Genetics of Hemophilia

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ABSTRACT

Hemophilia A (HA) is an X-linked bleeding disorder caused by heterogeneous pathogenic variants in the factor VIII gene (F8), located at Xq28, with an estimated incidence of 1 in 5,000–10,000 males. The molecular basis of HA is highly diverse, encompassing point variants, deletions, insertions, and structural rearrangements, with intron 22 and intron 1 inversions accounting for the majority of severe cases. A strong genotype–phenotype correlation exists, making molecular characterization essential for accurate diagnosis, prognosis, and management. This review examines the molecular basis of HA, including F8 gene structure and variant spectrum, patterns of inheritance (including rare symptomatic "hemophilic females"), and the evolution of genetic testing strategies - from Southern blotting and Sanger sequencing to next-generation sequencing (NGS).

To review the molecular basis of HA, highlight the associations of genotype–phenotype and, molecular (genetic) testing relics the definitive diagnostic tool for HA.

Literature on F8 gene structure, mutation spectrum, inheritance patterns, and diagnostic approaches was examined with emphasis on intron 22 and intron 1 inversions, which account for most severe cases. Analyzed the modern advanced techniques for the diagnosis and highlighted with target on intron 22 and intron 1 inversions.

HA demonstrates heterogeneous genetic mechanisms, including point mutations, deletions, insertions, and structural rearrangements. Strong genotype–phenotype correlations make molecular characterization essential for accurate diagnosis, prognosis, and management. Next-generation sequencing has significantly improved mutation detection rates, reduced turnaround time and cost, and enhanced understanding of inhibitor risk and reproductive options. However, a small proportion of cases remain molecularly unexplained.

HA is considered a genetic disease so the diagnosis of HA with the advanced techniques is critical for Genetic counseling, reproduction, profession and tragedies. Knowledge and preventive measures are the key factors for planning and policy.

KEY WORDS

Factor VIII (F8) gene, Genetic counseling, Hemophilia A, Intron 22 inversion, Next-generation sequencing, Prenatal diagnosis

INTRODUCTION

Hemophilia A is an X-linked bleeding disorder caused by heterogeneous mutations in the factor VIII gene (F8). The FVIII protein is required for circulation of the intrinsic coagulation path-way.[1] Hemophilia A, or congenital factor VIII deficiency, is the most common of the inherited bleeding disorders, its incidence is estimated to be between 1: 5,000 and 1: 10,000 in men.[2,3] Globally, the birth prevalence of Hemophilia A is estimated at 24.6 per 100,000 live male births, with 9.5 per 100,000 for severe cases.[4] Despite advances in molecular diagnostics and therapeutic interventions, disparities in access to care persist, particularly in low- and middle-income countries. The World Federation of Hemophilia (WFH) reports that nearly 75% of individuals with bleeding disorders worldwide remain undiagnosed or inadequately treated. These disparities are especially pronounced in South and Southeast Asia, where limited infrastructure, economic con-straints, and lack of awareness hinder early diagnosis and comprehensive management. A recent review of hemophilia care in Asia highlighted that the prevalence

ranges from 3 to 8.6 per 100,000 males, with Hemophilia A being five times more common than Hemophilia B.[5] In Nepal, Hemophilia A remains under recognized and underdiagnosed. With an estimated 3,000 individuals with hemophilia, only about 850 have been formally identified and registered by the Nepal Hemophilia Society (NHS) as of 2023 (NHS, 2023; WFH, 2022). The diagnosis of HA in Nepal has historically relied on phenotypic assays, such as the Activated Partial Thromboplastin Time (aPTT) and FVIII activity level measurements, which are available in only a handful of central hospitals.[6] Factor VIII (F8) is the only gene known to be associated with hemophilia A. 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.

Molecular Basis of Hemophilia A(HA)—F8 Genes and Variants:

HA is monogenic disorder caused from pathogenic variants in the F8 gene located at Xq28 of the X chromosome.[7] F8 gene is present in distal end of the long arm of the X-chromosome (Xq28) and spans 186 kb of genomic DNA. It consists of 26 exons that encode a 2351 amino acid precur-sor polypeptide. 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).[8]

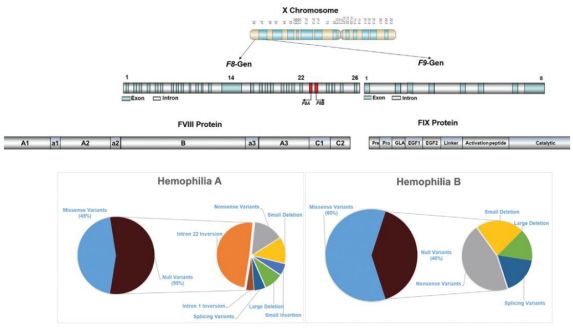

Figure 1. Prevalence and profile of genetic variants in hemophilia A and hemophilia B.

The molecular basis of HA is tremendously varied which has been characterized in numerous patients. Nearly all types of genetic alterations are detected in F8 gene point variants, deletions, in-sertions 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. F8 gene defects predicts the severity of the disease. The type of defects that cause a significant disruption/ absence of the FVIII protein or alteration of a major functional site are identified as null variants (intron 22 and intron 1 inversions, large deletions, nonsense variants, non-A-run small deletions, and splice site variants). Another group recapitulates the non-null vari-ants, which are largely missense variants and contribute to a moderate or mild phenotype in the ma-jority of cases. HA is highly heterogenous with pathogenic variants iden tified throughout the whole gene. According to their localization, the pathogenic variants can affect FVIII protein by diverse mechanisms. They can interfere with the binding of FVIII to different ligands of the coagulation cascade which can lead to decreased function or complete lack of FVIII synthesis or affect the expression, transport through the cell, activity, and stability of FVIII. 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%). Mostly, genetic defects are unevenly distributed along the F8 gene, four hot spots can be identified for recurrent occurrence of genetic changes: the two inversion hotspots, the CpG di-nucleotide hotspots, caused by spontaneous methylation-induced deamination of the5-methylcytosine.[9,10] Small deletions and insertions caused by polymerase slippage errors in series of adenine nucleotides.[11]

Intron 22 and Intron 1 Inversions: The two intronic regions of the F8 gene, intron 1 and intron 22, are frequently involved in pathological inversions resulting from recombination with homologous regions outside the F8 gene. The region homologous to intron 1 (int1h2) is located approxi-mately 140kb toward the telomere and is in the opposite orientation. Intrachromatid or intrachro-mosome homologous recombination of these two regions causes an inversion which shifts exon 1 of the F8 gene by around 140kb toward the telomere. This inversion causes 1 to 4% of all severe cases of HA.[12] Another most frequent inversion involves intron 22. Nearly 45% of severe HA is caused by an intrachromosomal inversion in intron 22. This happens because of two regions homologous to the sequences in intron 22 positioned about 500kb telomeric to the F8gene.[13,14] This inversion event occurs spontaneously in male germ cells with 10-fold higher rate than in females.[15]

Inheritance: Since F8 gene is located on the X chromosome, it is subjected to the unique inher-itance pattern of X-linked genes affecting almost exclusively males.[16] Such a male is called 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.[17] 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, known

as 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 grand children through the carrier daughter. However, 30% of cases in HA is identified as "sporadic".[18] The occurrence 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 genes, either in the affected child or in a female who gave birth to a hemophilia boy or her parents. Maternal mosaicism can also be a reason for the occurrence of such cases, where no mutation is detected in the mother by standard testing.[19] 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.[20,21] 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 con-sanguinity), (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 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.[22-25]

Genetic Studies

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. The human F8 gene was cloned and characterizedin 1982. In 1991, Higuchi et al. performed the first systematic analysis of complete F8 coding region, using denatur-ing gradient gel electrophoresis as a variant screening method but no causative variant was detected in about half of the severely affected patients.[26] Then, the F8 intron 22 inversion was discovered, accounting approximately 45% of the severe HA cases. Initially, the analyses were performed us-ing Southern blotting and long-distance PCR but now simplified technique of inverse PCR (in-verse shifting PCR [IS-PCR]) was established.[26-28]

Molecular genetic testing in hemophilia A

Targeted mutation analysis:

• An F8 intron 22-A gene inversion is described in nearly half of families with severe hemo-philia A. This inversion can be detected by Southern blotting or, more recently, by long range or inverse PCR.

• An F8 intron 1 gene inversion accounts for 2-3% of severe hemophilia A. This inversion is typically detected by PCR.[29,30]

Mutation scanning or sequence analysis: Sanger sequencing was the first applied method that in-volved amplification of F8 gene 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]

The mutation detection rate in individuals with hemophilia A who do not have one of the two common inversions varies from 75% to 98%, depending on the screening method used.[34] In severe hemophilia A, gross gene alterations (including large deletions or insertions, frameshift and splice junction changes, and nonsense and missense mutations) of F8 account for approximately 50% of mutations detected. In mild to moderately severe hemophilia A, missense mutations within the exons coding for the three A domains or the two C domains account for most of the mutations detected.[29,35,36] 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 F8 gene is approximately 95%. Still 5% remains unclear.[37] Although additional molecular and informatics research is needed, NGS is expected to explain cases without "detected" genetic alterations in the F8 gene, leading to better understanding of genotype/phenotype correlation and more effective clinical care (Fig. 2).

Test method	Mutations detected	Mutation detection frequency by test method	
		Probands with severe hemophilia A	Probands with mild to moderately severe hemophilia A
Targeted mutation analysis	*F8* intron 22-A gene inversion	48%	0%
	F8 intron 1 gene inversion	3%	0%
Mutation scanning or sequence analysis	*F8* sequence variants	43%	98%
Deletion analysis	*F8* exonic and large gene deletions	6%	< 1%

Figure 2. Molecular genetic testing and phenotype /genotype relation in hemophilia A.

Diagnostic Approach

The diagnosis of Hemophilia A traditionally begins with clinical evaluation and coagulation assays, including activated partial thromboplastin time (aPTT) and specific factor VIII activity measurements. However, to confirm the molecular etiology and guide personalized management, genetic testing has become indispensable. Accurate molecular diagnosis of hemophilia A is essential for identifying causative mutations in the F8 gene,

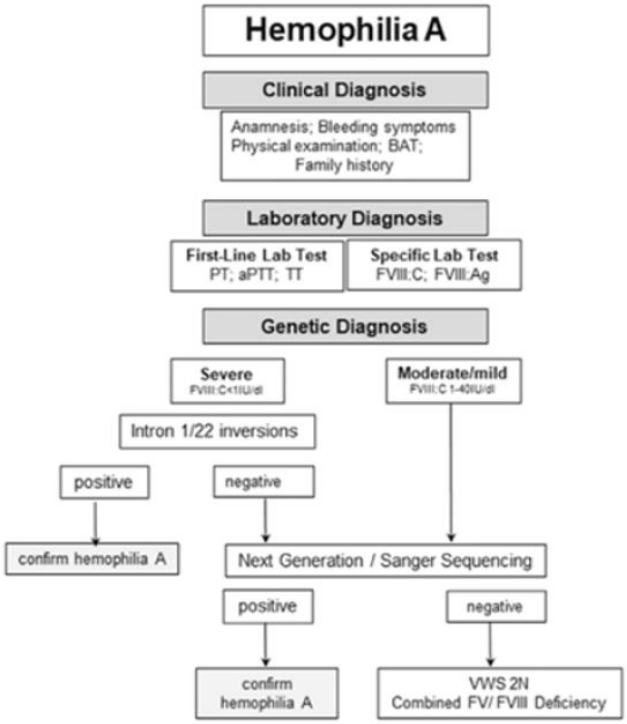

Figure 3. Diagnostic flowchart for hemophilia A. PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; BAT, bleeding assessment tool.

understanding genotype-phenotype correlations, and guiding clinical management. Diagnostic strategies have evolved from conventional methods to high-throughput genomic technologies. Advances in molecular diagnostics, particularly next generation sequencing (NGS) have revolutionized the detection of pathogenic variants in the F8 gene, enabling comprehensive analysis of both coding and noncoding regions.[38]

Clinical applications of hemophilia A molecular genetic testing

• Molecular genetic testing is performed on a proband to detect the family specific mutation in F8 in order to obtain information for genetic counseling of atrisk family members.

• It is indicated for prognostication in individuals who represent a simplex case (i.e., who are the only affected member in a family), identification of the specific F8 mutation can help predict the clinical phenotype and assess the risk of developing a factor VIII inhibitor

• Carrier testing for atrisk relatives requires prior identification of the disease causing mutations in the family

• Prenatal diagnosis and preimplantation diagnosis for at-risk pregnancies require prior identification of the disease-causing mutation in the family.[29,39]

Genetic Counseling and Reproductive Options

Genetic Counseling: Genetic counseling, provides individuals and families information about how genetic disorders are inherited and helps them make informed medical and personal decisions. It has become an integral part of comprehensive care for hemophilia. It is very important for girl and women who carry the disease and are emotionally burdened during reproductive decision-making., 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 preimplantation genetic diagnosis. As a consequence, the final decision of partners 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.[40] 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. While counseling families with newly diagnosed hemo-philia without family history of disease, especially when the mother is not a carrier, special attention is needed. In these cases, the disease can be caused by either a de novo variant in the F8 gene or to the presence of a mosaicism in the mother.[41,42] 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.[43,44]

Prenatal Diagnosis

Prenatal testing is indicated in families with severe or moderate forms of hemophilia, while in families with the mild phenotype such indication is rare. 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. 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. Another newer technique is Preimplantation Genetic Diagnosis (PGD). 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.[45]

CONCLUSIONS

Next Generation Sequencing has revolutionized DNA sequencing by allowing the simultaneous rapid exploration of multiple genes at manageable cost. 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.

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