Hemophilia and von Willebrand disease together account for the large majority of congenital bleeding disorders. Contemporary management, including development of safer clotting factor concentrates and increased emphasis on long-term follow-up in comprehensive hemophilia centers, has improved both quality of life and longevity for patients with congenital bleeding disorders. In addition to facilitating development of recombinant clotting factor concentrates, isolation and characterization of the respective genes have led to increasing availability of a repertoire of genetic tests that, although expensive, are critical for appropriate genetic counseling of affected patients and their family members. This article provides a practical approach to using genetic testing for hemophilia A and B.


Platelets are the cornerstone of primary hemostasis. Platelet adhesion to exposed subendothelial collagen is mediated by von Willebrand factor (vWF). The resulting platelet activation leads to 3 key events: release of platelet α and δ (dense) granule contents, which activates additional platelets; platelet aggregation, which is mediated by fibrinogen or vWF; and exposure of negatively charged platelet membrane phospholipids, which provides a surface for assembly of the procoagulant clotting factors.

The aim of secondary hemostasis is to form a fibrin clot. Vascular injury exposes tissue factor, which is normally sequestered from the circulation, resulting in activation of the procoagulant cascade (Figure 1). Tissue factor-activated factor VIIa complex activates factor X to factor Xa and factor IX to factor IXa. Factor IXa, with factor VIIIa as a cofactor, also activates factor X to factor Xa. Factor Xa, with factor Va as a cofactor, activates prothrombin (factor II) to thrombin (factor IIa). These reactions occur on the negatively charged phospholipids of activated platelets. In addition to feedback amplification actions, factor IIa cleaves fibrinogen to fibrin monomers, activates platelets, and activates factor XIII to factor XIIIa, which cross-links strands of fibrin monomers to form an insoluble hemostatic thrombus.

Bleeding disorders can be broadly classified as primary and secondary hemostatic defects. Primary hemostatic defects consist of quantitative and qualitative platelet disorders (congenital and acquired); however, the most common congenital bleeding disorder is thought to be von Willebrand disease (vWD.) Deficiencies of components of the procoagulant cascade are considered secondary hemostatic defects, of which hemophilia A and B are best characterized.

Advances in molecular genetics and sequencing of the human genome led to isolation and characterization of the respective genes. This not only allowed advances in development of recombinant clotting factor concentrates but also facilitated the availability of genetic tests. Thus, the role of the hemophilia center has expanded from clinical genetic counseling to ordering tests, interpreting results, and providing posttest counseling for families with hemophilia. This article provides a practical review of indications for and interpretations of genetic testing for hemophilia A and B.

**DISEASE SYNOPSIS**

**EPIDEMIOLOGY**

Hemophilia A and hemophilia B are single-gene, clinically indistinguishable, X-linked recessive bleeding disorders that are due to a deficiency in blood coagulation factor VIII and factor IX, respectively. Hemophilia A affects approximately 1 in 5000 live male births among all ethnic populations. Hemophilia B affects 1 in 30,000 live male births across all ethnic groups, and like hemophilia A, about 30% of cases are sporadic.

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DIAGNOSIS AND CLASSIFICATION

Hemophilia A. The diagnosis of hemophilia A is established on the basis of reduced or absent factor VIII activity and is classified on the basis of the level of factor VIII activity as severe (<1%), moderate (1%-5%), or mild (>5%-40%) disease, the estimated prevalence being 43%, 26%, and 31%, respectively.

Key Considerations. Four key considerations warrant attention in the diagnosis of hemophilia A. (1) Factor VIII is a labile protein; thus, factor VIII activity assays are ideally performed on fresh plasma samples. If this is not feasible, prompt freezing of the sample at –20°C is recommended; however, this may result in loss of factor VIII activity of 10% to 20%. Thus, mild to moderate reductions in factor VIII activity should be verified with a subsequent fresh plasma sample. (2) von Willebrand factor is a carrier protein for factor VIII; thus, reduced factor VIII activity should always prompt performance of vWF assays to exclude the possibility of vWD. (3) Rarely, mild hemophilia A with autosomal inheritance may actually reflect the presence of a specific subtype of vWD, type 2N (Normandy). This disorder occurs as a result of mutations in the factor VIII binding domain of vWF, resulting in defective binding of factor VIII to vWF. Typically, factor VIII activity is reduced, and vWF activity and antigenic assays are normal. (4) Blood for clot-based factor VIII activity is typically collected in citrate anticoagulant, which binds calcium. For blood samples with an increased hematocrit level (in which the relative plasma volume is decreased) or decreased hematocrit level (in which the relative plasma volume is increased), the volume of anticoagulant will need to be adjusted to maintain an optimal ratio of citrate volume to blood in order to obtain an accurate estimate of factor VIII activity. Typically, term neonates have an increased hematocrit level in umbilical cord blood samples, which may underestimate factor VIII activity.

Hemophilia B. As with hemophilia A, the diagnosis is established on the basis of plasma factor IX activity, which...
allows classification of hemophilia B as severe (<1%), moderate (1%-5%), or mild (>5%-40%) disease.6

Key Considerations. At least 3 key considerations warrant attention in the diagnosis of hemophilia B. (1) The diagnosis of hemophilia B relies on an accurate factor IX activity assay. Factor IX is a vitamin K–dependent protein6; thus, vitamin K deficiency and use of vitamin K antagonists such as warfarin should be excluded before the patient is diagnosed as having hemophilia B. (2) Factor IX levels in newborns and children are lower than adult reference ranges and typically increase progressively with age, a critical piece of information when interpreting factor IX levels in children and adolescents.11 (3) As with factor VIII activity assays, adjustment of citrate for hematocrit is necessary.

Clinical Features
In general, patients with hemophilia can present in 4 ways. (1) In familial hemophilia, carriers may undergo prenatal testing of male fetuses, or alternatively, factor VIII or factor IX activity assays performed on umbilical cord blood at the time of delivery may confirm the diagnosis. (2) Depending on severity, evaluation of excessive bleeding after circumcision may lead to the diagnosis of moderate or severe hemophilia. (3) For infants who do not undergo surgical procedures within the first 6 to 12 months of life, evaluation of either spontaneous or easy bruising as the child becomes more active typically leads to the diagnosis. (4) Rarely, in patients with mild hemophilia, the diagnosis may not be made until adulthood, when investigation of postoperative hemorrhage or evaluation of a prolonged activated partial thromboplastin time typically obtained by some surgeons as routine preoperative screening leads to the diagnosis.

Frequency and type of bleeding symptoms vary with severity of hemophilia. Patients with severe disease experience spontaneous bleeding that consists of hemarthrosis, soft tissue hematomas, and intracranial hemorrhage in addition to minor hemorrhage such as epistaxis and ecchymoses. Patients with moderate disease experience infrequent spontaneous bleeding; however, they typically experience bleeding after minor trauma. Patients with mild hemophilia generally experience hemorrhage after major trauma or surgery. Before the introduction of prophylactic clotting factor infusions, recurrent hemorrhosis resulted in progressive destructive arthropathy that necessitated early joint replacement. Since the introduction of prophylactic infusion therapy, patients lead a relatively normal life. Thus, contemporary management of hemophilia has led to an increase in median life expectancy from approximately 30 years in the 1980s to approximately 65 years.4

Management
The mainstay in the management of hemophilia consists of prophylaxis and treatment of bleeding episodes. In the past, recurrent bleeding resulted in frequent emergency department visits and hospitalizations; however, early studies of home self-infusion therapy programs showed clear benefits in reduced hospitalization and arthropathy.12 This led to programs of prophylactic infusions that further reduced morbidity and mortality of hemophilia.13 Substantial progress has been made since the introduction of cryoprecipitate for the treatment of bleeding.14 The development of plasma-derived clotting factor concentrates, introduction of home infusion programs, and eventual introduction of recombinant clotting factor concentrates have led to marked improvements in overall management of patients with bleeding disorders.

At the time of the initial diagnosis of mild or moderate hemophilia A, a desmopressin (1-deamino-/4-valine-8-D-arginine-/vasopressin) trial is performed. Factor VIII activity is checked before and 1 hour after an intravenous infusion of 0.3 µg/kg of desmopressin or intranasal desmopressin. Depending on the degree of increase in factor VIII activity, desmopressin may be used for management of minor hemorrhage. For prophylaxis and treatment of major hemorrhage and surgery, bolus or continuous intravenous infusion of factor VIII concentrates is considered the standard of care.

Complications of Hemophilia
Initial use of plasma-derived factor concentrates resulted in a high incidence of transfusion-transmitted viruses such as hepatitis15,16 and human immunodeficiency virus.17 Identification of pathogens, improvements in purification methods, and subsequent development of recombinant clotting factor concentrates have virtually eliminated this complication. Currently, the most serious complication of hemophilia is the development of inhibitor antibodies, predominantly against factor VIII, which occurs frequently in response to infusion of factor VIII concentrates in those with severe hemophilia and rarely in those with mild hemophilia A. The prevalence of factor VIII inhibitors is approximately 6%, with an annual incidence of 3.5 per 1000 patients with severe hemophilia A.18 Studies of predictors of development of inhibitors point to a genetic predisposition, with up to 30% of patients with the factor VIII inversion mutation and large deletions developing factor VIII inhibitors.19 Inhibitor antibodies against factor IX occur in some patients with severe hemophilia B in response to infusion of factor IX concentrates. More recently, it was recognized that patients with deletions of the factor IX gene are at high risk of developing inhibitors and anaphylactic responses to infusions of factor IX concentrates.20,22
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GENETIC BACKGROUND

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Located on the long arm of the X chromosome (Xq28), approximately 500 Mb telomeric to the glucose-6-phosphate dehydrogenase gene (Figure 2), the factor VIII gene’s structure is typical. It is 186 kilobase (kb) with 24 relatively short exons, ranging from 69 to 262 base pairs (bp), and 2 long exons, exon 14 (3106 bp) and exon 26 (958 bp). The resulting messenger RNA (mRNA) is approximately 9 kb, the coding sequence of which is 7053 bp. The intron-exon boundaries correlate roughly with the factor VIII domains. The introns are large (14-32 kb). Intron 22 is the largest (32 kb) and is of interest given that the most common mutation in hemophilia A occurs as a result of an inversion involving intron 22.

A CpG island in intron 22 acts as a bidirectional promoter for 2 additional genes. The first, termed factor VIII–associated gene A (F8A or Int22h-1), is an intronless gene approximately 2 kb within intron 22 that is transcribed in the opposite direction of factor VIII. The second, factor VIII–associated gene B (F8B), is 2.5 kb and transcribed in the same direction as factor VIII. The F8A and F8B transcripts originate within 122 bases of each other; however, the function of their potential protein products is unknown. The F8A sequence is replicated at least twice, approximately 500 kb telomeric to the factor VIII gene and close to the tip of the X chromosome termed Int22h-2 (proximal) and Int22h-3 (distal). Int22h-2 and Int22h-3 are about 100 kb apart and are transcribed in the same direction as the factor VIII gene and thus in the opposite direction of Int22h-1. These 3 homologous repeats are involved in the intron 22 inversion mutation, which is a frequent rearrangement of the factor VIII gene resulting in severe hemophilia A.

Factor VIII is predominantly expressed in the liver. The putative promoter region is located 300 nucleotides 5′ to the gene. The factor VIII gene encodes a precursor protein of 2351 amino acid residues consisting of a 19–amino acid leader peptide followed by 2332 amino acids in the mature protein. The mature protein can be divided into several homologous domains termed A1, A2, B, A3, C1, C2 (Figure 3). Proteolytic cleavage, by thrombin (factor IIa) or factor Xa in the presence of phospholipid surfaces,
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results in activation of factor VIII to factor VIIIa. The exact function of the B domain is unknown because it is cleaved during proteolytic activation. However, because B-domain-deleted factor VIII molecules are expressed at 5-fold to 10-fold higher levels than non–B-domain–deleted factor VIII molecules, the B domain may have a role in intracellular processing and/or secretion of factor VIII. Mutations in this region have been reported in hemophilia A.

von Willebrand factor–bound factor VIII is protected from inactivation by activated protein C. The putative vWF binding region on factor VIII is thought to be at the N-terminus of the light chain of factor VIII and in the C2 domain. The binding site for factor IXa has been localized to the A2 domain and regions of the light chain. In addition, the binding site for factor X is localized to the C-terminus of the A1 domain. Binding to phospholipids, which are important for factor X activation by factor IXa and factor VIIIa, occurs in the C1 and C2 domains of the light chain factor VIII. No deleterious mutations in hemophilia A have been identified in the inactivation cleavage sites of factor VIII.

Mutations in the Factor VIII Gene. The type of mutation in the factor VIII gene predicts disease severity. Mutations that result in a significant disruption of the factor VIII protein or alter an important functional site will result in severe disease, whereas mutations that alter apparently “minor” regions of the factor VIII protein result in mild to moderate disease. Typically, deletion of regions of factor VIII, insertions of fragments of DNA that disrupt the factor VIII protein, and mutations that result in premature termination of the synthesis of factor VIII, eg, nonsense mutations, will result in severe disease. Some missense mutations in regions of critical function also result in severe disease.

The molecular pathology of hemophilia A, which has been reviewed comprehensively, is addressed briefly herein. The most common mutation, found in approximately 40% of patients with severe hemophilia A, is the intron 22 inversion mutation. This mutation results when homologous recombination occurs between the F8A gene (Int22h-1) in intron 22 and 1 of 2 homologous regions (Int22h-2 or Int22h-3) telomeric to the factor VIII gene. This probably occurs as a result of folding over of the tip of the X chromosome. On unfolding, exons 1 to 22 are inverted and placed about 500 kb upstream of exons 23 to 26 and oriented in the opposite direction. Depending on which repeat the F8A pairs with, the inversion may be termed type I (distal) or type II (proximal). Rarely, type III inversion mutations occur in patients with a third extragenic copy of F8A. Given that most inversions originate in male meiosis, almost all mothers of patients with the inversion mutation are carriers, with the mutation having originated within the germline of the maternal grandfather.

An additional inversion of intron 1 of the factor VIII gene that affects up to 5% of patients with severe hemophilia A has been described. The large size of the factor VIII gene predisposes to the occurrence of deletions, which account for approximately 5% of characterized mutations. Typically, these result in severe disease with less than 1% factor VIII activity. However, specific deletions of 156 bp in exon 22 or the 294 bp of exons 23 and 24 are associated with moderate disease, likely because of in-frame splicing of exon 21 to exon 23 or exon 22 to exon 25.
Patients with large deletions are susceptible to formation of factor VIII inhibitors (antibodies) in response to therapy with factor VIII concentrates. In an analysis of the hemophilia A database, it was found that up to 40% of patients with deletions develop factor VIII inhibitors, whereas up to 60% of patients with single base pair changes resulting in nonsense mutations and 15% of patients with single base pair changes resulting in missense mutations develop inhibitors.29

Single base pair changes (resulting in missense, frameshift, or splice junction mutations), insertions, or duplications account for the balance of cases of hemophilia A that are spread throughout the factor VIII gene.3 Although the structure-function relationships of some of the missense mutations are known or can be deduced (eg, alteration of vWF binding site, thrombin cleavage site, etc), the structural consequences of most such mutations remain undefined. Currently, no deleterious mutations have been found in the promoter region. Mutations and polymorphisms in the factor VIII gene are cataloged in an international database available on the Internet and updated periodically at http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm.

**Polymorphisms in the Factor VIII Gene.** A polymorphism is an alteration in the DNA sequence of a gene resulting in 2 or more alleles with a frequency of 1% or greater in the population and typically does not result in disease; thus, such alterations are considered “neutral” changes in DNA. Polymorphisms present within the factor VIII gene (intragenic) or outside the factor VIII gene (extragenic) have been used to assign haplotypes (combinations of polymorphisms) for linkage analysis. The putative defective factor VIII gene can be tracked with polymorphisms that are closely linked to the gene. The carrier frequency of such polymorphisms varies depending on ethnicity of the study population and needs to be considered when studying patients of diverse ethnic origin. A complete listing of polymorphisms is available in the factor VIII mutation database (http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm).

**DETERMINATION OF FACTOR VIII INHIBITORS.** A single antibody test is sufficient to confirm the presence of factor VIII inhibitors. However, confirmation can be obtained by classical activation assays (eg, platelet factor 3 assay); activated partial thromboplastin time (aPTT) is prolonged in the absence of aPTT activators. A confirmatory assay is required if factor VIII levels are considered normal.

**DETERMINATION OF FACTOR IX INHIBITORS.** The factor IX gene is located on the long arm of chromosome (Xq27.1) and is 38 kb with 8 exons of varying lengths (25-1935 bp). The resulting mRNA is approximately 3 kb, of which the coding sequence is 1390 nucleotides. The intron/exon boundaries roughly correlate with the factor IX domains (Figure 4) and bear a high degree of homology to members of the vitamin K–dependent protein family (factor VII, factor X, and protein C). The factor IX gene encodes a precursor protein of approximately 454 amino acid residues consisting of a signal peptide, propeptide followed by a glutamic acid–rich domain, 2 epidermal growth factor (EGF) domains, an activation peptide, and a catalytic domain.

Activation of factor IX to factor IXa occurs by cleavage by factor VIIa–tissue factor or activated factor XI. This releases the activation peptide that results in a circulating light chain and a heavy chain connected by the disulfide bond. Numerous posttranslational modifications are necessary for its normal function, including tyrosine sulfation, serine phosphorylation, and O- and N-linked glycosylation. The relatively small size of the factor IX gene lends itself to detailed molecular analysis, which has been cataloged in a database that is updated regularly. The number system used subsequently can be accessed at http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html.

**Deleterious Mutations in the Factor IX Gene.** Most mutations are single base pair changes that result in missense, frameshift, or nonsense mutations. Short deletions (<30 nucleotides) account for approximately 7%, larger deletions approximately 3%, and insertions approximately 2% of mutations. Many of the single base pair changes occur at CpG doublets that are “hot spots” for mutation. However, a subset of repeated mutations is due to a founder effect, which typically results in mild disease. Mutations have been detected in all regions, including the poly(A) signal.

Missense mutations account for most mutations that typically result in mild disease unless the mutations occur in...
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residues critical for normal factor IX function. Some mutations in the promoter region of the factor IX gene result in a unique phenotype, termed hemophilia B Leyden, which is characterized by severe disease at birth with progressive amelioration of severity throughout adolescence and puberty. However, some promoter mutations (e.g., Brandenburg mutation at –26) in the factor IX promoter result in a severe lifelong disease state. Nonsense mutations in the signal peptide and propeptide regions lead to severe hemophilia B; however, missense changes leading to retention of factor IX within hepatic cells have been described (e.g., Ile-30 and Ile-19). Lack of cleavage of the propeptide leads to a dysfunctional factor IX molecule (e.g., Arg-4).

Mutations in the glutamic acid–rich domain disrupt γ-carboxylation (posttranslational modification) and calcium binding, the latter of which is important for normal factor IXa binding to collagen, activated platelets, and endothelial cells. Mutations in the EGF domains disrupt protein-protein interaction (factor VIIIa and factor VIIa-tissue factor), resulting in reduced activation of factor IX. Also, EGF mutations affect binding to calcium, which is essential to its procoagulant activity. Mutations in the catalytic domain typically disrupt the catalytic triad (His-221, Asp-269, and Ser-365), which is essential for factor IXa protease function.

An unusual factor IX variant due to mutation at Ala-10 is characterized by normal baseline factor IX activity. However, warfarin therapy results in a severe and disproportionate reduction in factor IX activity (typically <1%), which causes bleeding in patients being treated with warfarin who have an apparently therapeutic international normalized ratio. An indication of such a situation is a disproportionate prolongation of the activated partial thromboplastin time, which should prompt clotting factor assays.

**Polymorphisms in the Factor IX Gene.** Eight common polymorphisms have been described in different ethnic populations of European and African descent. These polymorphisms, however, are much less common in Asian and other populations. The most informative polymorphism documented in the Asian population is HhaI (allele frequency, 0.17); several recent reports have described additional polymorphic loci in these populations, facilitating molecular diagnosis of nonwhite carriers and patients with hemophilia B. A polymorphism within the factor IX coding region, Ala148Thr, occurs within the activation peptide. This does not correlate with factor IX activity or antigenic levels. The Ala allele occurs with a frequency of <0.3 in the white population; however, it is much less frequent in the African American population (0.053-0.15) and Asian population (<0.01).

**Inheritance of Hemophilia A and B**

Hemophilia A and B are X-linked recessive disorders in which males are affected and females are typically asymptomatic carriers of the hemophilia defect. Carrier females have a 50% likelihood of having either an affected son or a carrier daughter (Figure 5). Although most carriers are asymptomatic, some carriers have reduced factor VIII and factor IX levels and may have clinically important bleeding. The molecular basis in symptomatic females includes skewed lyonization of the normal X chromosome and Turner syndrome (XO karyotype), in which the dominant
mutant factor VIII or factor IX gene is responsible for production of the abnormal factor VIII or factor IX, respectively. Affected males cannot transmit hemophilia to male offspring, whereas all female offspring of patients with hemophilia will be obligate carriers of the hemophilia defect. In extremely rare cases, the offspring of an affected male and a carrier female will be an affected male.

**RATIONALE FOR GENETIC TESTING**

**GENETIC TESTING OF AFFECTED MALES**
The rationale for genetic testing of males with hemophilia is as follows.

1. Prenatal testing of male fetuses establishes a diagnosis of hemophilia, which is critical for management of labor and delivery.

2. Knowledge of mutation can predict severity of disease. In symptomatic carriers with no family history of hemophilia and thus no information on clinical phenotype, genotyping of the carrier and/or fetus or child can predict severity of disease.

3. It is important to differentiate mild hemophilia A from vWD type 2N. Options to achieve such a differentiation include assessment of factor VIII binding to vWF, genotyping the factor VIII binding domain of vWF, or factor VIII genotyping. Assessment of factor VIII binding to vWF or genotyping the factor VIII binding domain of vWF is a more cost-effective approach.

4. Knowledge of factor VIII or factor IX gene mutation is useful in estimating the risk of developing factor VIII\(^2\) and factor IX\(^2\) inhibitors.

5. Determining the genotype of patients with hemophilia A and hemophilia B is critical for cost-effective carrier testing of at-risk female family members.

**GENETIC TESTING OF AT-RISK FEMALES**
The rationale for genetic testing of females with hemophilia is as follows.

1. In familial hemophilia, paternity issues aside, genetic testing of obligate carriers (daughters and mothers of affected males) is typically not performed to establish carrier status but to establish genotype for possible future prenatal diagnosis.

2. In familial and sporadic hemophilia, genetic testing of at-risk female family members (e.g., sisters, maternal aunts, or maternal cousins of patients with hemophilia A) is performed to establish carrier status, which is critical for optimal prenatal counseling and testing if indicated.

3. Genotyping of symptomatic carriers with no family history of hemophilia (thus no information on severity of disease) will predict severity of hemophilia if the carrier were to have an affected male child.

**RATIONAL APPROACH TO GENETIC TESTING IN HEMOPHILIA A**

**APPROACH TO GENETIC TESTING OF AFFECTED MALES**

Genetic testing of affected males is as follows (Figure 6).

1. Confirm the diagnosis of hemophilia A.

2. Provide pretest counseling regarding the risks, benefits, potential outcomes, costs, and clinical utility of testing.

3. For severe hemophilia A, test for intron 22 inversion mutation, and if result is negative, follow up with testing for intron 1 inversion mutation.

4. If inversion mutations are not found and/or the patient has mild to moderate disease, analysis of the relevant regions (coding region, splice junctions, and promoter) of the factor VIII gene is warranted.

5. Posttest counseling is critical to review results of testing, utility of results, and clinical importance of results.

**APPROACH TO GENETIC TESTING OF AT-RISK FEMALES**

Although reduced factor VIII activity in at-risk females typically confirms carrier status, normal factor VIII activity does not exclude the possibility. Thus, molecular genetic
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1. Confirm the diagnosis of hemophilia A in family member.
2. Provide detailed pretest counseling to discuss estimates of risks of being a carrier, likelihood of finding a mutation, and clinical utility of genetic test results.
3. For at-risk females in whom the familial genotype is known, proceed with focused testing for the individual factor VIII mutation.
4. If the familial factor VIII genotype is unknown, attempt to obtain information on clinical phenotype.
5. If clinical phenotype is consistent with severe disease or is unknown, proceed with testing for the intron 22 inversion mutation, and if result is negative, test for the intron 1 inversion mutation.
6. If inversion testing is negative and/or phenotype reveals mild or moderate disease, proceed with analysis of the factor VIII gene (Figure 6).
7. Rarely, clinicians are faced with an adopted asymptomatic female who has no access to phenotypic or genotypic information; in fact, it may not be known whether there is a family history of hemophilia A or B. Clotting factor (VIII and IX) analysis should be performed. If results are normal, genetic testing poses a challenge; how-

FIGURE 7. Algorithm for approach to genetic testing of at-risk hemophilia A carrier. *Refer for linkage analysis if testing detects no mutation in proband or carrier.
ever, it may be reasonable to follow the testing algorithm outlined for hemophilia A (given the higher frequency compared to hemophilia B) and if results are negative, proceed with testing algorithm for hemophilia B.

8. Posttest counseling is critical to educate the patient about findings, clinical importance, and use in ongoing management.

RATIONAL APPROACH TO GENETIC TESTING IN HEMOPHILIA B

APPROACH TO GENETIC TESTING OF AFFECTED MALES

Genetic testing of affected males is as follows.

1. Confirm the diagnosis of hemophilia B.

2. Provide pretest counseling regarding the risks, benefits, potential outcomes, and clinical utility of testing.

3. For patients with mild hemophilia B, perform an analysis for the presence of the 3 founder mutations.

4. If founder mutations are not discovered and/or the patient has severe hemophilia B, proceed with analysis of the factor IX gene (Figure 8).

5. Posttest counseling is critical to review results of testing, utility of results, and clinical importance of such results.

APPROACH TO GENETIC TESTING OF AT-RISK FEMALES

Although reduced factor IX activity in at-risk females typically confirms carrier status, normal factor IX activity does not exclude the possibility. Thus, molecular genetic testing is the only option for diagnosis. Apart from the approximately 30% incidence of new mutations that lead to hemophilia in families with no history of hemophilia (sporadic disease), causative mutations in familial hemophilia remain constant within families. Thus, knowledge of the genotype of an affected male in the family facilitates focused carrier testing of at-risk females (Figure 9).

1. Confirm the diagnosis of hemophilia B in family member.

2. Provide detailed pretest counseling to discuss estimates of risks of being a carrier, likelihood of finding a mutation, and clinical utility of genetic test results.

3. For at-risk females in whom the familial genotype is known, proceed with focused factor IX gene testing.

4. For at-risk carriers in whom familial genotype is unknown and clinical phenotype is consistent with mild hemophilia B or clinical phenotype is unknown, proceed with testing for founder mutations.

5. If founder mutations are not discovered and/or clinical phenotype is consistent with moderate or severe disease, proceed with factor IX gene analysis.

6. For patients who do not have access to such information, analysis of the entire factor IX gene is feasible.

7. Posttest counseling is critical to educate the patient about findings, clinical importance, and use in ongoing management.

OVERVIEW OF METHODS FOR SPECIFIC GENETIC TESTS FOR HEMOPHILIA A

Reviews of genetic testing methods for hemophilia A have been published recently. In general, testing can be divided into direct and indirect testing, and selected laboratories offer prenatal testing. Screening methods specifically used in analysis of factor VIII gene mutations can be accessed at the factor VIII mutation database Web site (http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm).

GENETIC TESTING OF AFFECTED MALES

Apart from assigning haplotype for linkage analysis of family members, there is no role for indirect testing of the affected male. Thus, testing primarily consists of direct DNA-based analysis.

Direct DNA-Based Analysis. DNA-based analysis for the intron 22 inversion mutation is typically performed by the Southern blotting technique. Briefly, DNA extracted from either peripheral blood leukocytes or chorionic villus samples is cleaved by restriction endonuclease enzyme,
transferred onto a piece of nitrocellulose or nylon filter paper, and incubated with a radioactive-labeled probe. The labeled probe hybridizes to the region of interest and renders the bands radioactive that contain complementary sequences. The final bands are visualized by autoradiography. This technique detects the more common types I and II inversions and the rare type III inversion. Intron 1 inversion testing is also performed by the Southern blotting technique. A polymerase chain reaction (PCR)–based assay for detecting the intron 22 inversion has been described.

**Follow-up Testing of Patients Without the Inversion Mutations.** For patients with severe hemophilia A who do not have the inversion mutations or for patients with mild or moderate hemophilia, detection of alternative mutations is indicated. Insertions, deletions, missense mutations, or nonsense mutations can result in severe disease. Amplification of regions of the factor VIII gene by PCR and agarose gel electrophoresis allows assessment of the presence or absence and size of the amplified regions. This step detects insertions and deletions of the patient’s factor VIII gene. Further characterization of the deletion junctions may provide useful information for carrier testing.

**Detection of Point Mutations.** For the rest of the patients with hemophilia A, screening techniques, eg, single-stranded conformational polymorphism or proceeding with direct DNA sequencing of all the relevant regions of the factor VIII gene allows detection of point mutations. Because of the large size of the factor VIII gene (26 exons), this is a labor-intensive and expensive task; however, availability of automated methods has led to more efficient
testing. Alternative strategies include analysis of factor VIII mRNA by reverse transcriptase–PCR; however, although this is probably less labor intensive, it is likely not practical because of the instability of mRNA, unless the testing is done with a fresh peripheral blood sample that is processed promptly.

**GENETIC TESTING OF CARRIERS**

Direct DNA-based testing provides the most definitive information on carrier status. However, for selected deletions and in the small proportion of patients in whom mutations are not found, carrier testing would consist of linkage analysis.

**Direct DNA-Based Testing.** Southern blotting techniques can be used to detect the inversion mutations. Polymerase chain reaction amplification and sequence analysis for point mutations, deletions of portions of exons, and insertions provide information on carrier status. Carrier testing by direct techniques in which the proband has a deletion of whole exons poses a challenge. Given that females have 2 copies of the factor VIII gene, attempts at PCR amplification will not detect deletions because the normal factor VIII allele, if present, will be amplified. Thus, either linkage analysis or the Southern blotting technique using probes designed for different regions of the factor VIII gene is required.

**Indirect DNA Analysis (Linkage Analysis).** Linkage analysis is typically used when only the location of a gene is known but not the gene itself or when currently available direct genetic testing has been unable to detect a mutation, although the gene location has been identified. The principle underlying genetic linkage analysis is the tendency for polymorphisms (alleles) close together (also called haplotypes) on the same chromosome to be transmitted together as an intact unit through meiosis. Polymorphisms within the factor VIII gene (intragenic) or outside the factor VIII gene (extragenic) are typically single base pair changes or simple sequence repeats (CAn repeats). Testing for the presence of these haplotypes consists of relatively uncomplicated methods, such as PCR amplification and restriction enzyme digestion, that provide information on the presence of restriction fragment length polymorphisms. These restriction fragment length polymorphisms can then be tracked within family members, which provides an estimate of the risk of being a carrier.

Disadvantages of indirect testing include the need for DNA samples from multiple affected and unaffected family members and the requirement that the proband’s mother be heterozygous and thus “informative” for the polymorphism. The informativeness of the polymorphism implies that the DNA sequence at a locus differs on the maternally inherited and paternally inherited chromosomes. Most families (up to 90%) are informative with 1 or more DNA polymorphisms if both intragenic and extragenic polymorphisms are analyzed. The distance between the disease-causing mutation and the polymorphism, ie, the risk of recombination, may lead to false-positive or false-negative results. This risk is lowest if intragenic markers are used (<1%). A major drawback with linkage analysis is the possibility of unmasking nonpaternity in any given family. A thorough discussion of these advantages and disadvantages of linkage analysis is critical during the pretest counseling phase and should not be deferred to the posttest counseling phase.

**INTERPRETATION OF TEST RESULTS**

Reduced factor VIII activity is virtually diagnostic of hemophilia A if specimen artifact has been excluded. Given that vWF is a binding protein for factor VIII, a reduced vWF, as can be seen in vWD, typically results in a mild to moderate reduction in factor VIII activity and may lead to a false diagnosis of hemophilia A. Thus, obtaining vWF levels in all patients with reduced factor VIII activity is critical to exclude vWD. Rare instances of other genetic disorders that can have low factor VIII activity include vWD type 2N and rare combined deficiencies of factor V and factor VIII. The latter occurs as a result of mutations in LMAN-1 (formerly ERGIC-53), which is a protein necessary for efficient transport of factor V and factor VIII from the endoplasmic reticulum to the golgi. Factor V and factor VIII levels are typically in the 10% to 15% range. Molecular genetic testing is available only on a research basis.

Detection of a deleterious mutation assigns the genotype in the proband and confirms carrier status in the at-risk female. Certain well-defined mutations (eg, inversions, deletions, insertions, splice junction mutations, and nonsense mutations) have obvious deleterious effects. Determining the deleterious nature of previously uncharacterized missense mutations poses a challenge. However, the presence of such a mutation in hemizygous genes (factor VIII and factor IX) and the absence of additional mutations provide reasonable evidence of its deleterious nature. Other criteria typically considered include analysis of the degree of conservation of the respective residue among other species and the presence of a similar mutation in other patients. The best evidence of a deleterious nature of a missense mutation is in vitro confirmation of its effect on protein function.

**GENETIC COUNSELING**

**Counseling the Affected Patient.** As discussed previously, knowledge of the genotype does not affect routine clinical management of the patient. Established procedures
for evaluation and management of the patient with a new diagnosis and ongoing management with comprehensive hemophilia evaluations are still used. The predisposition to developing inhibitors in patients with certain genotypes may warrant closer follow-up and testing; however, currently, it is not considered standard practice to use factor VIII bypassing agents in previously untreated patients to reduce the risk of development of inhibitors.

Providing the patient with information on yield of genetic testing is critical. Up to 40% of patients with severe hemophilia A (<1% factor VIII activity) will be found to have the intron 22 inversion mutation, and up to 5% of patients with severe hemophilia A will have the intron 1 inversion mutation. For inversion-negative patients, 29% (271/943) will have deletions (small and large), 6% (57/943) will have insertions, and 66% (624/943) will have point mutations, including missense, nonsense, and splice junction mutations (http://europium.csc.mrc.ac.uk). A key point in providing counseling is to highlight the fact that, despite thorough evaluation of the factor VIII gene, approximately 2% of patients with hemophilia A will have no detectable mutations. In this situation, linkage analysis would need to be considered, which assumes that the causative defect in this subgroup of patients lies within or close to the factor VIII gene.

Counseling the Carrier. Most carriers have normal levels of factor VIII activity, and knowledge of their genotype will not affect routine management. Knowledge of carrier status provides essential information for prenatal counseling of the risk of having an affected male or a carrier female child and the decision to undergo prenatal testing. Typically, because the large majority of females are asymptomatic, prenatal testing of female fetuses is not recommended, whereas male fetuses may be at risk of being affected with potentially severe disease, thus providing rationale to pursue such testing.

The information gained from knowing whether a male fetus is affected can typically be used in 1 of 2 ways. The first issue is whether the carrier would want to continue the pregnancy. Although it is commonly believed that carriers would choose to undergo prenatal testing and would consider termination of the pregnancy, in 1 report of 35 carriers, 43% (15/35) chose prenatal testing; however, only 17% (6/35) would choose to terminate their pregnancies. The second issue is management of labor and delivery. Options include umbilical cord infusion before induction of labor or immediate infusion of factor VIII on delivery.

A complete listing of laboratories that offer genetic testing for hemophilia A and that are registered with GeneTests can be found on its Web site (www.genetests.org), and the accompanying GeneReviews is an excellent resource. In general, testing can be divided into direct and indirect testing, and selected laboratories offer prenatal testing.

OVERVIEW OF METHODS FOR SPECIFIC GENETIC TESTS FOR HEMOPHILIA B

As with hemophilia A, apart from assigning haplotype for linkage analysis of family members, there is no role for indirect testing of the affected male. Thus, testing consists primarily of direct DNA-based analysis. However, for selected deletions and in the small proportion of patients in whom the mutations are not found, carrier testing consists of linkage analysis.

Direct DNA-Based Analysis

Generally, most patients with hemophilia B have mild disease. Thus, because up to 25% of white patients with mild hemophilia B have 1 of 3 founder mutations (Gly60Ser, Ile397Thr, and Thr296Met), the first logical step is to perform limited testing for these founder mutations. For patients with hemophilia B in whom a founder mutation is not discovered, patients with moderate and severe hemophilia B, and at-risk carriers, the next logical step is screening or sequencing regions of functional significance in the factor IX gene. Although most patients with hemophilia B have 1 deleterious mutation, about 1% have double mutations. For probands with deletion of part of their factor IX gene, carrier testing by PCR amplification of individual exons will likely not be useful. Thus, either linkage analysis or Southern blotting technique is required.

Indirect DNA Analysis (Linkage Analysis)

As discussed previously, indirect DNA analysis of the proband does not provide any information on the proband’s genotype. However, it does assign a haplotype associated with the abnormal factor IX gene and is useful for carrier testing. Given the relatively small size of the factor IX gene, direct sequencing is feasible for carrier testing when the hemophilia B mutation is not a large factor IX gene deletion.

Genetic Counseling

Both pretest and posttest counseling are complex but are a critical component of genetic testing. The training, time, and effort needed for optimal communication of the effect of genetic testing on multiple aspects of the patient and family make this a daunting task for the untrained individual. In addition, psychosocial, economic, patient confidentiality, and health and life insurability issues (although beyond the scope of this article) are important and add another layer of complexity. Thus, although physicians, eg, hematologists, obstetricians, or other primary
care practitioners, may be knowledgeable about key issues, the time needed for such counseling in a busy clinical practice is not always available. Genetic counselors are likely best suited to provide such comprehensive counseling.

Counseling the Affected Patient. As discussed previously, knowledge of the genotype does not affect clinical management of the patient. Established procedures for evaluation and management of the patient with a new diagnosis and ongoing management with comprehensive hemophilia evaluations are still used. The predisposition to developing inhibitors and allergic reactions to infused factor IX concentrates in patients with certain genotypes may warrant closer follow-up and testing; however, currently, it is not standard practice to use bypassing agents to prevent the development of inhibitors. It is important to note that less than 1% of patients with hemophilia B will not have a detectable mutation in their factor IX gene.

Counseling the Carrier. Most carriers have normal levels of factor IX activity, and knowledge of their genotype will not affect routine management. Knowledge of carrier status provides essential information for prenatal counseling of the risk of having an affected male or a carrier female child and the decision to undergo prenatal testing. Typically, because the large majority of females are asymptomatic, prenatal testing of female fetuses is not recommended, whereas male fetuses may be at risk of being affected with potentially severe disease, thus providing rationale to pursue such testing.

The information gained from knowing whether a male fetus is affected can typically be used in 1 of 2 ways. The first issue is whether the carrier would want to continue the pregnancy. Although it is commonly believed that carriers would choose to undergo prenatal testing and would consider termination of the pregnancy, in 1 report of 35 carriers, 43% (15/35) chose prenatal testing; however, only 17% (6/35) would choose to terminate their pregnancies.40 The second issue is management of labor and delivery. Options include umbilical cord infusion before induction of labor or immediate infusion of factor VIII on delivery.46

A complete listing of laboratories offering hemophilia B genetic testing that have registered with GeneTests can be found on its Web site (www.genetests.org). In general, testing can be divided into direct and indirect testing, and selected laboratories offer prenatal testing.

GENETIC TESTING OF CHILDREN

Although a detailed discussion of genetic testing of children is beyond the scope of this review, this topic is a critical component of decision making.47 The 3 possible scenarios are (1) prenatal testing of the pregnant carrier, (2) testing of at-risk female family members who are minors, and (3) testing of the affected male to ascertain genotype to allow focused genetic testing of at-risk female family members.

Prenatal testing of male fetuses provides a diagnosis that substantially affects management of labor and delivery. Although the risk of perinatal hemorrhage is low, there is potential for a long-term effect, especially with intracranial hemorrhage,48 which can be minimized with infusion of the appropriate clotting factor concentrate.49 Patients are unlikely to have strong objections to such testing. The risks of prenatal testing of female fetuses must be carefully weighed in the context of its effect on labor and delivery. Currently, I do not recommend prenatal testing of female fetuses.

In general, testing of young at-risk female family members who have no immediate need for reproductive choices is discouraged until the individual reaches the age of majority (typically 18 years). Testing of the affected male purely for genotyping purposes does not affect the diagnosis of hemophilia; however, it needs to be undertaken after a thorough discussion of its usefulness.47

CONCLUSION

Genetic testing of families with bleeding disorders in general and hemophilia in particular is rapidly becoming part of routine care. Because of the substantial costs of testing, the onus of its optimal utilization rests with health care practitioners such as those in comprehensive hemophilia centers and genetic counselors and their interactions with patients and their family members. With judicious use of genetic testing, we can continue to improve the quality of life for patients with hemophilia.

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