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Review Article

Prenatal Diagnosis of Hemophilia A and B

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Abstract

Hemophilia A and B are inherited X-linked recessive bleeding disorder caused by deficiency of coagulation factor VIII (FVIII) or FIX. These are the most common hereditary hemorrhagic disorders known. These are globally distributed and hence are not limited by ethnicity or geographical borders. Both the disorders lead to life-threatening and often disabling condition. There have been major advances in molecular therapy in the last few decades which have improved the treatment for Hemophilia. But, still a lot needs to be done in this area in developing countries. Patients with Hemophilia rarely live beyond childhood in these countries. Hence, prenatal diagnosis aided by carrier detection is used extensively for the prevention of affected hemophilic child in developing countries. The diagnosis can be done using the direct method of mutation analysis or the indirect method of linkage analysis in the respective FVIII or FIX gene. Prenatal diagnosis of hemophilia A or B is possible by means of chorionic villus biopsy in the first trimester which traces the mutation or informative genetic markers. Today chorionic villus sampling is the most widely used method but amniotic fluid, fetal blood and pre-implantation genetic diagnostics can also be used in selected cases. Prenatal diagnosis must be preceded by adequate genetic counseling and risk assessment of the potential carrier and subsequent support during the diagnostic process.

Keywords: Hemophilia A and B; Factor VIII; Factor IX; Genetic analysis; Carrier detection; Prenatal diagnosis

Introduction of Hemophilia A & B

Hemophilia A and B are X-linked bleeding disorders caused by a defective or absent factor VIII (FVIII) or IX (FIX) that results in prolonged oozing after injuries, tooth extractions, or surgery, and delayed or recurrent bleeding prior to complete wound healing. The age of diagnosis and frequency of bleeding episodes are related to the factor VIII/IX clotting activity. In severe hemophilia, spontaneous joint or deep muscle bleeding is the most frequent symptom. Individuals with severe hemophilia are usually diagnosed during the first year of life; without prophylactic treatment, they have an average of two to five spontaneous bleeding episodes each month. Individuals with moderately severe hemophilia seldom have spontaneous bleeding; however, they do have prolonged or delayed oozing after relatively minor trauma and are usually diagnosed before age five to six years; the frequency of bleeding episodes varies from once a month to once a year. Individuals with mild hemophilia do not have spontaneous bleeding; however, without preventive treatment, abnormal bleeding occurs with surgery, tooth extraction, and major injuries; the frequency of bleeding may vary from once a year to once every ten years. Individuals with mild hemophilia are often not diagnosed until later in life. In any individual with hemophilia, bleeding episodes may be more frequent in childhood and adolescence than in adulthood. Approximately 10% of carrier females are at risk for bleeding (even if the affected family member is mildly Affected) and are thus symptomatic carriers, although symptoms are usually mild. These diseases have an incidence of 1 in 5000 and 1 in 25 000 male births and causes increased morbidity and mortality. No ethnic or geographic predisposition has been defined [1]. Both FVIII and FIX genes map to the long arm of X-chromosome at Xq28 and Xq27, separated by 35 cm [2–4]. Mutations in both the genes including a variety of deletions, insertions, missense, nonsense and splice site mutations, apart from the common intron 1 and 22 inversions in the FVIII gene, have been reported to cause the clinical phenotype [5, 6].

Management & associated problems

Treatment of manifestations: intravenous infusion of plasma-derived or recombinant factor VIII/IX concentrates within one hour of onset of bleeding.

Prevention of primary manifestations: For those with severe disease, prophylactic infusions of factor VIII/IX concentrate three times a week or every other day usually maintain factor VIII/IX clotting activity higher than 1% and prevent spontaneous bleeding.

Therapies under investigation: ongoing clinical trials for a longer-acting factor VIII concentrate.

Other: Vitamin K does not prevent or control bleeding in hemophilia A, cryoprecipitate contains factor VIII but does not undergo viral inactivation so is no longer used to treat hemophilia; no clinical trials for gene therapy in hemophilia are currently in progress although several improved approaches are in pre-clinical testing.

Factor VIII Gene Polymorphisms

The factor VIII gene contains several SNPs, many of which fall into the subcategory of restriction fragment length polymorphisms (RFLPs). In addition, two micro-satellites have been reported within the gene, one in nintron 13 [7], the other in intron 22 [8]. The intron 13 micro-satellite comprises the simple repeat (CA), whereas the intron 22 comprises (CA)n(CT)n. Preferably, verification of micro-satellite data should be sought from at least one other intragenic locus.

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The Xba-I and Msp-I RFLPs in intron 22 of the factor VIII gene occur within the int22h-1 sequence [9, 10]. The allele frequencies of some of the polymorphisms have been investigated in various populations and differences are seen.

The differences in allele frequencies between populations have implications for linkage studies in hemophilia A: the loci of greatest diagnostic potential may differ according to the ethnicity of the family to be investigated and this has to be borne in mind in assessing the investigative strategy. There is strong linkage disequilibrium between the intron 18 Bcl-I, intron 7 G/A, intron 19 Hind III, and intron 25 Bgl-I RFLPs [11, 12, 13]. The linkage disequilibrium in these cases is strong but not complete; therefore, additional informatively might be obtained from their inclusion in a linkage study. The intron 18 Bcl-I and intron 22 Xba-I A loci also show linkage disequilibrium [14]; however, this is less pronounced and their combined analysis provides much more informativity than either used on its own. Linkage disequilibrium, like allele frequencies, differs between ethnic groups and this should also be taken into account in assessing the linkage analysis strategy within a family.

When utilizing the indirect methods, the DNA of both parents of the suspected female carrier and possibly grandparents and other relatives is necessary in addition to that of the porosities and hemophiliac. The combined informativeness of the commonly used polymorphisms in the introns 19 (RFLP-Hind III) or 18 (RFLP-Bcl1) and VNTR-PCR in the introns 13 and 22 is about 80%. The polymorphisms in intron 7 and exons 14 and 26 may bring further information in some cases. Highly informative extragenous VNTR St14 (heterozygosity up to 90%) is burdened by high risk of recombination (3-5%) and is used in exceptional cases only.

Factor IX Gene Polymorphisms

The human factor IX gene contains several SNPs, most of which fortuitously fall into the subcategory of RFLPs. The MnlI polymorphism [15] is located in exon 6 (known as the Malmö polymorphism). There are two micro-satellite loci, one in intron 1 [16] (the "DdeI" polymorphism) [17] and one in the 3' untranslated region of exon 8 [18]. These are of the type (RY) n (R, purine; Y, pyrimidine). As seen for polymorphisms in the factor VIII gene, factor IX gene polymorphisms show ethnic variation; this in some instances is quite pronounced. The polymorphism is caused by variability in the number of A and B units present.

Molecular genetic testing

Clinical testing

- Targeted mutation analysis
- An *F8* intron 22-A gene inversion accounts for nearly half of families with severe hemophilia A [19]. This inversion can be detected by Southern blotting or, more recently, by long-range [20] or inverse [21] PCR.
- An *F8* intron 1 gene inversion accounts for 2%-3% of severe hemophilia A. This inversion is typically detected by PCR [22].
- F9 gene has no such inversions reported.
- Linkage analysis is used to track the disease-causing allele in a family and to identify the origin of mutations:
- Indirect mutation detection: Linkage studies are always based on accurate clinical diagnosis of hemophilia A/B in the affected family members and accurate understanding of the genetic relationships in the family. In addition, linkage analysis depends on the availability and willingness of family members to be tested and on the presence of informative heterozygous polymorphic markers. Use of up to five intragenic polymorphisms and one extragenic polymorphism is informative in approximately 80%-90% of families. Recombination events occur in up to 5% of meioses, but have not been observed between hemophilic mutations and intragenic sites.
- Identifying the origin of a *de novo* mutation: Among the nearly 50% of families with a simplex case of hemophilia A (i.e., occurrence in one family member only), the origin of a *de novo* mutation can often be identified by performing molecular genetic testing in conjunction with linkage analysis. The presence of the mutation on the affected individual's haplotype is tracked back through the parents and, if necessary, through maternal grandparents to identify the individual in whom the mutation originated.

Linkage Analysis in Haemophilia A and B

In essence, the practical approach involves the extraction of genomic DNA from anti coagulated whole blood obtained from relevant family members, amplification of DNA spanning the target polymorphic locus using the polymerase chain reaction (PCR), and then analysis of the PCR product using any of a variety of techniques to distinguish the alleles present [23] (for example, restriction digestion, single strand conformation polymorphism analysis, heteroduplex analysis [24], PCR product length analysis). For most of the polymorphic loci in both genes this is a straightforward task and is the mainstream approach in linkage analyses [25,26]. However, for the XbaI A and MspI A RFLPs in intron 22 of the factor VIII gene, the task is complicated by the extra genic homologues (XbaI B and C, and MspI B and C). Until recently, specific amplification of these loci in int22h-1 was not possible. However, the introduction of long distance PCR (LD-PCR), in which stretches of genomic DNA as long as tens of kilo bases can be amplified from small amounts of starting material, paved the way for the solution of specific amplification of the int22h-1 loci [27,28].

It is clear that PCR has provided a major step forward for the analysis of polymorphisms in the hemophilia. The traditional approach for RFLP analysis, using Southern blot, was lab our intensive, time consuming, hazardous, and required relatively large quantities of high molecular weight DNA. In comparison, PCR is rapid, non-laborious, non-hazardous, and requires only small quantities of DNA, which can be degraded to a considerable degree. However, the methods used for post-PCR product analysis differ enormously. At one end of the spectrum there are low tech approaches, such as agarose gel electrophoresis followed by ethidium bromide staining, whereas at the other there are the high tech approaches such as the Light Cycler TM or fluorescence based capillary electrophoresis. The choice within laboratories reflects many parameters that need not concern us here;

the important point is that post-PCR product analysis is flexible and amenable to the most up to date technologies in molecular biology.

Testing Strategy

Establishing the diagnosis of hemophilia in a proband requires measurement of factor VIII/IX clotting activity. Molecular genetic testing is performed on a proband to detect the family-specific mutation in order to obtain information for genetic counseling of at-risk family members. Carrier testing for at-risk relatives requires prior identification of the disease causing mutations in the family. Prenatal diagnosis for at-risk pregnancies requires prior identification of the disease-causing mutation in the family.

Testing of relatives at risk

Identification of at-risk relatives: A thorough family history may identify other male relatives who are at risk but have not been tested (particularly in families with mild hemophilia).

Early determination of the genetic status of males at risk: Either assay of factor VIII/IX clotting activity from a cord blood sample obtained by veni-puncture of the umbilical vein (to avoid contamination by amniotic fluid or placenta tissue) or molecular genetic testing for the mutations identified in the family can establish or exclude the diagnosis of hemophilia in newborn males at risk.

Determination of genetic status of females at risk: Approximately 10% of carriers have factor VIII/IX activity lower than 30%-35% and may have abnormal bleeding themselves. In a recent Dutch survey of hemophilia carriers, bleeding symptoms correlated with baseline factor clotting activity; there was suggestion of a very mild increase in bleeding even in those with 40% to 60% factor VIII/IX activity [29]. Therefore, all daughters and mothers of an affected male and other at-risk females should have a baseline factor VIII/IX clotting activity assay to determine if they are at increased risk for bleeding unless they are known to be non-carriers based on molecular genetic testing. It is recommended that the carrier status of a woman at risk be established prior to pregnancy or as early in a pregnancy as possible.

Prenatal testing

Since hemophiliacs require administration of FVIII/IX, which is expensive and not easily available, it is important to curb the disease. For this it is important to detect these patients prenatally and offer the option to abort the affected fetus. Amongst the current techniques available, direct sequencing is the most accurate way of prenatally diagnosing Hemophilia A. However, in countries with limited resources where direct sequencing is a costly option other alternative methods are used. Linkage analysis using restriction fragment length polymorphism and Variable number tandem repeats (VNTRs) markers such as CA repeats provides one such alternative method. Direct mutation detection of Intron 22, 1 inversion mutation by Polymerase chain reaction (PCR) or cordocentesis followed by measurement of factor VIII levels in fetal blood are also suitable economical options. Our previous experience has shown that linkage analysis with CA-13, CA-22, *Bcl*1 and *Xba*1 along with intron 22 and 1 inversion mutation provides informativity in 85% of the cases with family history and in 52% sporadic cases of hemophilia A.

Molecular genetic testing: Prenatal testing is available for pregnancies of women who are carriers if the mutation has been identified in a family member or if linkage has been established in the family. The usual procedure is to determine fetal sex by performing chromosome analysis of fetal cells obtained by chorionic villus sampling (CVS) at approximately 10-12 weeks gestation, by amniocentesis usually performed at approximately 15-18 weeks gestation. If the karyotype is 46, XY, DNA extracted from fetal cells can be analyzed for the known disease-causing mutation or for the informative markers. CVS generally is not done after the 13th week of pregnancy because there is more amniotic fluid around the baby which makes it harder to do the procedure.

Chorionic villus sampling (CVS)

Chorionic villi are tiny fingerlike growths found in the placenta. The genetic material in chorionic villus cells is the same as that in the baby's cells. The chorionic villus sample is collected by putting a thin flexible tube (catheter) through the vagina and cervix into the placenta. The sample can also be collected through a long, thin needle put through the belly into the placenta. Ultrasound is used to guide the catheter or needle into the correct spot for collecting the sample.

A small amount of placental tissue was removed and was washed two to three times with washing media prepared by diluting 10 times the stock solution [RPMI –1640, 10% sodium bicarbonate, antibiotic mixture of streptomycin (0.2% w/v) and penicillin (500000 units)] in distilled water with heparin (0.5% v/v). The CVS was then transferred to the transport media prepared by adding stock solution, distilled water and fetal calf serum in the ratio of 1:9:2 respectively with heparin (0.4% v/v). The CVS was then sending for DNA analysis. Maternal contamination was checked using VNTR 1 of vWF polymorphic loci [30]. PCR was performed according to Nakahori *et.al.* [31] to determine the sex of the fetus. There is a risk of miscarriage associated with CVS; however, it is very low - between one in 100 and one in 200[32]. The risk is only slightly higher than that of amniocentesis, mostly due to the naturally higher risk of miscarrying in the first trimester. The risk for either digital or limb deficiency after CVS is only one of several important factors that must be considered in making complex and personal decisions about prenatal testing.

Per-cutaneous umbilical blood sampling (PUBS)

A cordocentesis may be used if an amniocentesis or CVS have been unsuccessful, or the tests results from these tests were inconclusive. Cordocentesis can rapidly identify genetic disorders (hemophilia) within 24 hours. In hemophilia families, cord blood analysis is helpful in cases where (*a*) linkage analysis is not possible (proband unavailable for testing) or molecular markers for linkage are uninformative; and (*b*) inversion (intron 22 and intron 1) mutation is absent in severe Hemophilia A cases or in sporadic hemophilia families. Cordocentesis involves the doctor using ultrasound images to guide the insertion of a long, thin needle through the woman's belly and uterus and into the umbilical cord, at the end closest to the **placenta.** A small blood sample is then taken and the needle is removed. The baby's heartbeat is **continuously monitored** during the procedure, which can take up to 1 hour.

In this fetal blood sample is obtained by PUBS at approximately 18-21 weeks' of gestation. Factor VIII/IX assay is performed on plasma samples using an APTT based one-stage assay [33].

For the interpretation of factor VIII/IX level (activity and antigen) in umbilical cord blood sample it is necessary to consider its developmental changes; factor VIII level is physiologically low at the time of sampling. Contamination of the sample with the mother's blood or amniotic fluid has to be controlled. In the latter case, high amount of thromboplastin precludes correct determination of the factor VIII/IX activity, while the factor VIII/IX antigen remains unaffected. Estimation of the mother's blood admixture in the fetal blood sample is based on differential acidoresistance of fetal and adult red cells (Kleihauer test) and the fetal and adult hemoglobin levels are determined.

Cordocentesis has a miscarriage (or premature birth) rate of about 1 to 2% (1:100 to 1:50) as well as a small risk of infection. Sometimes the baby's heartbeat temporarily slows during the procedure.

Genetic counseling

Genetic counseling is the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions. Genetic counseling remains an important part of hemophilia care whether in countries at the forefront of diagnosis and treatment or those where there are little to no facilities for diagnosis or treatment. Comprehensive genetic counseling includes addressing a wide range of issues and offering a spectrum of diagnostic and carrier tests. Ethical considerations include human rights, issues surrounding consent, and those pertaining to confidentiality. These apply to genetic counseling in both national and international contexts and vary according to the legal and social customs in particular settings. Concerns about disclosure of personal information (such as the results of genetic testing) to others (family members, insurance companies, etc.) and maintenance of confidentiality can raise difficulties for some individuals because genetic testing and screening may reveal unexpected or awkward information, for example about paternity.

Perceptions of hemophilia

Perceptions of hemophilia are complex and have a major impact on the choices people make about having a child who might be affected. Personal beliefs and religious and cultural traditions contribute to these perceptions and influence general attitudes to illness, disability, and medical treatment. Because of their profound impact, these beliefs should *always* be explored with patients.

In many cultures it is not unusual for a couple's parents-in-laws to be very influential about the acceptance of disability, having children, and how hemophilia is managed. Often the in-laws are uninformed about the medical aspects of hemophilia. Sometimes a man with hemophilia is unwilling and unable to accept his hemophilia and thus is reluctant to tell his daughter that she is a carrier, fearing rejection by his partner and offspring. This may leave a daughter with unresolved issues and misconceptions about hemophilia. Or, the father with hemophilia may be willing to talk to his daughters but his wife resists due to the effect it may have on the daughters' chances of marriage. In other situations, family members may be ignorant of the possibility of inheritance.

Mode of Inheritance

Hemophilia is inherited in an X-linked manner.

Risk to Family Members

- Parents of a male probaThe father of an affected male will not have the disease nor will he be a carrier of the mutation.
- Women who have an affected son and one other affected relative in the maternal line are obligate carriers.
- If a woman has more than one affected son and the disease-causing mutation cannot be detected in her DNA, she has germ line mosaicism.
- One-third to one-half of affected males has no family history of hemophilia. If an affected male represents a simplex case (an affected male with no known family history of hemophilia), several possibilities regarding his mother's carrier status and the carrier risks of extended family members need to be considered:
- The mother is not a carrier and the affected male has a *de novo* disease-causing mutation. Somatic mosaicism may occur in as many as 15% of probands with a point mutation and no known family history of hemophilia A [34]; germ line mosaicism is rare.
- The mother is a carrier of a *de novo*, disease-causing mutation that occurred:
- As a germ line mutation (i.e., in the egg or sperm at the time of her conception and thus present in every cell of her body and detectable in her DNA);
- As a somatic mutation (i.e., a change that occurred very early in embryogenesis, resulting in somatic mosaicism in which the mutation is present in some but not all cells and may or may not be detectable in DNA); or
- As germ line mosaicism (in which some germ cells have the mutation and some do not, and in which the mutation is not detectable in DNA from her leukocytes).
- The mother is a carrier and has inherited the disease-causing mutation either from her mother who has a *de novo* disease-causing mutation or from her asymptomatic father who is mosaic for the mutation.
- The mother is a carrier of a mutation arising in a previous generation, which has been passed on through the family without manifesting symptoms in female carriers.

Overall, the mother has an approximately 80% chance of being a carrier when her son is the first affected individual in the family; however, the mother of a severely affected male with an intron 22 inversion has a 98% chance of being a carrier.

Sibs of a male proband

- The risk to the sibs depends on the mother's carrier status. If the proband's mother is a carrier, each male sib is at a 50% risk of having hemophilia A and each female sib is at a 50% risk of being a carrier.
- Germline mosaicism is possible, albeit uncommon. Thus, if an affected male represents a simplex case and if his mother

has a normal factor VIII/IX clotting activity and no evidence of her son's disease-causing mutation in DNA from her leukocytes, she is still at a theoretically increased, but low, risk of having additional affected children.

- All sibs should have factor VIII/IX clotting activity assayed unless mutation analysis confirms that they have not inherited the mutation in their family.

Offspring of a male proband

- All daughters will be carriers for hemophilia A of the same severity as their father's hemophilia.
- No sons will inherit the mutant allele, have hemophilia A, or pass it on to their offspring.

Other family members of the proband: The proband's maternal aunts and their offspring may be at risk of being carriers or being affected (depending on their gender, family relationship, and the carrier status of the proband's mother).

Carrier Detection

- Carrier testing by molecular genetic testing is clinically available for most at-risk females if the mutation has been identified in the family.Large deletions are not detectable by sequence analysis in females.
- When carrier testing is performed without the previous identification of the mutation in the family, a negative result in an at-risk relative is not informative.
- Factor VIII/IX clotting activity is not a reliable test for determining carrier status. It can only be suggestive, if low.

Related Genetic Counseling Issues

Family planning: The optimal time for determination of genetic risk, clarification of carrier status, and discussion of the availability of prenatal testing is before pregnancy.

DNA banking: DNA banking is the storage of DNA (typically extracted from white blood cells) for possible future use. Because it is likely that testing methodology and our understanding of genes, mutations, and diseases will improve in the future, consideration should be given to banking DNA of affected individuals. DNA banking is particularly relevant when the sensitivity of currently available testing is less than 100%.

- Indications for procedures and limitations of prenatal testingCounselors should discuss the prospective parents' degree of risk for transmitting genetic abnormalities based on factors such as maternal age, race, and family history.
- Prospective parents should be made aware of both the limitations and usefulness of either CVS or amniocentesis in detecting abnormalities.

Potential serious complications from CVS and amniocentesis

- Counselors should discuss the risk for miscarriage attributable to both procedures: the risk from amniocentesis at 15-18 weeks' gestation is approximately 0.25%- 0.50% (1/400-1/200), and the miscarriage risk from CVS is approximately 0.5%-1.0% (1/200-1/100).
- Current data indicate that the overall risk for transverse limb deficiency from CVS is 0.03%-0.10% (1/3,000-1/1,000). Current data indicate no increase in risk for limb deficiency after amniocentesis at 15-18 weeks' gestation.
- The risk and severity of limb deficiency appear to be associated with the timing of CVS: the risk at <10 weeks' gestation (0.20%) is higher than the risk from CVS done at greater than or equal to 10 weeks' gestation (0.07%). Most defects associated with CVS at greater than or equal to 10 weeks' gestation have been limited to the digits.

Conclusion

In conclusion, chorionic villus sampling and RFLP analysis provides a new alternative for women who wish to prevent the birth of a child with hemophilia, yet renounce second trimester abortion of an affected fetus. The decision to abort an affected fetus will always be a difficult one and will certainly not be acceptable to every woman at risk, regardless of the gestational age at the time of diagnosis. Therefore, improvements in the treatment of hemophilia and ultimately gene therapy remain the primary objectives in hemophiliac management.

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