary (cont)

• In cases of secondary erythrocytosis, an algorithmic approach can be used to direct appropriate testing

• This allow effective utilization of testing, and reduce unnecessary costs
Questions & Discussion