Molecular diagnosis of haemophilia and other bleeding disorders

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Bleeding disorders

Jayandharan et al, J Genet Synd Gene Ther, 2012
## General characteristics of inherited bleeding disorders

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Prevalence</th>
<th>Gene on chromosome</th>
<th>No of reported mutations in literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>1:1 million</td>
<td>4</td>
<td>240</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>1:2 millions</td>
<td>11</td>
<td>56</td>
</tr>
<tr>
<td>FV</td>
<td>1:1 million</td>
<td>1</td>
<td>133</td>
</tr>
<tr>
<td>FVII</td>
<td>1:500000</td>
<td>13</td>
<td>263</td>
</tr>
<tr>
<td>FV + FVIII</td>
<td>1:1 million</td>
<td>2, 18</td>
<td>52</td>
</tr>
<tr>
<td>FVIII</td>
<td>1:5000</td>
<td>X</td>
<td>~2700</td>
</tr>
<tr>
<td>FIX</td>
<td>1:25000</td>
<td>X</td>
<td>~1150</td>
</tr>
<tr>
<td>FX</td>
<td>1:1 million</td>
<td>13</td>
<td>112</td>
</tr>
<tr>
<td>FXI</td>
<td>1:1 million</td>
<td>4</td>
<td>195</td>
</tr>
<tr>
<td>FXIII</td>
<td>1:1 million</td>
<td>A subunit: 6</td>
<td>126</td>
</tr>
<tr>
<td>Glycoprotein Ib/IIIa</td>
<td>?1:1 million</td>
<td>17</td>
<td>171</td>
</tr>
<tr>
<td>BSS</td>
<td>1:1 million</td>
<td>17, 22,3</td>
<td>50</td>
</tr>
</tbody>
</table>
Hereditary bleeding disorders in India

Population ~ 1.2 billion

Incidence

250-60000 patients for each of the disorders

$\rightarrow 84\%$ - Severe; 16% mild\(^1\)

8-10 fold increased in populations practicing consanguineous marriage (South India)\(^2\)

1 CMC data unpublished
2 Mannucci et al, Blood, 2004
Molecular diagnosis of bleeding disorders
Haemophilia
- **Factor 8** gene defect $\rightarrow$ **haemophilia A**

- **Factor 9** gene defect $\rightarrow$ **haemophilia B**

FACTOR VIII AND FACTOR IX

FACTOR 8 GENE -186 Kb

26 EXONS

FACTOR 9 GENE - 34 Kb

8 EXONS

5'UTR- 150nt
3'UTR- 1806 nt

mRNA – 9010 nt

5'UTR- 29nt
3'UTR- 1390 nt

mRNA – 2803 nt

Pre A1 A2 A3 B C1 C2 C3

R372 R740 R1689

-19 +1 336 719 1691 2025 2332

R145 R180

-46 -19 1 40 85

Activation peptide

Catalytic

415

FACTOR VIII PROTEIN

FACTOR IX PROTEIN

Heavy chain
Light chain

N A1 A2 A3 C1 C2 Ca2+

N GLA EGF1 EGF2 S S Catalytic

C Heavy chain
Light chain
Gene defect $\rightarrow$ Qualitative (or) Quantitative change in protein $\rightarrow$ Haemophilia

$\text{FVIII:C or FIX:C -} \, 1-5\%$ - moderate

$\text{FVIII:C or FIX:C -} \, >1\%$ - severe

$\text{FVIII:C or FIX:C -} \, 5-40\%$ - mild

$\text{F8 or F9 gene mutation}$

Control of haemophilia

- Carrier detection

- Prenatal diagnosis
Who seeks genetic testing?

Eg: Pedigree with haemophilia

- Carrier status determination
- Prenatal diagnosis
Components of genetic testing

- Clinical history / phenotypic assays
- Pedigree analysis
- Genetic screening [Informed Consent]
- Counseling [Pre and post testing]
Genetics of haemophilia
X- linked inheritance

- Both VIII and IX deficiency are X linked
- Men are affected (25%)
- Women are carriers (25%)
Genetics of Haemophilia

Who is a carrier?

Eg: A Mother having a affected son
Genetics of Haemophilia

If the father is a haemophiliac, the daughters will be obligate carriers and all the sons would be normal.
Sporadic and Familial Haemophilia

- **Sporadic**: Only one known case in the family -
- **Familial**: More than one haemophiliac in the family exists -
Pedigree analysis

Obligate and Possible Carriers
Approach for genetic diagnosis of haemophilia

1. Linkage analysis
2. Direct mutation detection
Linkage analysis

-Track the defective chromosome

F8 gene
- Technically simple, low cost
- Multiple members and informativeness of polymorphism required
- Informative in ~80% families

F9 gene
- Technically simple, low cost
- Multiple members and informativeness of polymorphism required
- Informative in ~80% families

Linkage analysis

Methods

- PCR
- Restriction fragment length polymorphism (RFLP)
- Repeat sequence polymorphisms (microsatellites and VNTRs)
Direct Mutation detection

- Direct identification of disease causing mutation

- Highly sensitive

- Gold standard

- Expensive

Informative in >95% of families
PCR based direct mutation detection in haemophilia A

- Intron 22 inversions account for ~45-50% of severe haemophilia A phenotypes
- Intron 1 inversions account for ~2-5% of severe haemophilia A phenotypes

1. Lakich et al, Blood, 1993
DNA samples required for genetic diagnosis

Indirect
- Affected patient
- DNA samples

Direct
- Proband requesting genetic diagnosis
Chorionic villus sampling at 10-12 weeks for prenatal diagnosis

DNA

Genetic diagnosis
## Haemophilia

### Approach to molecular diagnosis at CMC, Vellore

<table>
<thead>
<tr>
<th>Factor 8 gene</th>
<th>Factor 9 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 1 &amp; 22 inversions</td>
<td>Direct mutation detection</td>
</tr>
<tr>
<td>Direct mutation detection</td>
<td></td>
</tr>
<tr>
<td>Indirect – Linkage analysis</td>
<td>Linkage analysis- not preferred</td>
</tr>
</tbody>
</table>

*(FVIII:C<1%)*
**Multiplex PCR and Conformation sensitive gel electrophoresis**

CSGE is a rapid, heteroduplex based detection method for mutation screening. The method relies on the differential migration of DNA heteroduplexes in comparison with homoduplexes during polyacrylamide gel electrophoresis under mildly denaturing conditions.

Algorithm for genetic diagnosis of bleeding disorders

[CMC, Vellore]

Common mutation
Haemophilia A* (Intron 1 & 22 Inversion)

(-)

Direct mutation detection
PCR-CSGE-Seq.

(-)

Uniplex PCR
- Fibrinogen deficiency
- Prothrombin deficiency
- Combined F5F8 deficiency
- Factor VII deficiency
- Factor X deficiency
- Factor XI deficiency
- Protein C deficiency
- Protein S deficiency
- Wiskott-Aldrich syndrome
- Bernard Soulier syndrome

Multiplex PCR
- Haemophilia A
- Haemophilia B
- Factor XIII deficiency
- Glanzmann Thrombasthenia

Indirect – Linkage analysis
Haemophilia A
Glanzmann Thrombasthenia
## Validation of molecular algorithms for genetic diagnoses

<table>
<thead>
<tr>
<th>Bleeding Disorder</th>
<th>Gene/ Chromosome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemophilia A</td>
<td>F8 / Ch.X</td>
<td>Jayandharan et al, Haemophilia, 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jayandharan et al, Haemophilia, 2005</td>
</tr>
<tr>
<td>Fibrinogen disorders</td>
<td>FGA/FGB/FGG-Ch.4</td>
<td>Sumitha et al, Haemophilia, 2013.</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>F2 / Ch.11</td>
<td>Jayandharan et al, J Thromb Haemost, 2005</td>
</tr>
<tr>
<td>Factor V+VIII deficiency</td>
<td>LMAN1-Ch.18</td>
<td>Jayandharan et al, Haemophilia, 2013.</td>
</tr>
<tr>
<td></td>
<td>MCFD2-Ch.2</td>
<td></td>
</tr>
<tr>
<td>Factor VII deficiency</td>
<td>F7/ Ch.13</td>
<td>Jayandharan et al, Haematologica, 2007</td>
</tr>
<tr>
<td>Factor X deficiency</td>
<td>F10 / Ch.13</td>
<td>Jayandharan et al, J Thromb Haemost, 2005</td>
</tr>
<tr>
<td>Factor XI deficiency</td>
<td>F11/ Ch.4</td>
<td>Jayandharan et al, J Thromb Haemost, 2005</td>
</tr>
<tr>
<td>Factor XIII deficiency</td>
<td>F13A/ Ch.6</td>
<td>Jayandharan et al, Thromb Haemost, 2006</td>
</tr>
<tr>
<td></td>
<td>ITGB3- Ch.17</td>
<td></td>
</tr>
<tr>
<td>Bernard Soulier syndrome</td>
<td>GP1BA-Ch.17</td>
<td>Sumitha et al, J Thromb Haemost, 2011</td>
</tr>
<tr>
<td></td>
<td>GP1BB-Ch-22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GP9-Ch.3</td>
<td></td>
</tr>
<tr>
<td>Wiskott Aldrich syndrome</td>
<td>WASP-Ch.X</td>
<td>David et al, Eur J Haematol, 2012</td>
</tr>
</tbody>
</table>
Setting up a genetic diagnosis facility

Laboratory space: ~750-1000 sq.ft

Personnel: 2 [Technician, research officer]

Equipments: PCR machine, electrophoresis, 4°C/-20°C storage, centrifuge, pH meter, waterbath, ?Centralized DNA sequencer

HR Training/ collaboration with established centers
EQAS scheme for genetic diagnosis of bleeding disorders

CMC-EQAS

RCPA Quality Assurance Programs
Conclusions

Molecular studies in bleeding disorders useful for:

✓ prevention and control

✓ Need to generate diagnostic algorithms to each population

✓ Can be done at significantly lower cost by process optimization