Objectives

1. Provide an explanation for why genetic methods can be used to predict blood group and platelet antigen status
2. Review limitations of serologic methods and explain the strengths of molecular methods for predicting antigen phenotype
3. Use case studies to illustrate when and how molecular testing can aid in assessing alloimmunization risk and identifying compatible blood products

Blood Group Antigens

Blood group antigens are also known as Human Erythrocyte Antigens (HEA). These molecules are encoded and/or regulated by genes. Many antigens are determined by variation in these genes, including Single Nucleotide Polymorphisms (SNPs). There are many genotyping methods for testing for genetic variants including SNPs. Thus, blood group antigens are inherited and can be predicted using molecular methods.

Human Platelet Antigens

All Molecular Testing Starts with DNA

- All nucleated cells (except germ cells) have the same DNA content, with the DNA in chromosomes which are found in the cell nucleus.
- The genes that are turned on and off differ depending on the cell type (red cell, skin cell, liver cell), but the DNA is the same.
- Mature RBCs have gone through a process of enucleation, where they have given up their nucleus, and thus carry no genomic DNA.
- White blood cells have nuclei and are therefore a source of DNA.

Polymerase Chain Reaction (PCR)
**Genotyping Methods: Resolution**

- **Low Resolution**
  - Gel-based methods
  - SSP-PCR for known SNPs
  - SNP Arrays (e.g., HEA)
- **Medium Resolution**
  - SNP Arrays (e.g., RHD and RHCE)
  - Taqman
  - MALDI-TOF
- **High Resolution**
  - DNA sequence analysis
  - Exon scanning
  - cDNA analysis
  - NextGen Sequencing

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**Challenges of Serologic Typing**

- Serologic methods vary
  - Tube
  - Gel
  - Solid phase
- Serologic reagents vary or are unavailable
  - Monoclonal
  - Polyclonal
  - Blends
  - Patient source
- Simple (e.g. Fy(a-b)) vs complex (e.g. RhD) epitopes
- Antigen variants can be missed (e.g. D, C, e, U variants)
- Expression level can hamper detection (e.g. Fy(b), Jk(a), weak D)
- Cross-reactivity (e.g. RHCE*ceHAR, RHCE*ceCF)
- Antigen typing can be hampered by antibody-coated cells
- Antigen typing can be hampered by recent transfusion

**Case 1: Donor in the “DARC”**

- Donor typing discrepancy
- Historically typed Fy(a-b-)
- Now typing Fy(a-b+)

*ACKR1 (aka FY, DARC) gene encodes the Fy antigens*

Using genotype to predict phenotype: FY

- Donor typing discrepancy
  - Historically typed Fy(a-b-)
  - Now typing Fy(a-b+)

*ACKR1 (aka FY, DARC) gene encodes the Fy antigens*
There is a variant in the gene promoter region (c.-67). It is found predominately in individuals of African descent. It is commonly found linked to the FY c.125A (on a FY*02 "backbone"). This SNV disrupts the binding site for the erythroid GATA-1 transcription factor. This results in loss of Fy(b) expression on the RBCs, while expression on tissue endothelium is not affected. Individuals who are Fy null due to homozygosity for this allele are resistant to malaria.

**Clinical importance:**
- African individuals with this SNP who type Fy(b-) still express Fyb in the tissues, and would not be expected to produce anti-Fyb.

Other FY variants influence Fy* & Fy# antigen expression

- There are SNPs in exon 2 that result in amino acid changes in the FY protein.
- These variants are usually found in individuals of European descent.
- They are commonly found linked to the FY c.125A (on a FY*02 "backbone").

- c.265C>T (p.R89C)
- c.298G>A (p.A100T)

The Fy (b*) antigen status may be missed serologically.

**Clinical importance:** depending on the antisera being used, samples with the FYX allele may type Fy(b-).

There are SNPs in exon 1 that result in amino acid changes in the Fy protein. These variants are usually found in individuals of European descent. They are commonly found linked to the FY c.125A (on a FY*02 "backbone").

- c.125G>T (p.D42N)
- c.125G>A (p.D42N)

Wy, there's more!

The Fy (b++) antigen status may be missed serologically.

**Clinical importance:** depending on the antisera being used, samples with the FYX allele may type Fy(b-).

Case 1: Donor in the “DARC”

Putting it all together:
- The donor sample was genotyped using an FDA-approved HEA panel.
- Include 3 SNPs in the ACKR1 gene.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>FYA/FYB</td>
<td>c.125 A/A</td>
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<tr>
<td>FY GATA</td>
<td>c.-67 c/c</td>
</tr>
<tr>
<td>FYX</td>
<td>c.265 C/T</td>
</tr>
</tbody>
</table>

Predicted Phenotype: Fy(a-b+)

Case 2: Watch out for the Kidd!

- 19 year old female.
- RBCs type Jk(a+) with alloanti-Jk^a.
- Requested HEA panel and JK gene variant testing.

Case 2: Targeted Genotyping

- PreciseType HEA Molecular BeadChip.
- Predicted patient to type Jk(a+b+)

But Remember Resolution?

- Low Resolution:
  - Gel-based methods
  - SSP-PCR for known SNPs
  - PCR-RFLP for known SNPs

- Medium Resolution:
  - SNP Arrays (e.g., RHD and RHCE)
  - Taqman
  - MALDI-TOF

- High Resolution:
  - DNA sequence analysis
  - Exon scanning
  - cDNA analysis
  - NextGen Sequencing

JK (SLC14A1)

JK (SLC14A1) c.838G/A (p.280 Asp/Asn)
Case 2: High Resolution Genotyping

Exon Scanning of coding exons

<table>
<thead>
<tr>
<th>Gene or Region</th>
<th>Method</th>
<th>Analyte</th>
<th>Result</th>
<th>Interpretation</th>
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<tr>
<td>JK</td>
<td>gDNA seq*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>130G/A</td>
<td>E44K</td>
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<tr>
<td>Exon 5</td>
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<tr>
<td>Exon 6</td>
<td>None</td>
<td>N/A</td>
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<td></td>
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<tr>
<td>Exon 7</td>
<td>588A/G</td>
<td>silent</td>
<td></td>
<td></td>
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<tr>
<td>Exon 8</td>
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<td></td>
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<tr>
<td>Exon 9</td>
<td>838G/A</td>
<td>D280N</td>
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</tr>
<tr>
<td>Exon 10</td>
<td>None</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 11</td>
<td>None</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Case 2: Assigning Alleles & Predicting Phenotypes

Genotyping identified a JK variant associated with weak antigen expression and typing discrepancies. It has also been associated with alloanti-JK.

Case 3: What about U?

– 45 year old female with sickle cell disease
– Admitted in sickle cell crisis
  • Hct 23.4% on admission
  • History of anti-U, -E and -K
  • Most recent transfusion was 7 years ago
– Serology on current admission:
  • All S-s-U- reagent panel cells nonreactive
  • one S-s- panel cell reactive (U antigen type not designated)
– Requested U- RBCs, specified no U+
  • 2 S-s-U- E- K- RBC units obtained via ARDP
  • Patient transfused
  • Patient discharged

U, the universal antigen

• S, s and U are encoded by glycophorin B
• The U epitope was mapped to amino acids 33-38 (near the membrane)
• In the 1960s, it was noted that S-s- RBCs were associated with U- status
• ~1% of African Americans are S-s-U-
• The U- phenotype is very rare (0.001%) in Caucasians
**GYPB encodes S, s and U antigens**

![Gene Diagram]

- **pseudoxon**
- **c.143T>C (p.Met48Thr)**
- **GENE**
- **mRNA**
- **polypeptide chain**
- **surface-expressed**

**Thr48**  
**s Antigen**  
**S Antigen**

**Predicted Phenotype Report for S-s-U-**

**INTERPRETATION:** The sample carries the GYPB deletion.

**Predicted phenotype:** S-s-U-

But, it’s not that simple…

**U Variant (U+VAR)**

- Variability of anti-U reactivity suggested heterogeneity of U
- Some anti-U was shown to detect an antigen on U- RBCs
- S-s- RBCs that are reactive with anti-U/GYPB express U+VAR
- S-s-U- individuals can make anti-U-like antibodies to U+VAR cells
- Historically, U+VAR was detected using serology
  - adsorption/elution with anti-U
  - PeG enhancement
  - limited by the anti-U specificity (anti-U vs. anti-U/GPB)
- The genetic bases of U+VAR was elucidated in 2003

**Genetic Determinant of U+VAR**

- **GYPB.P2 or GYPB IVS5**
- **c.143T p.Met48**
- **IVS5+5g>t**

**Nearly undetectable surface-expression**

**Predicted Phenotype Report for S-s-U+VAR**

- Variability of anti-U reactivity suggested heterogeneity of U
- Some anti-U was shown to detect an antigen on U- RBCs
- S-s- RBCs that are reactive with anti-U/GYPB express U+VAR
- S-s-U- individuals can make anti-U-like antibodies to U+VAR cells
- Historically, U+VAR was detected using serology
  - adsorption/elution with anti-U
  - PeG enhancement
  - limited by the anti-U specificity (anti-U vs. anti-U/GPB)
- The genetic bases of U+VAR was elucidated in 2003
Case 3: The Investigation

- Patient was genotyped -> RBCs predicted to be S-s-U-
- The blood suppliers were contacted regarding the U typing techniques.
  - One blood supplier tests all of their S-s- donors using molecular techniques. This unit was labeled “U negative.”
  - Second blood supplier tests S-s- donors using unlicensed, incompletely characterized anti-U and labels the unit as U- or U+VAR based upon this testing. The unit was labeled “U negative.”
- Donor was requested to provide blood sample
- Molecular testing predicted this donor to be U+

Response to U+VAR Transfusions

Antibody Strength Over Time

Survey Question

Q3: When U- units are requested, is molecular confirmation of antigen status of the patient requested?

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>28%</td>
<td>74%</td>
</tr>
</tbody>
</table>

Q4: When U- units are supplied, is molecular confirmation of antigen status of the donor provided?

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>58%</td>
<td>42%</td>
</tr>
</tbody>
</table>

Blood Centers and U

In response to this case, the American Red Cross Greater Chesapeake and Potomac region developed a survey in 2011 to assess how IRLs characterize S-s- donors as well as how they handle U- patients.

- 57 blood centers queried
  - 35 ARC regions
  - 17 non-ARC blood centers (US, Canada, Sweden, New Zealand)
- 34 blood centers (60%) responded
  - 25 ARC regions
  - 8 non ARC blood centers

Survey Question

Has the situation changed?

<p>| If you have identified anti-U in a S-s-positive or when U+ VAR requests made, what is your routine screening procedure? |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Year</th>
<th>Donors</th>
<th># U-</th>
<th># U+VAR</th>
<th>% U-</th>
<th>% U+VAR</th>
<th>% U- or U+VAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>17,750</td>
<td>33</td>
<td>69</td>
<td>0.2%</td>
<td>0.3%</td>
<td>0.5%</td>
</tr>
<tr>
<td>2012</td>
<td>21,400</td>
<td>54</td>
<td>73</td>
<td>0.2%</td>
<td>0.3%</td>
<td>0.6%</td>
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<tr>
<td>2013</td>
<td>22,856</td>
<td>61</td>
<td>65</td>
<td>0.3%</td>
<td>0.3%</td>
<td>0.6%</td>
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<tr>
<td>2014</td>
<td>20,725</td>
<td>58</td>
<td>88</td>
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<td>0.4%</td>
<td>0.7%</td>
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<tr>
<td>2015</td>
<td>25,800</td>
<td>68</td>
<td>88</td>
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<td>0.4%</td>
<td>0.7%</td>
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<tr>
<td>2016</td>
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<td>148</td>
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<td>0.5%</td>
<td>1.0%</td>
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<tr>
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<td>135</td>
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<td>0.5%</td>
<td>0.8%</td>
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<tr>
<td>2018</td>
<td>31,172</td>
<td>101</td>
<td>144</td>
<td>0.3%</td>
<td>0.5%</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

<1% of predominantly African American donors screened are U- or U+VAR
Less than half of these are S-s-U.
Identifying Antigen Negative Blood Donors

- Genotyping panels can be used to
  - Predict multiple antigens simultaneously
  - Predict phenotypes of multiple donors simultaneously
  - Predict antigens for which antisera are unavailable or unreliable
  - Efficiently identify donors/units to fill antigen-negative red cell requests
  - Identify donors/units for special recruitment and for American Rare Donor Program membership

Locating C- E- K- Fy(a-) Fy(b-) Jk(b-) Units

Antigens, Glycoproteins and Genes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Glyco</th>
<th>HGNC</th>
<th>Chromosome</th>
<th>Nucleotide</th>
<th>aa, Mature</th>
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<tbody>
<tr>
<td>HPA-1</td>
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<td>ITGB3</td>
<td>17</td>
<td>1767&gt;C</td>
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<td>ITGB3</td>
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<td>ITGB3</td>
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<td>ITGB3</td>
<td>17</td>
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<td>ITGB3</td>
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<td>T799M</td>
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<td>HPA-14w</td>
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<td>ITGB3</td>
<td>6</td>
<td>1909_1911delAAAG</td>
<td>K611del</td>
</tr>
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</table>

What about platelets?

https://clinmedjournals.org/articles/jbri/2-013-001.gif

Antigens, Glyco- HGNC Chromosome Nucleotide aa, Mature

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<th>Glyco</th>
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</tbody>
</table>

https://www.ebi.ac.uk/ipd/hpa/freqs_1.html
Clinical Scenarios that can involve HPA Incompatibility

- Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT)
- Post-transfusion Purpura (PTP)
  - Sudden, severe drop in PLT count 5-14 days post PLT transfusion
  - Rare
  - Most common cause is HPA-1a alloimmunization
- Platelet Refractoriness
  - Inadequate post PLT transfusion increment
  - More commonly caused by non-immune factors with ~20% due to immune mechanisms
  - Most common immune factor is alloantibodies to HLA Class I
  - Less common immune factors include ABO and HPA antibodies
- Transplant-associated alloimmune thrombocytopenia
  - HPA-1a and -5b have been implicated

FNAIT - Diagnosis Algorithm

FNAIT

- Caused by maternal alloimmunization to a paternally-inherited fetal PLT antigen
- Can occur in the first pregnancy
- Fetal thrombocytopenia can be associated with severe bleeding including intracranial hemorrhage
- Newborn PLT count often continues to drop after birth
- Diagnosis involves demonstrated paternal incompatibility and corresponding maternal antibody
- 1 in 1000 to 1 in 2000 HPA-1a positive infants born to HPA-1a negative moms are born with NAIT
- 80% of NAIT is caused by HPA-1a alloimmunization
  - Less common HPA-2, -3, -4, -5, -15

Case 4: NAIT?

- One month old male, Trisomy 21
  - Dad has 1 healthy child and 4 early pregnancy losses
  - Grade 1-2 germinal matrix hemorrhage
  - PLT AbID consistent with anti-HPA-5b
  - HPA genotyping identified HPA-3, -5 and -15 incompatibility
  - ABO incompatibility was also identified

Supplying HPA Negative PLTs

- There is a need to have platelets available that lack specific HPA
- Most commonly requested HPA negative PLT is for HPA-1a neg
- Availability of HPA-1a antigen negative units is challenging
- Serologic antigen typing can be performed
  - It is limited to HPA-1a only
- Platelets lacking HPA antigens other than HPA-1a are requested
  - These can be identified by genotyping
Utility of Genotyping for Predicting Antigen Status

Summary

- RBC genotyping can be useful to obtain a red cell phenotype in the face of serologic challenges
- There are many RBC genotyping methods, with varying levels of resolution
- RBC genotyping can be useful in resolving typing discrepancies
- RBC genotyping can be used to identify antigen negative and rare blood donors
- PLT genotyping can be helpful to investigate suspected NAIT cases and identifying HPA antigen negative PLT donors

Thank you for your attention!

Questions?

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