Genetic testing in the laboratory

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Disclosures for Martina Daly

In compliance with COI policy, ISTH requires the following disclosures to the session audience:

<table>
<thead>
<tr>
<th>Research Support/P.I.</th>
<th>No relevant conflicts of interest to declare</th>
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</thead>
<tbody>
<tr>
<td>Employee</td>
<td>No relevant conflicts of interest to declare</td>
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<tr>
<td>Consultant</td>
<td>No relevant conflicts of interest to declare</td>
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<tr>
<td>Major Stockholder</td>
<td>No relevant conflicts of interest to declare</td>
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<td>Speakers Bureau</td>
<td>No relevant conflicts of interest to declare</td>
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<tr>
<td>Honoraria</td>
<td>No relevant conflicts of interest to declare</td>
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<tr>
<td>Scientific Advisory Board</td>
<td>No relevant conflicts of interest to declare</td>
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</table>
Challenges of genetic testing in platelet disorders

- Variable expression of bleeding symptoms
- Overlap in symptoms between affected and unaffected individuals
- Similarities with other bleeding disorders
- Contribution of other factors to phenotypic expression
- Normal variation in platelet count and function
- Redundancy of platelet signalling pathways
Genetic testing in a platelet bleeding disorder – Why?

May aid diagnosis and clinical management of the patient

Counselling of affected family members

Reveals insights into platelet biogenesis and function

May identify druggable targets leading to more effective treatments
## Characteristics of inherited platelet disorders

<table>
<thead>
<tr>
<th>Patient history</th>
<th>Acquired</th>
<th>Inherited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset of bleeding symptoms</td>
<td>Recent</td>
<td>Life-long</td>
</tr>
<tr>
<td>Other changes in general health</td>
<td>Evaluate changes</td>
<td>No change</td>
</tr>
<tr>
<td>History of excessive bleeding after minor trauma, surgeries, childbirth?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Family members with bleeding or thrombocytopenia?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Previous normal platelet counts</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Response to treatment (steroids, splenectomy)</td>
<td>Increased platelets (~80%)</td>
<td>Variable/small effect</td>
</tr>
<tr>
<td>Response to platelet transfusions</td>
<td>Poor response/short lived</td>
<td>Good increment/normal survival</td>
</tr>
</tbody>
</table>
Classification of Inherited platelet disorders

Autosomal dominant, recessive or X-linked inheritance

Affect platelet production resulting in low circulating platelet counts and changes in platelet morphology, platelet function, or a combination

Adhesion defects

Receptors for soluble agonists and of proteins in signalling pathways

Secretion defects

Aggregation defects

Procoagulant activity

Thrombocytopenias
# Approaches for genetic testing

<table>
<thead>
<tr>
<th>Era</th>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990s</td>
<td>Candidate gene analysis</td>
<td>Phenotype gives clues to the underlying defects</td>
</tr>
<tr>
<td>2000s</td>
<td>Linkage analysis</td>
<td>Large families with several affected and unaffected members, or several families with similar phenotypes</td>
</tr>
<tr>
<td>2010s</td>
<td>Whole exome/genome analysis</td>
<td>No clues to underlying genetic defects</td>
</tr>
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</table>
## Candidate gene analysis

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Phenotype</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glanzmann thrombasthenia</td>
<td>Autosomal recessive; platelets aggregate only in response to ristocetin</td>
<td><em>ITGA2B, ITGB3</em></td>
</tr>
<tr>
<td>Bernard Soulier Syndrome</td>
<td>Autosomal recessive; Platelets fail to aggregate in response to ristocetin</td>
<td><em>GP1BB, GP1BA, GP9</em></td>
</tr>
<tr>
<td>ADP receptor deficiency</td>
<td>Decreased aggregation to ADP, which may be reversible</td>
<td><em>P2RY12</em></td>
</tr>
<tr>
<td>MYH9-related disorders</td>
<td>Autosomal dominant; macrothrombocytopenia; Döhle-like inclusions in neutrophils; can be accompanied by nephritis, hearing loss and/or cataracts</td>
<td><em>MYH9</em></td>
</tr>
<tr>
<td>Hermansky-Pudlak syndrome</td>
<td>Autosomal recessive; occulocutaneous albinism; reduction in δ-granules by EM; reduced ATP release</td>
<td><em>HPS1-9</em></td>
</tr>
</tbody>
</table>
Genetic diagnosis of Glanzmann thrombasthenia

- Homozygous $ITGA2B$ defect predicting
  - p.Tyr471Stop

- Homozygous $ITGA2B$ defect predicting
  - p.Ile596Thr
# Predicted effects of substitutions in αllb

<table>
<thead>
<tr>
<th>Predicted substitution</th>
<th>PolyPhen V2</th>
<th>SIFT</th>
<th>GVGD</th>
<th>SNPs&amp;GO</th>
<th>Mutation Taster</th>
<th>Pathogenic Predictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Asp396Asn</td>
<td>Damaging</td>
<td>Damaging</td>
<td>Less likely to interfere with function</td>
<td>Disease</td>
<td>Disease</td>
<td>4</td>
</tr>
<tr>
<td>p.Leu492Pro</td>
<td>Benign</td>
<td>Damaging</td>
<td>Most likely to interfere with function</td>
<td>Disease</td>
<td>Disease</td>
<td>4</td>
</tr>
<tr>
<td>p.Ile596Thr</td>
<td>Damaging</td>
<td>Damaging</td>
<td>Most likely to interfere with function</td>
<td>Disease</td>
<td>Disease</td>
<td>5</td>
</tr>
<tr>
<td>p.Asn670Lys</td>
<td>Damaging</td>
<td>Damaging</td>
<td>Most likely to interfere with function</td>
<td>Disease</td>
<td>Disease</td>
<td>5</td>
</tr>
<tr>
<td>p.Glu698Asp</td>
<td>Benign</td>
<td>Possibly damaging</td>
<td>Less likely to interfere with function</td>
<td>Neutral</td>
<td>Disease</td>
<td>2</td>
</tr>
</tbody>
</table>
Characterisation of αIIb variants

**Mock**

**WT**

**D396N**

**L492P**

**I596T**

**N670K**

**E698D**

Surface expression of αIIbβ3 (% of WT)

Ladder MOCK

WT  D396N  L492P  I596T  N670K  E698D

Pro-αIIb  136 kDa  
αIIb  125 kDa

β tubulin  55 kDa
Platelet disorders can be overlooked

Could a defect in the platelet response to ADP contribute to the diagnosis of type 1 VWD?
Segregation of VWF and P2RY12 defects in family with bleeding disorder

- I.1: not recruited
  - II.1: VWD
  - II.2: VWD
  - II.3: not recruited
  - II.4: VWD
  - II.5: VWD

- III.1: VWD
- III.2: not recruited

Legend:
- Homozygous wild-type
- Heterozygous P2RY12 520A>G, K174E
- Heterozygous VWF 5321T>C, L1774S
Platelets show reduced aggregation to ADP

- ADP 30 µM
  - Patient
  - Control

- ADP 10 µM
  - Patient
  - Control

- ADP 3 µM
  - Patient
  - Control

Aggregation (%) vs. Time (60s)
The K174E variant does not bind ADP
Genetic testing may aid management

Diagnosis of ITP; failed to respond to treatment; diagnosis revised to inherited thrombocytopenia

AML

Inherited thrombocytopenia

Inherited thrombocytopenia

Heterozygous MYH9 defect predicting p.Trp33Arg
## Risk of non-haematological features of MYH9-RD

<table>
<thead>
<tr>
<th></th>
<th>Nephropathy</th>
<th>Hearing loss</th>
<th>Cataracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3/MD interface</td>
<td>Low</td>
<td>Before 60 years in all cases</td>
<td>Low</td>
</tr>
<tr>
<td>R702 substitutions</td>
<td>Before 40 years in all cases; progression to ESRD in all cases</td>
<td>Before 40 years in all cases</td>
<td>Low</td>
</tr>
<tr>
<td>R1165 substitutions</td>
<td>Low</td>
<td>Before 60 years in all cases</td>
<td>Low</td>
</tr>
<tr>
<td>p.D1424H</td>
<td>High; progression to ESRD in minority of cases</td>
<td>Before 60 years in all cases</td>
<td>Probably higher</td>
</tr>
<tr>
<td>p.D1424N</td>
<td>Very low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>p.E1841K</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>NHT deletions</td>
<td>Very low</td>
<td>Low</td>
<td>Very low</td>
</tr>
</tbody>
</table>

Analysis based on 255 cases from 121 families

*Pecci et al, Hum Mutat, 2014*
Use of next generation sequencing and linkage analysis to map genetic defects

Next Generation Sequencing allows rapid and simultaneous analysis of large numbers of genes, exomes or genomes.

Very powerful when used in combination with linkage analysis to identify underlying genetic defects in platelet disorders.
Identification of causative gene in Gray Platelet Syndrome

11 unaffected and 5 affected members of 2 Native American families

Genome-wide SNP analysis using Affymetrix SNP array (906,600 SNPs)

Homozygosity mapping identified three candidate loci

Linkage analysis showed linkage to 1.7 Mb interval in 3p21 containing 74 genes

Next generation RNA sequencing of GPS platelets detected abnormal transcripts mapping to NBEAL2 (Kahr et al, 2011)

Sequencing of exomes from 4 unrelated GPS patients identified NBEAL2 as causative (Albers et al, 2011)

Sanger dideoxy sequencing of candidate genes reveals mutations in NBEAL2 (Gunay-Aygun et al, 2011)

Neurobeachin-like 2 (NBEAL2) encodes a BEACH-domain containing protein involved in granule development
Genetic testing in disorders where a candidate region is not known

Most challenging

Targeted analysis of platelet genes

Whole exome sequencing
Inheritance of platelet function disorder in family 1

- Bleeding symptoms & alopecia
- Eczema, recurrent viral infections
- Psoriasis
Platelet function disorder is characterised by defect in platelet granule secretion.
Inheritance of platelet function disorder in family 2

- Bleeding, alopecia, mild thrombocytopenia
- Infective endocarditis
- Eczema & colitis
Sequencing of 260 Platelet Genes

NGS of 260 platelet genes mapped to the human genome

Coverage ≥ 10

Novel variations

Exonic variations

Present in both Family members

1 variation in FLI1 c.1009 C>T; p.R337W

4 variations including one within FLI1 c.1028 A>G; p.Y343C
Role of FLI1 in megakaryocytopoiesis

ETS family member that regulates genes expressed during megakaryocytopoiesis
The R337W and Y343C FLI1 variants fail to transactivate *GP6*.

N=3, **p<0.01, *** p<0.001
Whole exome sequencing and targeted gene analysis pipeline

1. **WES of DNA from 12 cases with Gi signalling abnormalities**
   - Median variants/exome (range): 24,987 (24,244 – 25,732)

2. **Alignment of exome sequence to the human genome reference sequence**
   - Variants in 329 genes known or predicted to be associated with platelet count and/or function
   - Filter for novelty against EVS, 1000 genomes, dbSNP129, dbSNP132 and ‘in house’ database
   - Remove variants with MAF ≥ 0.01
   - Unique variants and variants with a MAF < 0.01
   - Predictions of pathogenicity using PolyPhen / SIFT / Mutation Taster / HSF / SplicePort / ASSP
   - Candidate gene defects

   - Median variants/exome (range): 337 (310 – 338)
   - Median variants/exome (range): 25.5 (20 – 41)
   - Median variants/exome (range): 4.5 (2 – 9)
### Candidate gene defects in cases with Gi receptor signalling abnormalities

<table>
<thead>
<tr>
<th>Variant type</th>
<th>Gi signalling abnormality (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hetero</td>
</tr>
<tr>
<td><strong>Missense</strong></td>
<td>44</td>
</tr>
<tr>
<td><strong>Indel/ frameshift</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Indel/ inframe</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>Splicing</strong></td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>63</td>
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</table>
DAVID – Database for Annotation, Visualization and Integrated Discovery

Enrichment analysis which clusters gene products in terms of their associated biological processes, cellular components and molecular functions by identifying significantly associated GO annotations
Functional annotation analysis of genes harbouring defects in cases with Gi receptor signalling abnormalities

<table>
<thead>
<tr>
<th>Annotation term</th>
<th>P value</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular signalling</td>
<td>$3.8 \times 10^{-3}$</td>
<td>13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of defects</th>
<th>Mutation type</th>
<th>Patient ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCY6</td>
<td>1</td>
<td>Missense</td>
<td>F8.1</td>
</tr>
<tr>
<td>P2RY12</td>
<td>2</td>
<td>Missense</td>
<td>F3.1, F12.1</td>
</tr>
<tr>
<td>TLR2</td>
<td>1</td>
<td>Missense</td>
<td>F2.1</td>
</tr>
<tr>
<td>PTGIR</td>
<td>1</td>
<td>Splicing</td>
<td>F3.1</td>
</tr>
<tr>
<td>RGS19</td>
<td>1</td>
<td>Missense</td>
<td>F9.1</td>
</tr>
<tr>
<td>PDZD3</td>
<td>1</td>
<td>Splicing</td>
<td>F5.1</td>
</tr>
<tr>
<td>ARHGEF12</td>
<td>1</td>
<td>Splicing</td>
<td>F3.1</td>
</tr>
<tr>
<td>PLA2G4C</td>
<td>2</td>
<td>Missense</td>
<td>F5.1, F11.1</td>
</tr>
<tr>
<td>PLCB3</td>
<td>1</td>
<td>Missense</td>
<td>F7.1</td>
</tr>
<tr>
<td>PRKD1</td>
<td>1</td>
<td>Splicing</td>
<td>F7.1</td>
</tr>
<tr>
<td>PTPRC</td>
<td>1</td>
<td>Splicing</td>
<td>F1.1</td>
</tr>
<tr>
<td>UNC13A</td>
<td>2</td>
<td>Inframe deletion, Missense</td>
<td>F1.1, F8.1</td>
</tr>
<tr>
<td>VAV2</td>
<td>1</td>
<td>Missense</td>
<td>F8.1</td>
</tr>
</tbody>
</table>

Leo et al, 2015
Genetic testing in disorders where a candidate region is not known

Most challenging

Targeted analysis of platelet genes

Whole exome sequencing
**ATCN1** mutations cause inherited macrothrombocytopenia

Capture exome sequence from genomic DNA (11 affected and 10 unaffected members of 6 families with autosomal dominant MTP)

Sequence on HiSeq 2000 and align sequence to human genome

Detect variants with >0.25 allele frequency and annotate with ANNOVAR

Remove silent variants and filter for novelty against dbSNP131 and ‘in house’ database

Final list of variants

Identification of co-segregating variants

Statistical analysis to identify genes that are significantly represented

Predictions of pathogenicity using PolyPhen2, SIFT, Mutation Taster, phyloP

Further studies to confirm pathogenicity of identified **ACTN1** variants

Most common form of MTP in Japan, accounts for 4% of cases

Kunishima et al, Am J Hum Genet, 2013
Challenges and considerations

Patients
Clinical history, platelet phenotyping
Affected and unaffected family members
Other families with similar defects

Exclude known gene defects
Gene panels

Whole exome or genome sequencing
Inheritance pattern
Autosomal recessive – focus on homozygous variants
Autosomal dominant – focus on heterozygous variants
? Compound heterozygous variants

Strategies for filtering genetic data
Focus on rare or unique variants
? Cut off for MAF
Exclude synonymous variants that are not predicted to disrupt
RNA splicing
? Non-coding variants
Challenges and considerations

Strategies for prioritising variants for downstream studies
Variants predicted to be deleterious (e.g. Combined Annotation Dependent Depletion, CADD)
Expression in platelets
Functional annotation analysis
Disease network analysis
Human phenotype ontology

Confirmation of pathogenicity of novel candidate gene defects in assays that mimic or replicate patient’s phenotype

Managing expectations
Patients and clinicians
“If you torture the data enough, nature will always confess”

Ronald Coase, 1910-2013
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UK GAPP Consortium

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