REVIEW

Molecular genetics of hemophilia A: Clinical perspectives

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Abstract Since the publication of the sequence of the factor VIII (*F8*) gene in 1984, a large number of mutations that cause hemophilia A have been identified and a significant progress has been made in translating this knowledge for clinical diagnostic and therapeutic purposes. Molecular genetic testing is used to determine the carrier status, for prenatal diagnosis, for prediction of the likelihood of inhibitor development, and even can be possibly used to predict responsiveness to immune tolerance induction. Phenotypic heterogeneity of hemophilia is multifactorial, mainly related to F8 mutation but other factors contribute especially to coinheritance of prothrombotic genes. Inhibitor development is mainly related to F8 null mutations, but other genetic and non genetic factors could contribute. This review will focus on the genetic aspects of hemophilia A and their application in the clinical setting and the care of patients and their families.

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1. Introduction

Hemophilia A (HA, OMIM 306700) is an X-linked bleeding disorder caused by heterogeneous mutations in the factor VIII gene (F8). The FVIII protein is required for propagation of the intrinsic coagulation pathway [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].

Factor VIII (F8) is the only gene known to be associated with hemophilia A. F8 maps to the 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 precursor polypeptide [4]. The mature FVIII protein consists of three homologous A domains, two homologous C domains and the unique B domain, which are arranged in the order A1-A2-B-A3-C1-C2 from the amino terminus to the carboxyl-terminal end. The different domains play an important role in the function of FVIII as each domain contains specific binding sites for different components of the clotting cascade [5,6]. Genetic defects can affect these interaction sites and cause HA [7].

Since the publication of the sequence of the F8 gene in 1984, a large number of mutations that cause HA have been identified. The most common is the intron 22 inversion and intron 1 inversion of the F8 gene, which occur in 40–50% and 5–7% of patients with severe HA, respectively [8,9]. The remaining cases are caused by numerous different mutations spread throughout the gene. The majority of these are point mutations or small rearrangement [9,10]. Over the last decades, rapidly increasing numbers of causative gene alterations have been described in different ethnic groups [11–16]. At present, more than 1209 mutations within the F8 coding and untranslated regions have been identified and listed in the F8 HAM-

STeRS mutation database: a comprehensive international database, HAMSTeRS (The Hemophilia A Mutation, Structure, Test and Resource Site), which lists hundreds of mutations yielding the hemophilia phenotype established and maintained in the United Kingdom [URL: http://europium.csc.mrc.ac.uk/].

2. Hemophilia A: diagnostic workup

A specific diagnosis of coagulation factor defect cannot be made on clinical findings. Clinical conditions suggestive of a coagulation disorder defect are demonstrated in Table 1. Laboratory tests are mandatory for specific diagnosis [2,17].

Table 1 Clinical conditions suggestive of a coagulation disorder defect [6].

- Hemarthrosis, especially with mild or no antecedent trauma
- Deep-muscle hematomas
- Intracranial bleeding in the absence of major trauma
- Neonatal cephalohematoma or intracranial bleeding
- Prolonged oozing or renewed bleeding after initial bleeding stops following tooth extractions, mouth injury, or circumcision[*]
- Prolonged bleeding or renewed bleeding following surgery or trauma[*]
- Unexplained GI bleeding or hematuria[*]
- Menorrhagia, especially at menarche[*]
- Prolonged nosebleeds, especially recurrent and bilateral[*]
- Excessive bruising, especially with firm, subcutaneous hematomas

[*] Any severity, especially in more severely affected persons.

Table 2 Severity classification of hemophilia A and related symptoms [6,20,21].

Severity	Clotting factor level % activity (IU/ml)	Symptoms	Usual age of diagnosis
Severe	<1% (<0.01)	Spontaneous bleeding, predominantly in joints and muscles	1st year of life
Moderately severe	1–5% (0.01–0.05)	Occasional spontaneous bleeding. Severe bleeding with trauma, surgery	Before age 5–6 years
Mild	>5–40% (0.05–0.40)	Severe bleeding with major trauma or surgery	Often later in life

Clinical severity does not always correlate with the in vitro assay result.

3. Laboratory diagnosis of hemophilia A

3.1. Coagulation screening tests

Evaluation of an individual with a suspected bleeding disorder includes: platelet count and platelet function analysis (PFA closure times) or bleeding time, activated partial thromboplastin time (APTT), and prothrombin time (PT). Thrombin time and/or plasma concentration of fibrinogen can be useful for rare disorders [18].

In individuals with hemophilia A, the above screening tests are normal, except prolonged APTT [6,19]. However, in mild hemophilia A, the APTT may be normal [20,21].

Other tests recently suggested for the assessment of the overall clotting function include the thrombin generation test, thromboelastogram and the clot wave form analysis [22].

3.2. Coagulation factor assays

Individuals with a history of a lifelong bleeding tendency should have specific coagulation factor assays performed even if all the coagulation screening tests are in the normal range [19,20]. Patients with mild hemophilia A may have normal FVIII coagulant levels by one stage clotting or chromogenic assay which may give five times higher than two stage assay test [23,24]. The normal range for factor VIII clotting activity is 50–150%. In hemophilia A, the factor VIII clotting activity is usually lower than 30–40% with a normal, functional von Willebrand factor level [6,18,25].

Classification of the severity of hemophilia A is based on in vitro clotting activity as shown in Table 2. Approximately 70% of hemophilics is classified as severe, though this number may represent an overestimate since severe hemophilics are more likely to seek medical care [2].

4. Approach to genetic diagnosis of hemophilia A

There are two different approaches to the genetic evaluation of hemophilia A [5,26–28].

1. Analysis of single nucleotide polymorphism or microsatellite variable number tandem repeat markers in the FVIII gene to track the defective X-chromosome in the family (linkage analysis).
2. Identification of the mutation in the FVIII or FIX gene (direct mutation detection).

4.1. Linkage analysis

This can be reliable in up to 99% when applied to those with more than one affected member (familial hemophilia) but can only exclude the carrier status in a female when applied to a family with no prior history of hemophilia (sporadic hemophilia) [29]. The key requirement for linkage analysis is the heterozygosity of the polymorphic marker in the mother of the index case. This requires a strategy for sequential analysis of different polymorphisms in FVIII gene depending on heterozygosity rates in the population [5,7]. In view of considerable ethnic and geographical variation in the allele frequencies of these polymorphisms, it is necessary to establish the informativeness of these polymorphisms in different populations [4,5,9,28].

4.2. Direct mutation detection

Direct detection of disease causing mutation is being increasingly used for genetic diagnosis of hemophilia. This approach has a near 100% accuracy and is informative in over 95% of families with hemophilia A [29]. It is equally efficient and sensitive in detecting mutations in both familial and sporadic hemophilia, even in the absence of a proband [30].

The strategy employed includes amplification of the FVIII gene by polymerase chain reaction (PCR) followed by detection of mutations by various screening methods or/and DNA sequencing [7,28,31].

For reasons of cost and wide applicability, a simple mutation screening method prior to sequencing provides a powerful and accurate tool for genetic diagnosis. Abnormal PCR product profiles are sequenced to identify the nucleotide change [5] .Various mutation screening techniques can be used, such as long distance polymerase chain reaction, multiplex ligation-dependent probe amplification, denaturing high performance liquid chromatography and direct sequencing [7,28–31]. Using combined strategy, the detection rate can be as high as 100%, 86% and 89% in patients with severe, moderate and mild HA, respectively [14].

5. Molecular genetic testing in hemophilia A

5.1. Targeted mutation analysis

- An *F8* intron 22-A gene inversion is described in nearly half of families with severe hemophilia A [6,8,11]. Lower values are reported in non Caucasians [9]. This inversion can be detected by Southern blotting or, more recently, by long-range or inverse PCR [11,31].
- An *F8* intron 1 gene inversion accounts for 2–3% of severe hemophilia A in Caucasians and up to 7% in Asian population [9].This inversion is typically detected by PCR [8,11,31].

5.2. Mutation scanning or sequence analysis

- 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 [6,29].

- 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 [10,11,32,33].
- 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 [8,10,11,32,34].

6. Clinical value of molecular genetic testing of hemophilia A

Establishing the diagnosis of hemophilia A in a proband requires measurement of factor VIII clotting activity, molecular studies are not indicated for diagnosis of hemophilia A [6]. The indications of molecular genetic diagnosis of hemophilia A and, their clinical applications are summarized in Table 3.

7. Genotype–phenotype relation in hemophilia A

All males with a F8 disease-causing mutation will be affected and will have approximately the same severity of disease as other affected males in the family. However, other genetic and environmental effects may modify the clinical severity [6,35].

It has been long recognized that 10–15% of patients with "phenotypically characterized" severe hemophilia (<1% clotting factor activity) have relatively mild disease clinically [30,36,37]. Not all these patients have frequent spontaneous bleeding, and even among those who bleed, the extent of joint

damage tends to vary considerably. The basis for this difference has not been completely understood [21,35,38].

7.1. Factor 8 gene mutation and clinical phenotype

The Factor 8 gene mutation is the most important determinant of the phenotype in hemophilia A [38,39]. Other contributing determinants of the clinical phenotype are summarized in Table 4.

Generally, it has been demonstrated that the most frequent mutations in F8C are intron 22 and 1 inversions, which occur in approximately 50% and 5% of patients, respectively, with a severe phenotype. Large gene deletions are observed in approximately 5% of alleles from patients with severe hemophilia A. The remaining severe cases and all moderate and mild cases result from numerous point mutations and small insertions/deletions, which are de novo mutations in one-third of cases [30,40]. Point mutations leading to new stop codons are all essentially associated with a severe phenotype, as are most frameshift mutations. (An exception is the insertion or deletion of adenosine bases resulting in a sequence of eight to ten adenosines, which may result in moderately severe hemophilia A [40,41]. Splice site mutations are often severe but may be mild, depending on the specific change and location [5,6,8]. Missense mutations occur in fewer than 20% of individuals with severe hemophilia A but nearly all of those with mild or moderately severe bleeding tendencies [8,32,42].

Severe hemophilia with mild bleeding phenotype is described in non-null F8 mutations [39].

The data suggest that the spectrum of gene defects in different populations is heterogeneous. There is no hotspot of mutation in the F8 gene, except the intron 22 and intron 1 inversion, even in patients from different areas of a same country [15]. Different non inversion mutations in F8 gene have been described in different populations, and these relatively frequent, population-specific, mutations mainly missense mutations, together with the *de novo* alterations can lead to significant differences in the spectrum of *F8* mutations among different populations [43].

Published data of the The Italian AICE-Genetics hemophilia A database [8] where the factor VIII gene (*F8*) was analyzed in 1296 unrelated patients with hemophilia A revealed that *F8* mutations were identified in 874 (89%), 146 (89%), and 133 (94%) families with severe, moderate, or mild hemophilia A, respectively. Mutations predicting a null allele were responsible for 80%, 15%, and less than 1% of cases of severe, moderate, or mild hemophilia A, respectively. In severe HA, F8 intron 22 and 1 inversions occurred in 52% and 2%, respectively, large and small deletions in 1% and 10%, respectively, non sense mutations in 9%, splice site mutation in 4%

Table 3 Clinical applications of hemophilia A molecular genetic testing [4,11,15].

- 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 at-risk 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 at-risk 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

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Table 4 Molecular genetic testing and phenotype /genotype relation in hemophilia A [6].

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%

and small insertions in 6%. Missense mutations accounted for 68% and 80% of F8 mutations in moderate and severe HA, respectively.

Chen et al. 2010 [9] tested 115 HA patients from 91 unrelated families in Taiwan, found Intron 22 inversion in 27.8% of the total and 36.7% of severe HA patients while intron 1 inversion comprised of 7.6% of severe patients, values different from Caucasian population. The only female patient with severe HA was found to have heterozygous non-sense mutation (c.6683G > A) of exon 24.

7.2. Coinheritance of thrombophilia genes and clinical phenotype in hemophilia A

In severe hemophilia, heterozygosity for thrombophilic genes may play a role in the milder clinical presentation [35,44]. Coinheritance of prothrombotic genes in hemophilia resulting in milder phenotype has been described including protein C, protein S and antithrombin deficiencies, heterozygosity for factor V Leiden, PT20210A and for tethylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphisms [44–46].

Ettingshausen et al. 2001 [47] studied 92 patients with severe hemophilia A, and reported 10 cases with associated genetic thrombophilic factors (6 FV Leiden, 3 PT20210A, 1protein C type I deficiency), they had delayed onset of symptoms (0.9 vs. 1.6 years). Other studies described protective effect of gain-of-function gene mutations (factor V Leiden [48] and prothrombin G20210A mutation [48–50]) for annual bleeding frequency and severity of the hemophilic arthropathy.

It has been suggested that the prothrombotic mutation may compensate for the low factor VIII level, resulting in more efficient thrombin generation and ensuing attenuation of clinical symptoms [44,46].

However, significant association between co-inheritance of prothrombotic genes and mild hemophilia phenotype has not been confirmed by other studies [37,51]. Prothrombotic risk factors seem to influence phenotype but they can account for only a small part of the heterogeneity .It is suggested that the origin of the large heterogeneity of phenotypes in severe hemophilia is multifactorial [35].

On the other hand, the association of this prothrombotic mutation with other acquired or inherited thrombophilic factors might overcome the congenital bleeding tendency in hemophiliacs, thereby increasing the risk of thrombotic complications [46].

7.3. Other possible hemophilia A phenotype modifiers

Inter-individual variance in the pharmacokinetics (PK) of FVIII is well described. In patients with hemophilia a clear association was demonstrated between blood group and von Willebrand factor level and their FVIII half-life. Patients with blood group O and a low von Willebrand antigen level have a significantly decreased FVIII half-life and significantly lower annual clotting factor consumption [25,52].

The role of the fibrinolytic pathway in the clinical heterogeneity of hemophilia phenotype have been suggested [53,54]. Grünewald et al. 2002 [53] hypothesized that ineffective hemophilic hemostasis in response to trauma evokes a protracted stimulation of the entire hemostatic system, including costimulation of fibrinolysis. The association of a more intensely hemorrhagic phenotype with a paradoxical hyperstimulation of the fibrinolytic system resembles a vicious circle, where bleeding seems to cause predisposition to more bleeding. Whether these differences can also explain the heterogeneity of phenotypes has not yet been established [25,54].

Other authors [38] suggested that mediators of the inflammatory response in the synovium are likely to impact the severity of joint damage and partially contribute to the variability in the severity of arthropathy in hemophilia patients.

8. Genetic aspects of inhibitor development in hemophilia A

The production of neutralising antibodies in response to infused factor VIII has always been of considerable interest, principally because it is a major complication of replacement treatment [55]. The cumulative risk of inhibitor development in previously untreated patients (PUPS) was reported to range from 0% to 38.7% depending on type of factor VIII product used [56,57]. Inhibitors develop more commonly in severe hemophilia than in mild/moderate disease, and it is potentially a major complication of gene therapy [8,5].

Evidently, the mutation underlying the hemophilia is important [5,8]. Mutations of F8 gene associated with the absence of a gene product, such as deletions or nonsense mutations, confer a high risk for inhibitor production; mutations associated with the presence of a gene product (even very low amounts of the protein) confer a low risk for inhibitor production [8,59,63–65]. However, in reality, the situation is not so clear cut. Among patients with identical mutations, some may produce inhibitors and others may not. Clearly other factors are implicated [5,63,64]. Margaglione et al. 2008 [8] reported that patients who had severe hemophilia A and mutations predicting a null allele developed inhibitors more frequently (22% to 67%) than patients with missense mutations (5%). Both genetic and non genetic factors could be involved in inhibitor development in HA (Table 5).

8.1. Genetic factors involved in inhibitor development

Several genetic factors, such as a positive family history of inhibitors, ethnicity, FVIII genotype, and certain polymorphisms in immune modulatory genes, are associated with the risk of inhibitor development [35,64].

Table 5 Genetic and non-genetic factors influencing the development of inhibitors in hemophilia A patients [59–63].

Genetic risk factors
- Type of F8 mutation
- HLA class II polymorphism
- Ethnicity
- Immunogenecity
- Family history of inhibitors

Non-genetic risk factors
- –Immunological factors
- –Surgery and trauma
- –Treatment-related factors
    - –Age at 1st exposure
    - –Modality of FVIII infusion (continuous infusion)
    - –Intensive treatment with FVIII concentrates
    - –Type of FVIII given
    - –Changes of FVIII concentrates

Studies of the correlation of the genetic defects with the clinical course revealed that the type of F8 mutation represents the most important genetic predisposing factor for inhibitor formation, the most severe complication of treatment with factor VIII concentrates [36]. Large deletions, nonsense mutations and inversions are associated with a higher risk of inhibitor development in an Italian study [65], explaining increased risk in patients with inhibitor family history.

Another large study in the Netherlands [58] found that, in patients with severe HA, splicing errors presented the highest frequency of inhibitors, ahead of inversion of intron 1 and of intron 22, nonsense mutations and large deletions. The lowest inhibitor frequency in severe HA was found in patients with missense mutations and small deletions/insertions. Their results suggest that complete absence of FVIII because of null mutations, including splice site mutations, or the absence of a second transcript result in an increased risk of inhibitor development.

However, concordance family studies showed that factors other than F8 mutations are involved. An emerging role is investigated for polymorphisms of immune-regulatory genes that may increase (IL-10 and TNF-alpha) or reduce (CTLA-4) inhibitor risk and whose heterogeneous ethnic distribution may correlate to the higher inhibitor risk in non-caucasian patients [61,62,66,67].

A role for FVIII haplotypes, particularly in black hemophiliacs, has been recently proposed. Viel et al. [68] suggested that mismatched factor VIII replacement therapy may be a risk factor for the development of anti-factor VIII alloantibodies in black population.

A weak association between human MHC (HLA) class II genotype and the development of inhibitor antibodies against factor VIII was reported; slightly more pronounced in patients with the intron 22 inversion [5]. The interaction between F8 genotype and HLA haplotype has been suggested as possible determinant factor of inhibitor development in hemophilia [69]. Other studies failed to demonstrate relation between HLA class I and II and inhibitor development in hemophilia A [70].

8.2. Non genetic factors involved in inhibitor development

Some clinical features of inhibitors in hemophiliacs remain incompletely explained by genetic predisposition [71].

The observation of hemophilic monozygotic twins discordant for inhibitors points out the interplay of non-genetic factors. Theoretically, challenges of the immune system brought about by infections, vaccinations, and tissue damage in association with FVIII exposure have the potential to generate signals that activate the antigen-presenting cells [71,72], ultimately promoting the immune response against FVIII. The influences of treatment-related cofactors, such as age at first exposure, type of product used, mode of delivery, intensity of replacement, and treatment modality, have been reported in clinical studies [60,70,73].

8.3. Genetic factors and response to immune tolerance in hemophilia patients with inhibitors

Immune tolerance induction (ITI) is an important line of management of inhibitor development in hemophilia patients [57].

The role of F8 genetic profile in predicting response to immune tolerance induction (ITI) in inhibitor patient is suggested [40].

Recently, an Italian Study Group [65] (AICE PROFIT) proved that F8 mutations known to be associated with a high risk of inhibitor development (large deletions, inversions, nonsense mutations and splice site mutations) had significantly lower ITI success rate than patients with lower-risk F8 defects (small insertions/deletions and missense mutations). On multivariate analysis, the mutation risk class remained a significant predictor of success, as were inhibitor titer at ITI start, and peak titer during ITI. The study concluded that ITI success is influenced by F8 genotype.

9. Diagnosis and morbidity of female carrier of hemophilia A

Approximately 10% of females with one F8 disease-causing mutation and one normal allele has a mild bleeding disorder [4,6]. It has been estimated that for each male with hemophilia, there are five potential female carriers [74].

Pedigree analysis and clotting factor VIII levels were previously used to diagnose carriership for hemophilia [4]. By pedigree, a "definite" carrier is the daughter of a hemophiliac, the mother of two hemophiliacs and the mother of a hemophiliac with family history of hemophilia traceable in the female line [27]. In the early 1980s, it became possible to ascertain the carrier status by means of DNA analysis, which has evolved from haplotyping to mutation analysis offering certainty about the carrier status [75]. During the last 3 decades, genetic counseling, carrier testing, and prenatal diagnosis of hemophilia have become an integrated part of the comprehensive care for hemophilia [4,74].

Female carriers are expected to have a plasma concentration of factor VIII corresponding to half the concentration found in healthy individuals, which is generally sufficient for normal hemostasis. However, in carriers a wide range in clotting factor levels is seen, from very low, resembling affected males, to the upper limit of normal [76]. This range has been attributed to the phenomenon of lyonization, random X-chromosome inactivation, which takes place in the early embryonic life [1,27].

In the study of Plug at al 2006 [77], the median clotting factor level of carriers was 60% (range, 5–210%) and in non-carriers 102% (range, 45–328%). Their findings suggest that not only clotting factor levels are at the extreme of the distribution, resembling mild hemophilia, but also mildly reduced clotting factor levels between 40% and 60% are associated with bleeding. Ay et al. [76] reported that FVIII levels are lower in carriers compared to non-carriers [74%(51–103)vs. 142%(109–169)]. The type of FVIII gene mutation do not influence FVIII levels and Carrier status is the major determinant of a carrier's FVIII plasma level. Factors known to influence FVIII levels in the general population do not significantly affect FVIII activity in carriers.

Carrier women will benefit from knowledge of both their genetic (mutation present or not) and their phenotype (level of plasma factor activity) status [4,74]. Carriers of hemophilia A with clotting factor levels of less than 60% often have an increased bleeding tendency. When a FVIII level of less than 60% is found, a carrier should be considered and treated as a (mild) hemophilia patient. Carriers with clotting factor levels of less than 30% should be regularly seen at a hemophilia treatment centre [78].

The heterogeneity in FVIII levels is particularly important for the pregnant carrier for at least two major reasons: First, hemophilia carriers have been reported to be at a significantly higher risk for primary and secondary postpartum hemorrhage. Second, the risk for hemorrhage also extends to a hemophiliac infant born to the carrier, particularly with respect to scalp and intracranial bleeds [79].

10. Prenatal diagnosis of hemophilia A

As the severity of hemophilia remains stable within an individual family, the partners can base their decision on their own experience with the disease, while they are informed by a clinician about progress in hemophilia treatment. Prenatal testing is generally indicated in families with severe or moderate forms of hemophilia. In families with the mild disease such indication is rare [27,80].

10.1. Molecular genetic testing

Prenatal testing can be done for carrier women if the mutation is identified in a family member or if linkage has been established in the family [4]. The fetal sex is identified by chromosome analysis of fetal cells obtained by chorionic villus sampling (CVS) at approximately 10–12 weeks' gestation or 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 F8 disease-causing mutation or for the informative markers [6].

10.2. Percutaneous umbilical blood sampling (PUBS)

If the disease-causing F8 mutation is not known and if linkage is not informative, prenatal diagnosis is possible using a fetal blood sample obtained by PUBS at approximately 18–21 weeks' gestation for assay of factor VIII clotting activity [6,27].

Invasive sampling such as chorionic villus sampling (CVS) or amniocentesis (AMC) carries about 1–2% risk of fatal and non-fatal complications to the fetus [27]. Hence, efforts are on to develop prenatal diagnostic strategies either by using circulating fetal cells or fetal DNA from maternal blood [80].

Fetal sex assessment by detecting specific Y chromosome sequences in maternal blood has high accuracy from the seventh week of gestation [81]. Recently, Cell-free fetal nucleic acids (cffNA) detected in the maternal circulation during pregnancy, potentially offer an excellent method for early non-invasive prenatal diagnosis (NIPD) of the genetic status of a fetus. Using molecular techniques, fetal DNA and RNA can be detected from 5 weeks gestation. This method can be used for non invasive fetal sex determination [82,83].

10.3. Preimplantation genetic diagnosis (PGD)

PGD is recently available for families in which the disease-causing mutation has been identified in an affected family member [84,85]. Although financial implication is considerable, yet for couples who do not want to go through the trials and tribulations of termination of pregnancy in case of an affected fetus, these techniques remain the techniques of choice for prenatal diagnosis [80].

Factor VIII DNA microarray analysis is reported as an alternative gene mutation analysis approach that has a high sensitivity and reproducibility in molecular diagnosis of hemophilia, however, expensive the technique is [86]. A recent study [80] suggested the advantage of gene microarray analysis in prenatal diagnosis of hemophilia, not only by identifying the highly heterogeneous mutations but may also be useful in studying the effect of various ameliorating or epistatic genetic mutations/polymorphisms simultaneously, providing a wide range of options to the genetic counselors, and the couples opting for prenatal diagnosis.

11. Gene therapy in hemophilia A

Hemophilia is a very good candidate for use of gene therapy protocols because it is a monogenic disease, and even low expression is able to achieve reversion from a severe to a moderate phenotype [56,87]. Gene therapy for hemophilia is justified because it is a chronic disease and because a very regular factor infusion is required that may involve fatal risks and because it is very expensive [87].

Several strategies have been proposed for gene therapy for hemophilia. These strategies are based on both *in vivo* and *ex vivo* approaches. The *in vivo* delivery studies using non-viral or viral vectors, such as, AAV(adeno-associated viral vector), and retroviral have demonstrated very encouraging preclinical data and early-phase clinical trials were safe [87–89]. However, to achieve the therapeutic success of these strategies, there remain challenges on both efficacy and safety issue such as potential side effects related to vector-mediated cytotoxicity, unwanted immunological responses and the risk of insertional mutagenesis [90,91].

Ex vivo delivery of therapeutic transgenes provides a safer strategy by avoiding systemic distribution of viral vectors [90]. A clinical trial that used autologous skin fibroblasts, genetically modified with the FVIII transgene, implanted into the greater omentum of severe hemophilia A patients, was well tolerated and a safe procedure [92]. However, elevation of FVIII levels was modest and short term, it was suggested that the viability of the transplanted cells as well FVIII expression levels is a major obstacle of this strategy. The use of hematopoietic stem cells (HSC) [93,94] and autologous endothelial progenitor cells [95] provides an alternative strategy to deliver the therapeutic coagulation factor [90, 96].

Recently suggested new approaches of gene therapy in hemophilia are Platelet-based gene therapy aiming at delivery of clotting factors to vessel injury sites by platelets [97,98], and intraarticular gene therapy targeting protein expression in affected hemophilic joints [90].

A recent approach is the novel concept of continuous expression of activated FVII from a donated gene for the treatment of hemophilia, based on the fact that infusion of recombinant human activated factor VII (FVIIa), proved effective in inducing hemostasis in severe hemophilia [99,100]. Compared to factor VIII, FVIIa as a potential transgene, is unlikely to induce a harmful immune response because all hemophilia patients should be fully tolerant to it, and it controls hemostasis regardless of F8 inhibitors status. The use of FVIIa as the transgene and gene therapy as the delivery method is suggested as future therapy [101,102].

Gene therapy has recently been investigated for the management of the problem of inhibitor development in hemo-

philia patients, yet animal studies are still in early phases [56,103].

In conclusion, the rapidly proceeding advances in the technology of genetic diagnosis of hemophilia in the last decades give the patients and their treating physicians better options to anticipate disease severity and the possibility of complications. This offers better options for genetic counseling, disease prevention, planning of patient therapy, and better detection rate and care of carriers and their offsprings.

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