Optimizing Approaches to Molecular Testing for Inherited Bleeding Disorders

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Queen’s University
Kingston, Canada

ISLH, May 2017
Molecular Characterization of The Human Hemostatic Cascade
Isolation and characterization of a cDNA coding for human factor IX

(cDNA hybridization/DNA sequence analysis/blood coagulation)

KOTOKU KURACHI AND EARL W. DAVIE

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Contributed by Earl W. Davie, July 29, 1982

and George Brownlee, Oxford University

Factor IX Gene Cloned in Nov 1982
Factor IX Gene 34 kb: Xq27

FIX mRNA ~1.3 kb
Characterization of the human factor VIII gene


Departments of Molecular Biology and *Protein Biochemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

The complete 186,000 base-pair (bp) human factor VIII gene has been isolated and consists of 26 exons ranging in size from 69 to 3,106 bp and introns as large as 32.4 kilobases (kb). Nine kb of mRNA and protein-coding DNA has been sequenced and the mRNA termini have been mapped. The relationship between internal duplications in factor VIII and evolution of the gene is discussed.

and Genetics Institute

Factor VIII Gene Cloned in Nov 1984
Factor VIII Gene 184 kb: Xq28

FVIII mRNA ~9 kb

F8A  F8B
Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene.

Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization.

Cloning and characterization of two cDNAs coding for human von Willebrand factor.

VWF Gene: Chromosome 12p

178 kbp
What is the role for molecular testing in the diagnosis of disorders of hemostasis?
In the vast majority of cases -

a) The diagnosis

and

b) The clinical management

of inherited bleeding disorders should utilize conventional clotting tests.
• 9 month old boy with a large soft tissue bleed

• 16 year old girl with menorrhagia

• 21 year old male with post-tonsillectomy bleeding

• 69 year old male with new extensive bruising
• 9 month old boy with a large soft tissue bleed
  a) Severe hemophilia A

• 16 year old girl with menorrhagia
  b) Type 1 VWD

• 21 year old male with post-tonsillectomy bleeding
  c) Mild hemophilia B

• 69 year old male with new extensive bruising
  d) Acquired hemophilia A

All timely, inexpensive and precise phenotypic diagnoses
Molecular Testing for Inherited Bleeding Disorders

1. Confirmation of uncertain phenotypic diagnosis
2. Molecular analysis as the preferred diagnostic test
3. Supplementary genotype-phenotype information
4. Differentiation of genocopies
Molecular Testing for Inherited Bleeding Disorders

Confirmation of the phenotypic diagnosis

a) Mild quantitative deficiencies

eg. mild FIX/mild FXI deficiency

b) Equivocal functional/structural results

eg. Equivocal VWF:RCo and/or multimer analysis
Type 2 von Willebrand Disease

- VWF:RCo: VWF:Ag ratio <0.6 (2A, 2B, 2M)
- Abnormal VWF multimer pattern (2A, 2B)
- RIPA +ve (2B)
- FVIII level 5-30% (2N)
Location of Type 2 von Willebrand Disease Mutations
Molecular Testing for Inherited Bleeding Disorders

Molecular Analysis as the Preferred Diagnostic Test

a) Prenatal diagnosis

a) Carrier detection
Spectrum of Hemophilia Mutations

> 2,100 different $F8$ mutations

http://hadb.org.uk/WebPages/PublicFiles/MutationSummary.htm

> 1,100 different $F9$ mutations

http://www.factorix.org/
National Hemophilia Genetic Testing Programs

1. Canada

   >4,000 diagnostic reports in past 16 years

2. USA

   My Life Our Future Project: >5,000 patients diagnosed
Factor VIII Intron 22 Inversion

[Diagram showing the inversion of intron 22 in the Factor VIII gene.]
45% of Severe Hemophilia A
Unusual Test Samples

Tsar Nicholas II, Tsarina Alexandra and Family
The Royal Hemophilia Mutation
Rogaev et al. Science October 2009

F9 Intron 3 Splice Acceptor Mutation
Royal Family F9 Splicing Mutation
Molecular Testing for Inherited Bleeding Disorders

Supplementary Genotype – Phenotype Information

- FVIII/FIX Inhibitor risk
- Hemophilia B Leiden
# FVIII Genotype and Inhibitor Risk in Severe Hemophilia A PUPs

<table>
<thead>
<tr>
<th>Type of Mutation</th>
<th>Risk (Approx.)</th>
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<tbody>
<tr>
<td>Multi-domain deletions</td>
<td>~60-80%</td>
</tr>
<tr>
<td>Light chain nonsense mutns</td>
<td>30-40%</td>
</tr>
<tr>
<td>Intron 22 inversion</td>
<td>20-25%</td>
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<tr>
<td>Single domain deletions</td>
<td>15-25%</td>
</tr>
<tr>
<td>Small non-A run insertions/deltns</td>
<td>15-20%</td>
</tr>
<tr>
<td>Heavy chain nonsense mutns</td>
<td>10-20%</td>
</tr>
<tr>
<td>FVIII missense mutns</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Small A run insertions/deltns</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Splicing mutns</td>
<td>&lt;5%</td>
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</tbody>
</table>
Enhanced Inhibitor Risk F8 Mutations in Non-Severe Hemophilia A

Inhibitor Risk at 50 EDs

- Trp2229Cys - 41.7%
- Arg2159Cys - 39.4%
- Asp2074Gly - 21.2%
- Arg593Cys - 18.3%
Post-Pubertal Changes to F.IX in Normal Subjects and Hemophilia B Leiden Patients

![Graph showing post-pubertal changes to F.IX in normal subjects and hemophilia B Leiden patients. The graph plots Age (years) on the x-axis and FIX (%) on the y-axis. The graph shows two lines: one for normal FIX and another for Hemophilia B Leiden FIX. The normal FIX line remains nearly constant at 25%, while the Hemophilia B Leiden FIX line increases from 25% to 100% over the age range of 5 to 40 years.](image-url)
Hemophilia B Leiden Mutations

- DBP/C/EBP
- AR/HNF4
- HNF4/COUP-TF/ARP/C/EBP

5’ -220 -190 3’

-26 -21 -20 -19
-6 -5
+6 +8 +11 +13

Gene
Differentiation of Genocopies

(identical phenotypes due to mutations in different genes)
Differentiation of Genocopies
(identical phenotypes due to mutations in different genes)

1. Type 2B VWD vs Platelet-Type VWD

Genetic Locus: \( VWF \) vs \( GPIb\alpha \)

Therapy: VWF/FVIII concentrate vs Platelets
Differentiation of Genocopies
(identical phenotypes due to mutations in different genes)

2. Type 2N VWD vs Mild/Moderate Hemophilia A

Genetic Locus:  \( VWF \)  vs  \( F8 \)

Therapy:  VWF/FVIII concentrate  vs  FVIII concentrate
DDAVP  vs  DDAVP
Molecular Analysis

of the

Rare Inherited Bleeding Disorders
<table>
<thead>
<tr>
<th>Protein</th>
<th>Prevalence of Severe Factor Deficiency</th>
<th>Size of Gene (kb)</th>
<th>Number of Exons</th>
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<tr>
<td>FVIII</td>
<td>1 in 10,000</td>
<td>186 kb</td>
<td>26</td>
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<tr>
<td>FIX</td>
<td>1 in 30,000</td>
<td>33 kb</td>
<td>8</td>
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<tr>
<td>VWF</td>
<td>1 in 100,000</td>
<td>175 kb</td>
<td>52</td>
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<tr>
<td>FXI</td>
<td>1 in 1,000,000</td>
<td>23 kb</td>
<td>15</td>
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<tr>
<td>FVII</td>
<td>1 in 1,000,000</td>
<td>12 kb</td>
<td>9</td>
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<tr>
<td>FX</td>
<td>1 in 500,000</td>
<td>22 kb</td>
<td>8</td>
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<tr>
<td>Fibrinogen</td>
<td>1 in 1,000,000</td>
<td>FGA 8kb FGB 8 kb</td>
<td>FGA 7 FGB 8 FGG 11</td>
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<tr>
<td>FV</td>
<td>1 in 1,000,000</td>
<td>80 kb</td>
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<td>FXIII</td>
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<td>Subunit B 28 kb</td>
<td>Subunit B 12</td>
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<tr>
<td>Fibrinogen</td>
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<td>Prothrombin</td>
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<td>Factor V</td>
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<td>FV &amp; FVIII</td>
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<td>Factor VII</td>
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<td>Factor X</td>
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<td>Factor XI</td>
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<tr>
<td>Factor XIII</td>
<td>121</td>
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**Rare Inherited Bleeding Disease Mutations**
A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders


Blood 2016 127:2791-2803
A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders

63 Bleeding and Platelet Disorder (BPD) gene platform - NGS

300 samples (260 unrelated subjects)

Group 1: Known pathogenic variants - 159
Group 2: Suspected pathological phenotypes - 61
Group 3: Bleeding of uncertain pathogenesis - 76
Group 4: Unaffected relatives - 4
Technical evaluation of the ThromboGenomics 63 gene platform
A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders

Group 1: Known pathogenic variants - 159
All pathogenic variants detected (100% sensitivity)

Group 2: Suspected pathological phenotypes - 61
56/61 pathogenic variants identified (92%)
(26 factor deficiencies, 4 Glanzmann, 1 BSS, 9 Hermansky-Pudlak, 4 May-Hegglin)
A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders

Group 3: Bleeding of uncertain pathogenesis - 76

Pathogenic variants identified in 8/76 (11%)

• 2 variants in a coagulation factor that partially explain the phenotype

• 6 pathogenic variants in MYH9, PROC, PROS1, RUNX1, SERPINC1, and TUBB1,
A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders

Group 3  -  Bleeding of Uncertain Pathogenesis

Other 68 (89%) cases

either phenotype misinterpreted or…

Other, novel genetic loci implicated
Guidelines for investigating causality of sequence variants in human disease

D. G. MacArthur et

Nature April 24th 2014

The bio-informatic challenge of assigning causality to sequence variants.
Conclusions

1. Diagnosis of Inherited bleeding disorders will continue to rely critically on standard coagulation test performance.

2. Molecular diagnostic testing for these conditions can provide supplementary information in addition to confirming the phenotypic diagnosis.

3. The recent introduction of high-throughput NGS-based genetic testing for these conditions may find a place in routine diagnosis of these conditions depending upon the cost of these tests.

4. Importantly, NGS-based genomic strategies will be invaluable for characterizing the genetic basis for currently unresolved inherited bleeding problems.