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Insights into the Molecular Genetic of Hemophilia A and Hemophilia B: The Relevance of Genetic Testing in Routine Clinical Practice

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Abstract

Hemophilia A and hemophilia B are rare congenital, recessive X-linked disorders caused by lack or deficiency of clotting factor VIII (FVIII) or IX (FIX), respectively. The severity of the disease depends on the reduction of coagulation FVIII or FIX activity levels, which is determined by the type of the pathogenic variants in the genes encoding the two factors (*F8* and *F9*, respectively). Molecular genetic analysis is widely applied in inherited bleeding disorders. The outcome of genetic analysis allows genetic counseling of affected families and helps find a link between the genotype and the phenotype. Genetic analysis in hemophilia has tremendously improved in the last decades. Many new techniques and modifications as well as analysis softwares became available, which made the genetic analysis and interpretation of the data faster and more accurate. Advances in genetic variant detection strategies facilitate identification of the causal variants in up to 97% of patients. In this review, we discuss the milestones in genetic analysis of hemophilia and highlight the importance of identification of the causative genetic variants for genetic counseling and particularly for the interpretation of the clinical presentation of hemophilia patients.

Keywords
- genetic analysis
- hemophilia
- carriers
- next-generation sequencing

Zusammenfassung

Hämophilie A und B sind seltene angeborene, rezessive X-chromosomale Erkrankungen, die durch einen Mangel an Gerinnungsfaktor VIII (FVIII) bzw. IX (FIX) verursacht werden. Der Schweregrad der Erkrankung hängt von der Verringerung der Gerinnungsaktivitäten von FVIII bzw. FIX ab, die durch die Art der pathogenen Varianten in den Genen für die beiden Faktoren (*F8* bzw. *F9*) bestimmt wird. Die molekulargenetische Analyze wird häufig bei vererbten Blutungsstörungen eingesetzt. Die Ergebnisse ermöglichen eine genetische Beratung der betroffenen Familien und helfen dabei, eine Verbindung zwischen dem Genotyp und dem Phänotyp zu erstellen. Die genetische Analyze der Hämophilie hat sich in den letzten Jahrzehnten enorm verbessert. Viele neue Techniken und Modifikationen sowie Analysesoftware sind inzwischen

Schlüsselwörter
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- Hämophilie
- Konduktorinnen
- “next-generation” Sequenzierung

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verfügbar, so dass die genetische Analyze und die Interpretation der Daten schneller und genauer erfolgen kann. Fortschritte bei den Strategien zur Erkennung von genetische Varianten erleichtern die Identifizierung der ursächlichen Varianten bei bis zu 97% der Patienten. In dieser Übersicht werden die Meilensteine der genetischen Analyze der Hämophilie erörtert und die Bedeutung der Identifizierung ursächlicher genetischer Varianten für die genetische Beratung und insbesondere für die Interpretation des Krankheitsbildes von Hämophiliepatienten hervorgehoben.

Introduction

Hemophilia A (HA) and hemophilia B (HB) are rare bleeding disorders caused by genetic defects in the genes encoding coagulation factor VIII (FVIII) and factor IX (FIX), leading to deficiency or absence of either of the two coagulation proteins. Classification of the severity of hemophilia is based on the amount of residual FVIII or FIX activity and it is strongly dependent on the inherited genetic alterations. The severe form is defined as a factor level less than 1 IU/dL of normal, the moderate form as a factor level of 1 to 5 IU/dL, and the mild form with factor levels greater than 5 and less than 40 IU/dL.[1] Hemophilia is a leading disease model in the field of human molecular genetics. The identification of the causative genetic defects in affected individuals is important for family counseling and has a predictive value for bleeding tendency and inhibitor risk. Genetic testing has increased the number of families with an identified defect and improved carrier testing and prenatal diagnosis. This review will consider the role and the place of molecular genetic analysis in the diagnostic algorithm for hemophilia and highlights the benefits for clinical practice, especially with the introduction of next-generation sequencing (NGS) to the routine settings.

1. Inheritance

Since both *F8* and *F9* genes are located on the X chromosome, they are subject to the unique inheritance pattern of X-linked genes affecting almost exclusively males.[2] Such a male is termed hemizygous and has the full phenotype of the disease. Females carry two alleles for X chromosome genes; so, the defect in a single allele can be compensated by the normal allele and these heterozygous carriers remain with a healthy phenotype in most cases. In 1961, Mary Lyon proposed that in the cells of female mammals, one of the two X chromosomes is randomly inactivated in early embryonic stage, so that both males and females have a single active X chromosome. This hypothesis provided an improved understanding of the basic mechanisms responsible for X-linked diseases.[3,4] A male's X chromosome is transmitted to his daughters and the Y chromosome is transferred to his sons. If an affected male has a child with a healthy female, none of his male offspring will be affected, but all of his female offspring will be carriers, termed obligate carriers. Owing to the X-linked inheritance of the disease and in combination with a detailed family history, it is possible to identify obligate carriers. If a female carrier has a child with a healthy male, each male offspring has a 50% chance of being affected, and female offspring has a 50% chance of being a carrier. Thus, the disease can be transmitted from affected males to male grandchildren through the carrier daughter.

Approximately 30% of cases, in both HA and HB, are described as "sporadic"[5] as the newborn baby is the first hemophiliac in this family. The manifestation of the disease without a family history can be explained with the possibility of the occurrence of a *de novo* genetic defect in the *F8*/*F9* genes, either in the affected child or in a female who gave birth to a hemophilia boy or her parents. Not rarely, family history remains unknown due to lack of information or the occurrence of few male births in the family. Maternal mosaicism can also contribute for the occurrence of such cases, where no mutation is detected in the mother by standard techniques.[6] Therefore, a negative family history is insufficient to rule out hemophilia when clinical presentation is suggestive of an excessive bleed tendency.

Rarely, females may also have symptomatic hemophilia designated as "hemophilic females." These females are diagnosed as having hemophilia, with FVIII levels comparable to those of males. Approximately 250 women and girls with a severe or moderate phenotype have been reported in the literature.[7,8] The phenotype of such females may result from complex genetic causes, which can be summarized as follows: (1) *homozygosity in a female* (two identical hemophilia alleles due to consanguinity), (2) *compound heterozygosity* (two different hemophilia alleles inherited from each parent), (3) *hemizygosity* (one hemophilia allele and no normal allele due to chromosomal aberrations as in Turner syndrome or mosaic Turner syndrome) (4) *heterozygosity with skewed X chromosome* (a nonrandom inactivation in favor of the hemophilia allele,[9–11] and (5) *genetic causes other than hemophilia*—Women with decreased FVIII levels also may have combined factor V (FV) and FVIII deficiency, an autosomal recessive disorder characterized by mildly decreased levels of both FVIII and FV, caused by variations in the *LMAN1* and *MCFD2* genes.[12]

2. Molecular Basis of HA and HB—*F8*/*F9* Genes and Variants

HA and HB are monogenic disorders raised from pathogenic variants in either the *F8* or *F9* gene located at Xq28 and Xq27 of the X chromosome, respectively.[13] *F8* gene is extremely

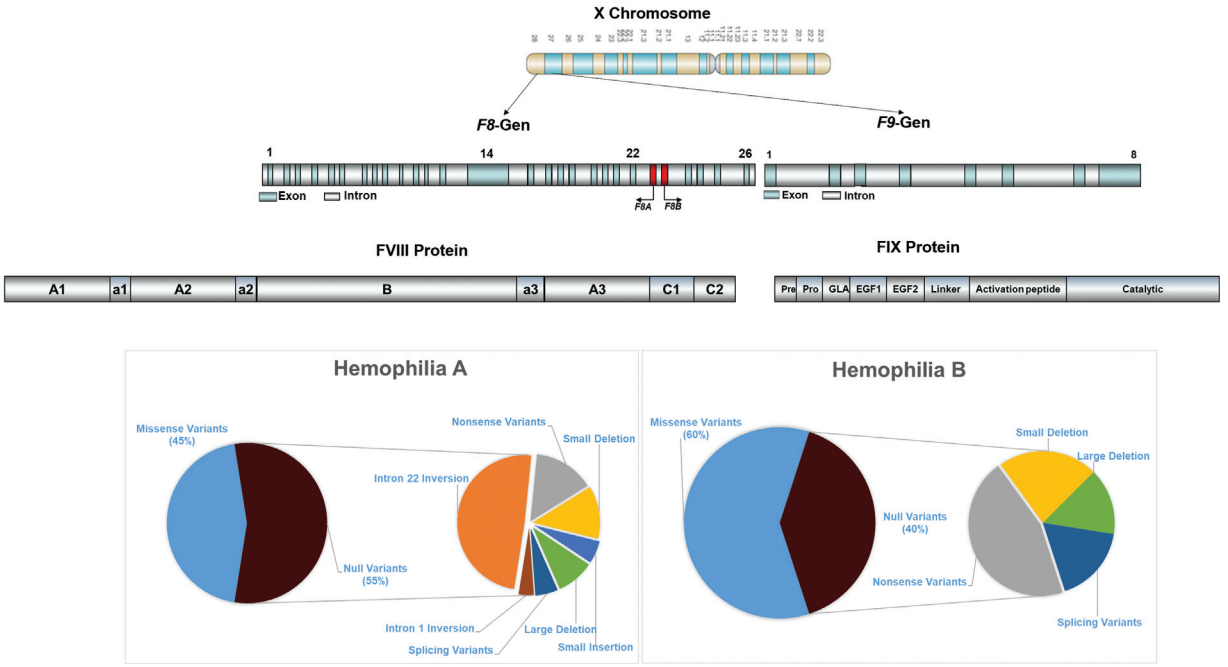

Fig. 1 Prevalence and profile of genetic variants in hemophilia A and hemophilia B.

large (~ 180 kb) and structurally complex (26 exons), while *F9* gene is considerably smaller (~ 34 kb) and structurally simpler, containing only eight exons. Additionally, the *F8* gene contains two nested genes, *F8A* and *F8B*, in the region of intron 22, which largely contribute significantly to the occurrence of the most common genetic defect in severe HA—intron 22 inversion (►**Fig. 1**).

The molecular basis of HA and HB is extremely diverse which has been characterized in several thousands of patients. Although they occur with different frequency in HA and HB, nearly all types of genetic alterations are detected in both *F8* and *F9* genes—point variants, deletions, insertions, and rearrangements/inversions. Hemophilia is one of the few genetic diseases where a good correlation between genotype and phenotype is observed. The type of genetic defects strongly correlates with the plasma residual factor activity, bleeding tendency, and severity of symptoms in hemophilia patients.

Spectrum of Genetic Variants in HA

The type of genetic defect in the *F8* gene predicts the severity of the disease. Defects that cause a significant disruption/absence of the FVIII protein or alteration of a major functional site are known as null variants (intron 22 and intron 1 inversions, large deletions, nonsense variants, non–A-run small deletions, and splice site variants). The second group summarizes the non-null variants, which are mainly missense variants and contribute to a moderate or mild phenotype in the majority of cases.

HA displays heterogeneity, with pathogenic variants identified throughout the whole gene. Depending on their localization, the pathogenic variants can affect FVIII protein by different mechanisms. They can interfere with the binding of FVIII to different ligands of the coagulation cascade and lead to decreased function or complete lack of FVIII synthesis or affect the expression, transport through the cell, activity, and stability of FVIII. The European Association for Hemophilia and Allied Disorders (EAHAD) variant database has recorded more than 3,000 causative genetic alterations in the *F8* gene. In general, the most prevalent types of genetic defects across all disease severities are the missense variants. While for mild/moderate phenotypes, the highest prevalence of missense type is observed; in severe phenotypes they are much less presented. Severe HA phenotypes are associated with null variants in approximately 80% of index patients. The overall prevalence of genetic variants in HA can be summarized as follows: missense variants (45%), nonsense variants (8%), inversions (29%), small deletions (6%), duplications (3%), insertions (2%), and large deletions (7%). Although most genetic defects are irregularly distributed along the *F8*, four hot spots can be identified accounting for recurrent occurrence of genetic changes: the two inversion hot spots, the CpG dinucleotide hotspots, caused by spontaneous methylation-induced deamination of the 5-methylcytosine,[14,15] and small deletions and insertions caused by polymerase slippage errors in series of adenine nucleotides (A stretches).[16] This subgroup of small deletions/duplications, located at stretches of adenines, is associated with a mitigated phenotype of severe HA and rare bleeding. Endogenous restoration of the reading frame by polymerase errors during DNA replication/RNA transcription, resulting in small amounts of endogenous FVIII protein, could explain the observed phenomena.[17]

Intron 22 and Intron 1 Inversions

The two intronic regions of the *F8* gene, intron 1 and intron 22, are of particular interest owing to their frequent

involvement in pathological inversions resulting from recombination with homologous regions outside the *F8* gene. The region that is homologous to intron 1 (int1h2) is located approximately 140 kb toward the telomere and is in the opposite orientation. Intrachromatid or intrachromosome homologous recombination of these two regions causes an inversion that displaces exon 1 of the *F8* gene by approximately 140 kb toward the telomere.[18] This inversion causes 1 to 4% of all severe cases of HA.

However, the most frequent inversion involves intron 22. Approximately 45% of severe HA is accounted by an intrachromosomal inversion in intron 22 which occurs due to two regions homologous to the sequences in intron 22 that are positioned approximately 500 kb telomeric to the *F8* gene.[19,20] This inversion event occurs spontaneously in male germ cells with 10-fold higher rate than in females.[21]

Several gross complex rearrangements in *F8* have been reported and due to their highly deleterious nature, these defects are associated with severe hemophilia phenotype.[22,23]

HB Genetic Variant Spectrum

HB is also genetically heterogeneous, with a predominance of missense variants, without a common inversion event analogous to the inversions in the *F8* gene. These variants account for approximately 65% of all unique genetic variants in HB. The pathogenic defects occur throughout the whole *F9* gene including the promoter and 3′UTR but with the highest concentration in the largest exon 8. In contrast to the *F8* gene, missense variants account for majority of cases with severe HB and for the larger proportion of pathogenic defects in mild/moderate phenotypes. The genetic variants are reported as nonsense (18%), small deletions (9%), small insertions (2%), splice variants (6%), and large deletions (5%). In severe HB, the proportion of null mutations is approximately 35%, while it is 80% in severe HA. The FIX EAHAD Variant Database currently reports more than 1,000 unique variants.

HB Leyden: Although rare, noteworthy is the existence of a particular unique variant of HB that initially is presented with severe or moderate phenotype (depending on the variant), followed by normalization of the FIX levels in the puberty—HB Leyden.[24] The molecular mechanism likely involves variants in the promoter region of the *F9* disrupting the binding sites to one of three transcription factors—HNF4a (hepatic nuclear factor 4a), C/EBP (CCAAT enhancer-binding protein), or ONECUT1/2. This apparent "cure" of HB with age is thought to be related to the rising postpubertal growth hormone levels. Similar molecular mechanisms have not yet been identified in HA patients.[25]

3. Genetic Analyses

In hemophilia, molecular testing remains the only way of providing an accurate diagnosis, overcoming the variability and inconsistent data obtained by the other methods based on the quantification of coagulation factors. Moreover, genetic analyses enhance the diagnostic precision, especially in milder bleeding phenotypes, contributing to correct treatment decisions.

Early genetic diagnosis of hemophilia was primarily focused on carrier detection[26] using a genetic marker that cosegregates with hemophilia in the family. Restriction fragment length polymorphism (RFLP) analysis was applied to trace the affected chromosome and predict the carrier status of females. Although this was the only existing approach, the procedure was not successful in many cases due to lack of informative evidence of the analyzed polymorphism, high proportion of sporadic cases, lack of index patients, and need of several family members for segregation analysis.[27] Additionally, RFLPs analyses were performed by Southern blotting, a laborious and time-consuming method.

The human *F8* gene was cloned and characterized in 1982. In 1991, Higuchi et al performed the first systematic analysis of complete *F8* coding region, using denaturing gradient gel electrophoresis as a variant screening method but no causative variant was detected in about half of the severely affected patients.[28] Shortly after, the *F8* intron 22 inversion was discovered, which accounts for approximately 45% of the severe HA cases. Initially, the analyses were performed using Southern blotting and long-distance PCR until a simplified technique of inverse PCR (inverse shifting PCR [IS-PCR]) was established.[29,30]

The advent of sequencing technologies that allow direct characterizing of variations has helped overcome many of the limitations of previous techniques. Direct sequencing, either Sanger or NGS, has increased the sensitivity of screening techniques and is currently the gold standard for molecular genetic analysis.

Sanger sequencing was the first applied method that involved amplification of *F8*/*F9* genes by polymerase chain reaction (PCR) as short DNA segments (200–1,000 nucleotides) followed by sequencing and alignment of resulting sequences with the human genome consensus sequence using dedicated software. Currently, Sanger sequencing is considered a time-consuming and rather expensive method with low throughput.

In recent years, NGS became available in diagnostic laboratories and began to be used for genetic analysis of bleeding disorders. The implementation of NGS in routine diagnostics allowed the parallel analysis of a large number of genes, which greatly reduced the workload and costs compared with Sanger sequencing and provided the result within a short and clearly defined time period.[31–33] Moreover, the NGS technique enables the analysis of large deletions and duplications without a need for the conventional gene dosage analysis using multiplex ligation-dependent probe amplification (MLPA). As the correct interpretation of genetic variants and their pathogenicity evaluation is crucial for diagnosis and appropriate genetic counseling, standards and guidelines for the interpretation and classification of gene variants have been suggested. The identified genetic variations are categorized as "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," and "benign" based on evidence for a set of criteria and the strength of those criteria.[34]

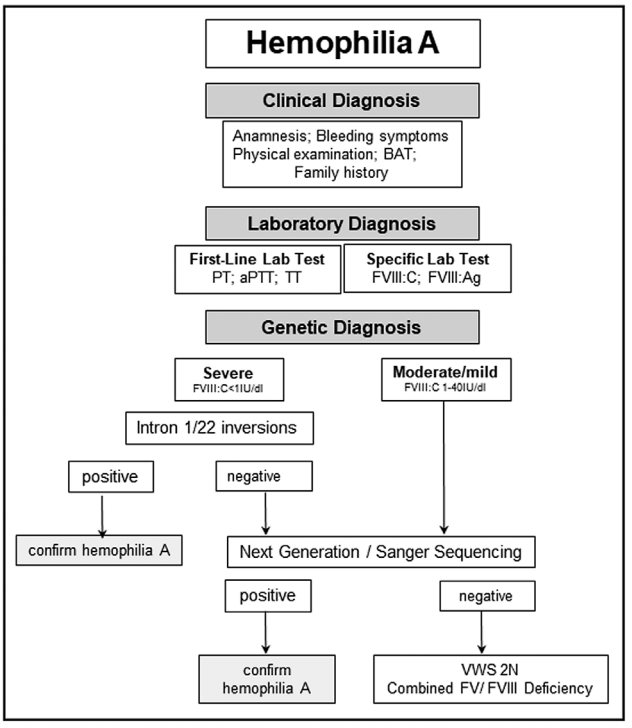

Fig. 2 Diagnostic flow chart for hemophilia A. PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; BAT, bleeding assessment tool; VWS 2N, von Willebrand syndrome type 2 Normandy.

Currently, sequencing of coagulation *F8* or *F9* genes in the hemophilia patient and the determination of carrier status is a fairly trivial procedure, which allows for adequate genetic counseling. The inclusion of NGS in the routine diagnostic algorithm improved the molecular testing in terms of speed, costs, and efficiency.[35]

In severe HA patients, molecular diagnosis starts with screening for intron 22 and intron 1 inversions, followed by direct sequencing of coding regions and splice sites of the *F8* in patients negative for the inversions (►Fig. 2). In mild/moderate HA and HB patients, the genetic analyze is initiated directly with detection of molecular defects by direct sequencing (►Figs. 2, 3).

Implementation of NGS in the routine diagnosing settings for HA/HB has both strengths and limitations.[36] A limitation may be considered the inability of NGS to detect gross genetic rearrangements, such as large inversions, which conventionally require specific techniques for the analyses. Large deletions/duplications of one or more exons can be as efficiently identified with NGS as with MLPA.[37]

Although NGS is of great benefit for hemophilia patients and their family, the inevitable discovery of genetic variants not related to the hemophilia, the so-called unsolicited, incidental or secondary findings, presents a potential challenge. This is a complex issue with many confounding factors, involving ethics and legitimacy.[38] Nowadays, there is a consensus that if these variants are not known to be connected with any phenotype they are of no further concern. If these variants are associated with a clinical phenotype, particularly a severe one, then these incidental findings become relevant and should be reported. Particularly important and difficult is the decision whether to report or not pathogenic variants in genes (*RUNX1*, *ETV6*, and *ANKRD26*) associated with increased risk of malignancy development. Thus, it is very essential to note that using multigene panel analyses, HA/HB patients should be explicitly informed about the possibility of unexpected findings, giving them the choice to decide whether to be informed about such variants or not.[39]

The success rate in identifying pathogenic variants in inherited bleeding disorders depends on both the type of molecular analysis performed and the genetic heterogeneity of the disease. Despite remarkable improvements in screening technologies, the detection rate of causative defects in both *F8* and *F9* genes is approximately 95%. Still, in 5% of cases with reduced FVIII/FIX levels, the genetic defect remains unclear.[40] The missing 5% may be in part due to phenotypic misdiagnosis of HA and von Willebrand disease type 2N (VWD 2N) as the clinical presentation is quite similar. In rare cases, combined FV/FVIII deficiency is associated with pathogenic variants in the *MCFD2* and *LMAN1* genes that contribute to the low FVIII levels. Sporadic cases of multiple vitamin K–dependent coagulation factor deficiency (VKCFDs) involving prothrombin, FVII, FIX, and FX might be misdiagnosed as HB.[41] Pathogenic HA/HB variants outside the region of the *F8/F9* genes might remain undetectable with the current testing procedures. Although additional molecular and informatics research is needed, NGS is expected to elucidate cases without "detected" genetic alterations in the *F8/F9* genes, leading to better understanding of genotype/phenotype correlation and more effective clinical care.

4. Genetic Counseling and Reproductive Options

Genetic Counseling

HA and HB, as congenital disorders, have a significant medical, psychological, and emotional impact on both affected males and female carriers. The possibility of transmitting the genetic disorders to offspring may lead to feelings of guilt and self-blame in parents and may have a strong impact on reproductive decision-making.[42,43] Genetic counseling, as a process that provides individuals and families with information about how genetic disorders are inherited and helps them make informed medical and personal decisions, has become an integral part of comprehensive care for hemophilia. This is of extreme importance especially for girls and women who carry the disease and are emotionally burdened during reproductive decision-making. In that context, genetic counseling of hemophilia involves three main aspects: identifying females who are at increased risk of either having hemophilia or passing it to the offspring; providing knowledge about the genetic aspects of the disease and its consequences for offspring; and offering information and education of prenatal diagnosis and reimplantation genetic diagnosis. As a consequence, the final decision of partners

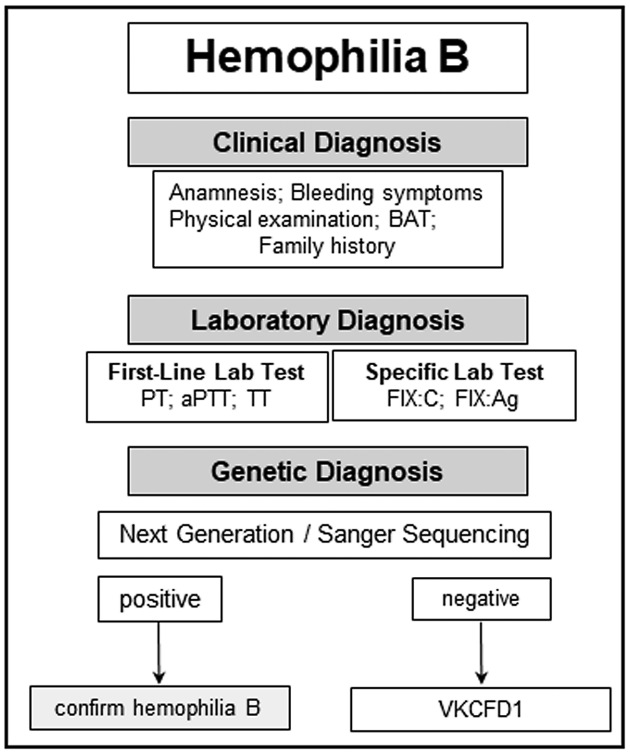

Fig. 3 Diagnostic flow chart for hemophilia B. PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; BAT, bleeding assessment tool; VKCFD1, vitamin K–dependent clotting factors-1.

can be based on genetic evidence and counseling from one side and from their own experience with the disease on the other, as the severity of hemophilia remains stable within an individual family.[44]

Nowadays, the causative genetic variant is routinely identified in hemophilia families, followed by genetic counseling, determination of carrier status in women at risk, so that informed reproductive decisions can be made in affected families. Large studies exploring the attitude of women from hemophilia families to become pregnant and childbearing showed quite controversial opinions that depended firmly on women's experiences with hemophilia in their families. The quality of life in hemophilia family strongly influenced this decision. In families that experienced a satisfactory quality of life, the women were more readily to have children of their own compared with women coming from families that faced difficulties with acceptance and managements of the disease.[45–47] In general, women wished to be well informed before making a reproductive decision.

Genetic counseling is of great importance, as it offers women the opportunity to be professionally informed about the existing probability of having a hemophilic son and the current prevention procedures. Accurate knowledge of the genetic defect in the family allows prenatal diagnosis to be performed at different stages: on the germ line of the female carrier for preimplantation genetic diagnosis (PGD), on chorionic villi during the first trimester of pregnancy, or on amniocytes at later stages of pregnancy.[48–50] The optimal time to determine genetic risk, clarify carrier status, and discuss the availability of prenatal/preimplantation genetic testing is before pregnancy. Males with hemophilia also need genetic counseling, including education about the genetics of their disease, their risk of affecting grandchildren, and the possibility that their daughters may have bleeding symptoms. Therefore, it is appropriate to offer genetic counseling (including discussion of potential risks to offspring and reproductive options) to both hemophilia males and female carriers.

Special attention is required when counseling families with newly diagnosed hemophilia without family history of disease, especially when the mother is not a carrier. In these cases, the disease can be attributed either to a de novo variant in the *F8*/*F9* genes or to the presence of a mosaicism in the mother.[51,52] Mosaicism is the presence of cell lineages with different genotypes in different tissues of an individual. It may be somatic or germline specific or both; both may have an impact on reproductive outcomes. In this case, the pathogenic variant is present only in some of the germline cells and therefore cannot be detected in peripheral white blood cells, which are typically used to perform genetic analysis.[53,54] The possibility of mosaicism in a family with a hemophilia patient without a family history can never be excluded. Genetic counseling has to be made with extreme caution and the parents must be informed that there is a possibility of having a second child with hemophilia, even if their own genetic test is negative.

Prenatal Diagnosis

Prenatal diagnostics are primarily used to guide obstetric management and to enable parents to be psychologically prepared for raising an affected child.[55] Prenatal testing is indicated in families with severe or moderate forms of hemophilia, while in families with the mild phenotype such indication is rare. Knowledge of fetal status can guide management decisions during childbirth, including avoidance of procedures and instrumentation that can cause neonatal trauma, such as forceps or vacuum-assisted extraction in vaginal and caesarean deliveries. Molecular genetic testing of at-risk female relatives to determine their genetic status is most informative if the pathogenic variant has been already identified in the proband.

In families with severe hemophilia, prenatal diagnosis is sometimes performed with the aim of terminating a pregnancy in the case of an affected child.[45] Quite several studies revealed that the choices regarding prenatal diagnosis is again based on experiences with the clinical severity, complications, and management of hemophilia within the family. Religious beliefs and prenatal diagnosis experiences of family members could also affect the decision-making. In some women, there are concerns about prenatal diagnosis when the potential risk of the procedure is taken into consideration and the fear when results became known.[45,56] From emotional relief and happiness in confirmation of expecting a healthy child to sadness, disappointment, and burden of subsequent decisions in diagnosis of an affected child could be disclosed.

Prenatal diagnosis from chorionic villus biopsy or amniocentesis is widely available. It can be performed between the 11th and 14th weeks of gestation and enables early genetic diagnosis with subsequent possibility of termination of pregnancy. There are cases in which pregnant women undergo prenatal diagnosis only to know whether or not can expect a male fetus affected with hemophilia. In this situation, mothers should be well informed about the procedure-related risk (miscarriages). Recently, noninvasive testing of fetus cells present in maternal plasma has been performed as early as 10 weeks of pregnancy, although it has been more successful later in pregnancy.[57] These tests may reduce the number of women undergoing invasive procedures when pregnancy termination is not their option, but it does not yet provide a definitive diagnosis. Invasive testing for prenatal diagnosis is becoming less common and is often performed to help with psychological preparation rather than to help the choice for terminating the pregnancy if the fetus is affected by severe hemophilia.

Improvements in care and clinical outcomes of hemophilia have been paralleled not only with advances in methods of prenatal diagnosis but also in attitudes to decisions about carrying out prenatal diagnostic tests for hemophilia. PGD and noninvasive prenatal testing are among the latest methods of prenatal diagnosis available to couples. PGD offers the possibility to select unaffected embryos but implies that the pregnancy occurs through in vitro fertilization procedures. One extensive study showed that hemophilia carriers have positive attitude toward PGD, except those who ejected it based on religious beliefs.

5. Benefits of Genetic Testing in Clinical Practice

Reaching a definitive molecular diagnosis is important for a variety of reasons. Currently, molecular diagnosis of HA and HB allows prediction of likelihood of inhibitor development associated with specific pathogenic variants and response to immune tolerance induction. It enables the clinician to tailor clinical management and discuss the prognosis with the patient. Identification of family-specific genetic variant enables carrier testing of female relatives. Without genetic testing, it can be challenging to establish the exact cause of a bleeding in mild phenotype with similar clinical and laboratory features (HA vs. VWD 2N).

Inhibitor Prediction

Alloantibodies (inhibitors) against FVIII or FIX represent the major complication in hemophilia care, as they render classical substitution therapy ineffective. Inhibitors occur at a frequency of 20 to 30% in severe HA and approximately 3 to 5% of those with severe HB.[58,59] The frequency of inhibitor formation varies depending on various environmental (treatment regimen, type of concentrate) and genetic factors (race/ethnicity, family history, type of genetic defect).[60] The *F8* gene variant is a major contributor to genetic risk factors for inhibitor development.[61,62] The correlation between the genetic variant and inhibitor development is well established. Variants predicted to cause complete absence of FVIII are associated with a higher risk, whereas variants that lead to some protein synthesis are associated with a lower risk. Moreover, patients carrying large multidomain deletions are at the highest risk (up to 80%), while patients bearing missense variants have the lowest risk (<5%). Interestingly, all other types of genetic variants are associated with a median risk of inhibitor formation ranging between 17 and 41%.

Molecular analysis may predict the risk of inhibitor development, not only in the case of severe HA but also in mild HA. It is well known that some missense variants are associated with occurrence of inhibitors in mild HA, while this has never been reported in patients with mild HB.[63] An alternative pathologic mechanism may underlie inhibitor development in these patients. Certain missense variants may alter the immunogenicity of the FVIII protein and induce an inhibitor response against the altered epitope.[64] The higher prevalence of less severe genetic variants (missense) in HB and the less common presence of truncated protein compared with HA may partly explain the lower prevalence of inhibitors in severe HB. Inhibitors in nonsevere HB are almost absent.[59,65] Identification of underlying genetic variant, especially in HB, is important not only for the prediction of the risk of inhibitor formation but also the possibility of anaphylactic reaction occurring after infused replacement therapy.[62]

Carriers

Due to the recessive X-linked inheritance pattern, women in hemophilia families may be heterozygous for the genetic defect referred to as "carriers of hemophilia." Knowing mother's carrier status and genotype related to disease severity can help in evaluating the mother's personal bleeding risk and in planning treatment. Approximately one-third of hemophilia carriers bleed and require treatment. They experience more spontaneous and provoked hemorrhages than noncarriers experience, and are at higher risk of prolonged bleeding after operations, tooth extractions, and tonsillectomy. The risk is highest in those with the lowest clotting factor levels.

Owing to the X-inactivation phenomena, pedigree analysis and clotting FVIII or FIX levels alone are not sufficient to diagnose carriership for hemophilia. While genetic testing is not mandatory for women who are obligate carriers, it is the only way to identify carriers in other scenarios. Genetic testing for carriers is a complex issue, raising several ethical and cultural concerns. Main concerns include disclosure of genetic confidentiality, psychosocial stigma, and emotional burden of future disability.

Severe Hemophilia with Mild Bleeding Phenotype

Although the severity of hemophilia is defined by overall bleeding manifestations, the individual clinical phenotype varies within each group. However, the severity and frequency of bleeding may be different in hemophiliacs with the same factor activity and genetic defect.[66] A mild bleeding tendency is reported in 10 to 15% of severe hemophilia patients. The basis for this heterogeneity in the clinical expression of severe hemophilia is still poorly understood. One possible explanation is the nature and location of the

genetic defect. Small deletion/insertions within polyA runs of exon 14 of the *F8* are theoretically associated with null variants and FVIII:C of less than 1%, but the hemophilia phenotype is associated with reduced bleeding tendency due to restoration of the reading frame in these specific nucleotide sequences.[16] The evidence that some *F8/F9* genetic variants are independent predictors of mild bleeding pattern in severe hemophiliacs may have implications for patient management, as it would influence the choice and timing of onset of prophylaxis and/or the adoption of individualized dose-escalating regimens. These findings are in addition to a lower risk of inhibitor development associated with less severe gene defects, and strengthen the recommendation for early molecular diagnosis to plan different therapeutic strategies in children.

Another possible explanation for the mitigation of the bleeding phenotype in severe hemophilia includes the effect of genetic variants in other loci. By simultaneously detecting pathogenic variants in many genes in a single test, it has been possible to detect genetic variants in one or more other genes along with those in the F8/F9 genes which may lead to phenotypes with milder symptoms. It has been suggested that the mild hypercoagulable state associated with gain-of-function thrombophilic variants such as FV Leiden or prothrombin G20210A may protect hemophiliacs from excessive bleeding; however, the clinical significance of these variants is still controversial.[67] Other coagulation abnormalities, related to natural anticoagulants (deficiencies of antithrombin, protein C, protein S), platelet disorders, and fibrinolytic factors, have been assumed to modulate the bleeding phenotype.[68–71] With the application of NGS in routine diagnostic, identification of such coincidental inheritance is rather straightforward due to the use of multiple gene panels. Such findings need to be interpreted carefully and might provide more evidence to elucidate this phenomenon.

Differential Diagnosis between Mild/Moderate Hemophilia A and VWD

Bleeding phenotypes in VWD are heterogeneous, and most of the forms can be distinguished from HA by measuring VWF antigen and activity levels. However, these functional assays frequently display considerable interlaboratory variability, and a definitive diagnosis cannot be achieved. Differentiating VWD-2N from mild/moderate HA is crucial for effective genetic counseling and therapy.[72] These important findings prompted a change in the therapeutic approach and genetic counseling.[73] Based on these and previously published results, it is necessary to consider VWD 2N in patients with low FVIII:C in the context of the failure to identify a hemophilic variant, FVIII:C/VWF activity ratio less than 0.9, or inadequate response to recombinant FVIII treatment.[35,74] Simultaneous genetic analyses of the *F8/VWF* with NGS yield a definitive diagnosis in such cases.

Discrepancy between Factor VIII Activity Assays

Regardless of the scenario that leads to the suspicion of hemophilia, the definite diagnosis is made by measurement of residual FVIII and FIX clotting activity (FVIII:C and FIX:C). These measurements can be performed using either one-stage or chromogenic coagulation assays. Results from the various FVIII:C assays are usually equivalent in patients with HA, although several reports have revealed systematic assay discrepancies in FVIII:C in one-third of patients with nonsevere HA as part of HA phenotype. This phenomenon becomes clinically significant when it affects diagnosis of HA or assessment of disease severity.[75,76] The reported data confirm that the discrepancy in FVIII:C assay is a fairly common event in nonsevere HA and has genetic background. The profile of genetic defects associated with FVIII:C discrepancy is linked to the class of missense defects. All genetic variants associated with discrepancy phenomena, where one-stage assay shows higher values then the chromogenic assay, are localized in the A1, A2, and A3 domains at the interface between the subunits (A1–A2, A1–A3, A2–A3). These defects lead to a decreased stability of the A2 domain in the activated FVIII protein, resulting in destabilization of the FVIII protein and premature loss of cofactor activity. Discrepancies characterized by higher FVIII:C measured by chromogenic assay involved genetic defects affecting either thrombin, VWF, or FIX binding sites.

Conclusions

High-throughput NGS has revolutionized DNA sequencing by allowing the simultaneous rapid exploration of multiple genes at manageable cost.[32] In hemophilia, molecular testing remains the only way of providing an accurate diagnosis, overcoming the variability and inconsistent data obtained by the conventional methods based on the quantification of coagulation factors. Understanding the basic genetics of hemophilia has contributed to clinical care by providing information leading to advanced treatment, better testing, and more accurate information for patients and families. Genetic testing is indicated for family planning, carrier testing, and prenatal diagnosis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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