Welcome to Mayo Medical Laboratories Profiles in Genetics. These presentations provide short discussion of current genetics topics and may be helpful to you in your practice. This presentation discusses the various options for prenatal diagnostic testing.
How to Order Genetic Testing for Hemophilia A or B: Part 2

Things you should know before you order a genetic test for hemophilia
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Disclosure

• None

I have nothing to disclose.
In part 1 of this talk, I covered when it is appropriate to test for hemophilia. Today, in part 2, I'll discuss the 2 pieces of information you need to choose the correct genetic test. I've simplified this information for anyone who is not a practicing hematologist or geneticist, but a referral to either of these specialized fields should be a consideration when evaluating someone with an apparent bleeding disorder because bleeding is a very nonspecific symptom that can be caused by a great number of things besides hemophilia. You can learn more about the great number of things that can cause abnormal bleeding in part 1 of this talk.

In part 2, we are focusing on the 2 critical pieces of information you need before you start ordering genetic testing for hemophilia.
Things You Need To Know Before Ordering Genetic Testing For Hemophilia

• #1:
  • Whether your patient or their family has a history of hemophilia A or B
  • You should determine if you are dealing with hemophilia A or hemophilia B prior to ordering genetic testing.

This is thing #1. I know this might seem obvious, but it turns out it is not. Unless the circumstances are pretty extraordinary, you need to know if you are dealing with hemophilia A or hemophilia B, prior to genetic testing.

The purpose of genetic testing is not to help distinguish between hemophilia A and B, because that can be easily and more cheaply accomplished with factor studies in affected males.
The purpose is to molecularly confirm the diagnosis and identify the specific mutation causing hemophilia for both prognostic and genetic counseling purposes.

When someone orders genetic testing for both hemophilia A and B on the same patient, at the same time, it indicates that someone somewhere misordered these tests; mistaking them for basic factor clotting activity tests perhaps, or that someone is confused about the use of genetic testing in hemophilia.

And, again, this is how you tell if you are dealing with hemophilia A or B. A deficiency in factor VIII indicates hemophilia A. A deficiency in factor IX indicates hemophilia B.
Things You Should Know Before You Order Genetic Testing For Hemophilia

#2:
- The severity of hemophilia affecting the patient or their family, which is based on factor levels in affected males in that family (note, these ranges apply to either hemophilia A or B):
  - Mild hemophilia: 0.05-0.4 IU/ml (5%-40% factor activity)
  - Moderate hemophilia: 0.01-0.04 IU/mL (1%-<5% factor activity)
  - Severe hemophilia: <0.01 IU/mL (<1% factor activity)
  - Normal range: >0.4 IU/ml (>40% to 150%)
  - >30%: usually no bleeding symptoms

The second thing you should know prior to ordering genetic testing, particularly if you are testing for hemophilia A, is the severity of the hemophilia in the family in question, which is conveniently based on factor clotting activity.

Speaking in general, and beyond the purposes of genetic testing, this information about clinical severity is important for the patient and the family to know so they have a realistic idea of bleeding risk and prognosis. However, it is also good to know when you are ordering genetic testing for hemophilia, especially in the case of hemophilia A, as we shall see.
So, to review…
You must know 2 things prior to ordering genetic testing for hemophilia.
1. A vs B
2. The severity of the disease, which is based on factor levels.
Let's say you've got these 2 pieces of information and that you are dealing with hemophilia B.
If You Are Dealing With Hemophilia B

- Genetic testing for hemophilia B is relatively straightforward
- If a familial mutation is not known, genetic testing typically begins with sequencing of the $F9$ gene

If, after a thorough evaluation of your patient’s clinical and family history, you find that you are dealing with hemophilia B and the familial mutation has not yet been identified, then you can confidently proceed to full-gene sequencing of the $F9$ gene.
What does full-gene sequencing mean? Sequencing is the genetic testing methodology used to read the DNA sequence of a gene right down to the base pairs. Sequencing reads the sequence of these base pairs and looks for small alterations in this genetic code that could change the function or expression of the gene.

Full-Gene Sequencing

- Good for small mutations (missense, nonsense, splicing mutations, very small deletions, duplications, or insertions)
- Typically cannot pick up large deletions or duplications
- Methods include Sanger sequencing (shown below) or next-generation sequencing
Most mutations that cause hemophilia B are little alterations in genetic code, which is why sequencing will detect pathogenic mutations in 97% to 100% of individuals with a clinical diagnosis of hemophilia B.

However, while this method allows us to see small mutations in the DNA sequence, it is too fine grain a method for much larger mutations, such as deletions, that completely eliminate exons, genes, or a part of chromosome, which are also a cause of hemophilia B, but are far more rare than the small mutations we discussed earlier.

Thus, if sequencing is negative or inconclusive, you then move on to deletion and duplication analysis.
This testing requires a different method to look for these gross deletions. One such method is MLPA, or multiplex ligation-dependent probe amplification, which uses oligonucleotides, which are short synthetic DNA strands designed to match up with a particular segment of DNA on a gene. This flags whether that segment of DNA is there as it should be or if it has been deleted.
If You Are Dealing With Hemophilia B

• Deletion and duplication analysis (e.g., MLPA) will detect pathogenic deletions or duplications in 3% of individuals affected with hemophilia B

• Targeted mutation analysis can be used to test for any known familial mutation (i.e., a mutation that has been identified in an affected family member)

So, to review, when you are dealing with hemophilia B, and the familial mutation has not yet been identified, you should order full-gene sequencing of the *F9* gene, with reflex to deletion and duplication analysis if sequencing comes back negative or inconclusive.

Once this mutation has been confirmed, other at-risk family members can order testing looking for that specific mutation rather than analyzing the full gene.
If You Are Dealing With Hemophilia A

• Genetic testing for hemophilia A is a little more complicated and requires knowledge of the severity of hemophilia A in the family.
  • Mild hemophilia A: 0.05-0.4 IU/mL (5%-40% factor VIII)
  • Moderate hemophilia A: 0.01-0.04 IU/mL (1%-%<5% factor VIII)
  • Severe hemophilia A: <0.01 IU/mL (<1%)
  • Normal range: >0.5 IU/ml (50%-150%)
  • >30%: usually no bleeding

Now, if you are dealing with hemophilia A and the familial mutation is yet unknown, you definitely need to know the severity of the disease in the family in order to choose the right initial test. Again, severity is based on factor clotting activity in an affected males of the family.
If you are dealing with mild or moderate hemophilia A, your job is relatively easy, because you can confidently start with full-gene sequencing of the F8 gene and reflex to deletion duplication analysis if sequencing is negative or inconclusive because, again, most mutations that cause mild or moderate hemophilia A are point mutations detectable by sequencing only.
If You Are Dealing With Severe Hemophilia A

- Sadly, genetic testing for SEVERE hemophilia A is not as straightforward due to large, recurrent mutations known as inversions that are a common cause of severe hemophilia A.

However, if you are dealing with severe hemophilia A, this is where things become a little complicated and strange.

Many times severe hemophilia A is caused by a specific type of mutation that would not be detectable either by regular full-gene sequencing, or standard deletion and duplication analyses.

These recurrent mutations are called inversions.
Inversions

- Inversions are large rearrangements in DNA whereby a segment of DNA is flipped, or rotated 180 degrees, before being reinserted back into the genomic sequence.

What are inversions? They are basically long stretches of genetic code that are flipped around and inserted back into the chromosome.

Yes, this actually happens.
Inversions—How Does This Happen?

- Through “intrachromosomal homologous recombination”

- There are sections of the $F8$ gene in which the order of nucleotides (the “sequence”) is similar to (ie, homologous to) the order of nucleotides outside of the gene and in other parts of the chromosome

- For example…

Sequence “Int1h2” outside the gene contains sequence that is very similar to “int1h1” inside the first exon of the $F8$ gene

How?

In the case of severe hemophilia A, this happens through a process called intrachromosomal homologous recombination.

Basically, there are stretches of genetic code that lie way outside of the gene that are fairly similar to segments of code inside the gene.
During meiosis, the great genomic reshuffling that happens during the creation of eggs and sperm, homologous chromosomes from Mom and Dad are supposed to pair up, intersect with each other (“cross-over”), and recombine to create the unique genome of the germ cell.

- Mistakes happen, eg, a chromosome can pair up with itself rather than with the homologous chromosome with which it’s supposed to pair.

During meiosis, the great genomic reshuffling that happens during the creation of eggs and sperm, homologous chromosomes from Mom and Dad are supposed to pair up, intersect with each other or “cross-over,” and recombine to create the unique genome of the germ cell.

But, mistakes can happen. For example, a chromosome can pair up with itself rather than with the homologous chromosome with which it’s supposed to pair.
Intrachromosomal Homologous Recombination

- If that happens, the chromosome recombines with itself
- The section of DNA sequence outside of the $F8$ gene lines up and recombines with the section of DNA within the $F8$ gene
- The inverted sequence basically rips the gene apart, the $F8$ gene becomes completely nonfunctional and produces no factor VIII

If that happens, the chromosome recombines with itself. A part of the section of DNA that was located outside of the gene is accidently inserted inside of the gene. The part of the gene this insertion replaces ends up far, far away from its other half. So, this insertion basically rips the gene apart and it becomes completely inactive. There is no factor VIII coming out of that gene ever and no factor VIII levels means this mutation can only cause severe hemophilia A in a male, not moderate or mild.
Intrachromosomal Homologous Recombination

- Yes, this actually happens
  - In fact, these inversions account for the majority of mutations causing severe hemophilia A
- There are 2 recurrent inversions in severe hemophilia A
  - Intron 1 inversions account for 2%-3% of cases of severe hemophilia A
  - Intron 22 inversions account for 48% of cases of severe hemophilia A

Now, these inversions are certainly bizarre, but they are also recurrent, meaning these intra- and extrageneic homologous regions near the F8 gene cause enough of a problem during meiosis that we see the mutation pop up time and again.

In fact, most of severe hemophilia A is caused by either an inversion in intron 1 or intron 22. And, because these inversions are not amenable to either full gene sequencing or standard deletion and duplication analysis, if you are dealing with severe hemophilia A, it is recommended you start with a different genetic test specifically designed to detect these inversions.
If You Are Dealing With Severe Hemophilia A

- If a familial mutation is not known, genetic testing typically begins by looking for an inversion in introns 1 and 22
  - This requires a different methodology from sequencing and deletion and duplication analysis
  - At Mayo, we use a PCR-based “inverse shifting” method (IS-PCR)
  - Intron 1 and Intron 22 inversion testing can be ordered separately, but they typically ordered together since they both use the same testing method

At Mayo, we use a PCR-based inverse shifting method to detect these inversions and we typically test for the intron 1 and intron 22 inversions simultaneously because, together, they account for just over half of all mutations that cause severe hemophilia A. Again, if you are dealing with mild or moderate hemophilia A, you do not have to worry about these inversions.
We don’t talk about these inversions in hemophilia B because they are very, very rare for that disorder. So why should this happen more frequently in hemophilia A? That’s a good question. It definitely has to do with presence of those disperse homologous sequences in and outside of the \( F8 \) gene and likely the location of the \( F8 \) gene on the far, far tip of the long arm of the X-chromosome. Since it is at the end, it might be more likely than other parts of the chromosome to fold over and recombine with itself.
So, to review, if you are ordering genetic testing for severe hemophilia A, these are the steps we recommend.

If You Are Dealing With Severe Hemophilia A

1. Start with inversion testing
2. If no intron 1 or intron 22 inversion is found, reflex to full-gene sequencing of $F8$
   - Sequencing will detect a pathogenic mutation in about 49% of affected males and in 43% of carrier females
3. If no mutations identified with sequencing, reflex to deletion and duplication analysis of $F8$
   - Deletion and duplication analysis will detect a pathogenic mutation in about 6% of affected males and carrier females
Those, my friends, are the basics of ordering genetic testing for hemophilia. If you want to go above and beyond this and be a superstar at ordering genetic testing, you should always submit your patient’s clinical and family history to the lab performing the testing.

Personally, knowing how often these tests are misordered, I become anxious when a client fails to submit patient information to us. Genetic testing is expensive, and the results can be life-changing. Providing the patient information allows us to ensure that the correct test is being performed on your patient.

We welcome the opportunity to discuss your patient and your concerns about selecting the right test with you.
This concludes Part 2. I appreciate your attention.