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The Role of Genetic Diagnosis in Hemophilia A

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Hemophilia A is a rare X-linked congenital deficiency of clotting factor VIII (FVIII) that is traditionally diagnosed by measuring FVIII activity. Various mutations of the *FVIII* gene have been reported and they influence on the FVIII protein structure. A deficiency of or reduction in FVIII protein manifests as spontaneous or induced bleeding depending on the disease severity. Mutations of the *FVIII* gene provide important information on the severity of disease and inhibitor development. *FVIII* mutations also affect the discrepant activities found using different FVIII assays. *FVIII* activity is affected differently depending on the mutation site. Long-range PCR is commonly used to detect intron 22 inversion, the most common mutation in severe hemophilia. However, point mutations are also common in patients with hemophilia, and direct Sanger sequencing and copy number variant analysis are being used to screen for full mutations in the *FVIII* gene. Advances in molecular genetic methods, such as next-generation sequencing, may enable accurate analysis of mutations in the factor VIII gene, which may be useful in the diagnosis of mild to moderate hemophilia. Genetic analysis is also useful in diagnosing carriers and managing bleeding control. This review discusses the current knowledge about mutations in hemophilia and focuses on the clinical aspects associated with these mutations and the importance of genetic analysis.

Key words: Hemophilia A, Genetic testing, Factor 8, Mutation

REVIEW ARTICLE

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INTRODUCTION

Hemophilia A is the most common inherited bleeding disorder and is caused by an abnormal or decreased activity of factor VIII (FVIII) protein. The *FVIII* gene is located on the X chromosome, and hemophilia A is an X-linked recessive disorder that predominantly affects males [1]. Its prevalence is 1 in 5,000–10,000 male births. Females are commonly heterozygote carriers; however, although rare, they can develop hemophilia as a result of X-chromosome inactivation, such as in Turner's syndrome, or if both parents carry abnormal genes [2].

Hemophilia A is diagnosed via factor assays to identify FVIII deficiency. The plasma concentrations of FVIII correlate with the degree of bleeding, which is classified as mild, moderate, or severe, and is related to the factor levels (mild, 6%–40% of normal; moderate, 1%–5% of normal; severe, < 1% of normal) [3].

According to the 2019 annual report of the Korea Hemophilia Foundation (KHF), there were was 1,746 patients with hemophilia A in Korea in that year [4]. Given the size of the Korean population, this is much fewer than expected. Many hemophilia patients do not register for the KHF because KHF registration is not obligatory. Therefore, a national registration system is needed to properly measure the incidence of hemophilia in the Korean population.

Moreover, a high proportion of these enrolled patients had severe hemophilia, with only 11% having mild disease. Generally, mild hemophilia is diagnosed only when an injury or medical intervention results in prolonged bleeding. A patient who is unaware that he has hemophilia may ignore the symptoms until they become severe or until complications develop.

The development of inhibitors is the most serious complication of FVIII replacement therapy; however, fewer hemophilia patients in Korea have inhibitors than in other countries [5]. Therefore, a more accurate diagnostic method, such as genetic analysis, is needed for the proper diagnosis and treatment.

This review discusses the current knowledge about mutations in hemophilia A, and focuses on the clinical aspect associated with these mutations, and the importance of genetic analysis.

STRUCTURE OF THE *FVIII* GENE

Hemophilia is caused by mutation of the *FVIII* gene, which spans 187 kb on the long arm of the X chromosome (Xq28) and contains 26 exons. FVIII protein has three distinct domain arranged as (NH_2) A_1-A_2-B-A_3-C_1-C_2 (COOH) [6]. The protein folds into a heterodimer in which the heavy (A_1-A_2-B domains) and light (A_3-C_1-C_2 domains) chains are linked by metal ion-dependent bonds [7]. This heterodimer circulates in a complex with von Willebrand factor (VWF) [8]. A_3-C_1-C_2 effectively competes with activated FVIII (FVIIIa) for binding to activated factor FIX (FIXa). The C_2 domain also contributes to VWF binding, which is essential for stabilizing FVIII in the circulation [9].

Various mutations of the *FVIII* gene can influence on the protein structure, reduce the synthesis or secretion of FVIII, and impair FVIII activity. A deficiency of or reduction in FVIII protein results in loss of normal hemostasis and manifests as spontaneous or induced bleeding depending on the disease severity. Mutations of the *FVIII* gene seem to be correlated with clinical findings and provide important information on the severity of disease and inhibitor development.

MUTATIONS OF THE *FVIII* GENE

More than 2,000 unique variants of the *FVIII* gene have been reported in the FVIII variant database [10]. The majority (66.5%) of hemophilia A cases are associated with single nucleotide variants (SNVs). Of these, missense mutations are the most common type in hemophilia A. Nonsense and splice site variants are more frequently associated with severe disease [11]. Severe hemophilia A is also associated with a higher proportion of deletions than mild or moderate hemophilia A.

Although mutations causing hemophilia A are scattered throughout the *FVIII* gene, there are two hotspots for recurrent genetic changes, inversion of introns 22 and 1 [12]. The size of the introns in the *FVIII* gene is variable and intron 22 is the largest. Intron 22 is the hotspot for the most common genetic defects leading to severe hemophilia A. Intron 1 is another hotspot, and mutations there cause severe hemophilia A in 1%-6% of patients [13].

Long-range PCR is generally used to detect these two inversions. If they are not present, full mutation screening of the *FVIII* gene was done using direct Sanger sequencing, covering all exons, intron and the promoter region. Copy number variant (CNV) analysis, such as array comparative genomic hybridization and multiplex ligation-dependent probe amplification, has recently been used to detect large deletions or duplications.

The routine order of genetic analysis for hemophilia A is based on the disease severity: severe cases first typically undergo intron 22 and 1 inversion analysis with long-range PCR before CNV analysis or direct sequencing. These routine molecular methods can identify pathogenic variants in approximately 95% of hemophilia A cases [14]. Next-generation sequencing such as whole genome sequencing, provides an enhanced opportunity to characterize molecular defects in patients, particularly in those whose molecular defect has not yet been determined.

In South Korea, it has been reported that intron 22 inversion and point mutations account for about 90% of all hemophilia mutation [5,15]. Therefore, in case of severe hemophilia A, long-range PCR analysis of the intron 22 inversion mutation should be performed at first. If intron 22 inversion is negative, direct sequencing analysis can be performed. MLPA can be useful for detecting large dosage mutations. In most cases, it is difficult to routinely perform genetic testing to diagnose hemophilia A except for research purposes.

The variant types except for the inversion of introns 22 and 1 span a broad spectrum. The American College of Medical Genetics and Genomics and the Association for Molecular Pathology have published guidelines for the clinical interpretation of novel genetic variants [16]. Therefore, there is a need to re-evaluate and update previous genetic reports in the hemophilia databases.

FVIII INHIBITOR DEVELOPMENT

Inhibitor formation is the most severe treatment complication and occurs in 20%–45% of previously untreated patients with severe hemophilia A. The risk factors for inhibitor development are divided into treatment-related (number of exposure days, intensity of exposure, replacement product type, and age

at first exposure) and patient-related (family history of inhibitors, severity of hemophilia, and FVIII mutation) factors [17].

Large deletions and nonsense mutations are associated with an increase in inhibitor development. Inhibitors are more common in patients with mutations within domains A_3, C_1, and C_2. In South Korea, high inhibitor risk is associated with large deletions and point mutations in domains A_3 and C_2 [5].

In severe hemophilia A, the patient's immune system drives inhibitor development, whereas in mild cases, other mechanisms may underlie inhibitor development. In particular, missense mutation in domains C_1 or C_2 may alter the immunogenicity of FVIII protein, leading to inhibitor formation [18]. Therefore, it is important to perform mutation screening in all novel hemophilia cases to predict and manage the risk of developing inhibitors. There may also be a role for genotyping in predicting the outcome of immune tolerance induction and for future gene-based hemophilia therapies [19].

FVIII ACTIVITY DISCREPANCY

FVIII mutations also help to explain the discrepant activities found using different FVIII assays. The one-stage clotting assay (OSA) measures the ability of reference plasma to shorten the activated partial thromboplastin time (APTT) of hemophilic plasma. This method is simple, rapid, and widely used for clinical monitoring. The chromogenic substrate assay (CSA) is an indirect method of measuring FVIII activity and is a two-stage clotting assay similar to the OSA. First activated factor X (FXa) is generated, then it is reacted with a chromogenic substrate and subjected to photometric measurement. This method does not require FVIII-deficient plasma and has lower inter-laboratory variability than the OSA; however, it is technically complex and more expensive than the OSA.

In most cases of severe hemophilia A, the FVIII activity measured with both assays is low enough that the correct diagnosis will be made. However, some patients with mild hemophilia A show FVIII activity within the normal range when it is measured with one assay but lower FVIII activity when it is measured with the other [20]. In these patients, the diagnosis may be missed if only one assay is used. This can lead to failure to diagnose hemophilia A or the severity of the disease being classified incorrectly.

Depending on the mutation site, FVIII activity is affected differently, influencing the OSA or CSA results. Dissociation of the A_2 subunit correlates with inactivation of FVIIIa and this mutation is related to plasma FVIII activity discrepancies between OSA and CSA [21]. Mutations located in the three A-domains are associated with lower levels in the CSA, whereas mutations affecting thrombin cleavage sites or FIX binding sites, such as the C-domains, have lower values in the OSA.

Replacement therapy with plasma-derived FVIII (pdFVIII) or recombinant FVIII (rFVIII) concentrates requires accurate potency determination to ensure optimal safety and efficacy. The OSA and CSA are used to assign potency and the FVIII activities indicated by the two assays are often comparable. However, discrepancies have been reported in some patients with specific mild hemophilia A phenotypes [20] and in patients being treated with pdFVIII and rFVIII products that modify the B-domain in FVIII [22]. The CSA is reported to be less affected by these modifications than OSA. Product manufacturers should provide appropriate guidance on recommended monitoring methods of factor activity and the laboratory has to prepare these methods. When the genetic analysis is routinely performed in new patient of hemophilia A, the mutation screening could confirm whether the mutation is known to cause discrepancies in OSA or CSA.

MUTATIONS IN HEMOPHILIA A CARRIERS

Although FVIII levels are decreased in carriers, factor levels and coagulation tests such as APTT are not good predictors of bleeding [23]. FVIII activity shows considerable variability and normal FVIII activity does not rule out carrier status. Labarque et al. [24] proposed that a cut-off value of 0.9 for the ratio of FVIII activity to the vWF antigen level may predict carrier status. The ratio is somewhat useful, but with few applications. It is also difficult to differentiate between hemophilia A and von Willebrand disease based on factor levels and coagulation test results alone. A molecular diagnosis is important for distinguishing between bleeding disorder with similar clinical presentations but different underlying genetic causes.

Identification of causative *FVIII* gene mutations can provide useful information for carriers and family members with a history of hemophilia A [12]. Hemophilia A carriers are predominantly female, and postpartum hemorrhage is the most serious of the reported abnormal bleeding patterns. Awareness among pregnant women and healthcare providers could lead to safer childbirth and appropriate management of postpartum bleeding. Therefore, genetic analysis is important for diagnosing carriers and for proper bleeding management [25].

CONCLUSIONS

It is important to incorporate the knowledge of molecular and genetic aspects of hemophilia into routine clinical care. Genetic analysis of the *FVIII* gene is useful for diagnosis of hemophilia A as well as for the selection of therapeutic regimens, such as FVIII concentrates or recombinant substitutes, by confirming susceptibility to inhibitor development. Advances in molecular methods for detecting mutations, such as next-generation sequencing, may enable active genetic analysis in the treatment of hemophilia patients. Furthermore, genetic screening of hemophilia carriers may predict disease and help prevent bleeding.

CONFLICTS OF INTEREST

There are no potential conflicts of interest relevant to this article.

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