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REVIEW ARTICLE

HETEROGENEITY OF HAEMOPHILIA A GENETIC AND ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF FACTOR VIII INHIBITORS.

Roswati MN

Faculty of Medicine, University Kuala Lumpur Royal College of Medicine Perak

Corresponding Author
Dr. Roswati M Noor
UniKL Royal College of Medicine Perak, No. 3, Jalan Greentown, 30450 Ipoh, Malaysia.
Email: roswati@unikl.edu.my

Abstract

Haemophilia A (HA) is a rare disease by definition, as it affects 1 in 5,000 male livebirths, but it is the commonest among inherited bleeding disorders. There are 1,295 HA cases registered in hospitals under Ministry of Health (MOH) for Factor VIII (FVIII) replacement therapy and 83 (6.4%) are identified to have FVIII allo-antibodies or inhibitors, which is an under-reported figure as studies have shown that 30% of HA develop inhibitors. With the recent adoption of prophylactic FVIII replacement therapy, this treatment modality will greatly improve the quality of life for HA. Unfortunately, 30% of HA develop inhibitors and this causes management of HA with inhibitors very challenging with progressive arthropathy, disability and increase in treatment costs. There have been many studies on mechanisms of inhibitor development, but the pathogenesis is not being fully elucidated. Risk factors to inhibitor development are grouped into genetic or non-modifiable and environmental risk factors. FVIII gene mutation has been consistently identified to be the most important risk factor. This review paper discusses on the genetics and environmental risk factors on inhibitor development in HA, towards better understanding in predicting which HA have the highest risk and lowest risk of inhibitor development and strategized appropriate treatment modality.

Keywords: Haemophilia A, Factor VIII inhibitors, Genetic risk factors, Environmental risk factors.

Prevalence of Haemophilia A

Haemophilia A (HA) is an X-linked recessive disorder, affecting only the boys and manifested as spontaneous musculoskeletal bleeds due to absence or dysfunctional FVIII coagulation protein. Mean prevalence of HA in Malaysia has increased from 5.6 per 100,000 males in 1998 to 6.6 per 100,000 males in 2006, with a mean of 5.9 ± 0.4 per 100,000 males[1]. There are 1,295 HAs registered with Ministry of Health (MOH) in 2017 with 83 (6.4%) of them are having FVIII inhibitors[2]. HA occurs 1 in 5,000-10,000 live male births around the globe and there is no ethnic predilection. Prevalence of HA in high income countries is 12.8 ± 6.0 per 100,000 males and the rest of the world is 6.6 ± 4.8 per 100,000 males. Discrepancy between these 2 categories of countries are due to under reporting of HAs, for example, the number of HAs registered with Haemophilia Federation of India is only about 10% of the expected cases, and similarly in Malaysia and South Africa, the numbers of HA reported are < 50% of the expected cases[3]. Canadian Haemophilia Registry has 3006 HA registered in 2018[4], with 381 HA are in the age group of 65 years and above. A review by Pratap et al, 2019[5] identified that the challenges faced by HA in Canada are of under-diagnosing of HA, ageing community of HA and inhibitor development.

There has been a great improvement in the treatment of HA in Malaysia, the latest being the use of prophylactic treatment with FVIII concentrates as recommended by MOH in 2018. With this treatment strategy, the cost to purchase FVIII concentrates will be increased due to the increase in volume purchased by MOH but there will be reduction in healthcare costs as this will be offset by the resultant reduction in major bleeding episodes, reduction in corrective surgeries for damaged joints and overall improvement in quality of life for children and adults with HA. The economic costs from reduction in absenteeism from work and school will, in totality, save costs to the nation. Nevertheless, there is still a big problem with HA on FVIII replacement therapy, where 20%-35% of severe HAs develop inhibitors[6]. Development of inhibitors is understandably caused from allo-immunization to exogenous FVIII since there is no circulating endogenous FVIII protein. This is not seen in all severe HAs. Inhibitors are also seen in 3-13% of mild and moderate severity HAs[7] where there is endogenous low level production of FVIII. Pathogenesis of inhibitor development is still not well understood. Presence of inhibitor in HA will cause neutralization of the administered FVIII concentrates. This leads to increase in cost of treatment by more than 3 fold as higher doses of FVIII is needed to overcome the antibodies for effective haemostasis.

Factor VIII biology and genetics

Absence of FVIII results in impaired thrombin generation and produces less stable fibrin clots. Severity of bleeding depends on FVIII levels measured by clotting activity in the plasma. Most severe has <1% FVIII activity or equivalent to <0.01 IU of clotting activity per 1 ml of plasma. Moderate has 1-5% of FVIII activity while mild HA has 5-40% of FVIII activity. Severe haemophilia manifested as spontaneous bleeding into muscles and joints, while moderate and mild HA, bleeding is triggered by injury or trauma that leads to prolonged bleeding with poor wound healing. Treatment is by replacement therapy with FVIII concentrates.

FVIII is produced mainly by the liver sinusoidal endothelial cells. The sites of FVIII synthesis was detected using complementary DNA (cDNA) probe which hybridized to messenger RNA (mRNA) of FVIII in tissues and it was discovered that not only most of FVIII mRNA is produced in the liver sinusoidal cells, but there exists expression of FVIII mRNA in isolated hepatocytes, lymph nodes and kidney cells, but not in white blood cells or cultured endothelial cells[8].

Located at Xq28 chromosome[9], the Factor VIII gene spans about 180kb with 26 exons and 25 introns. The exons ranges from the smallest exon 5 with 69 base pairs (bp) to the biggest 3.106

kilobase pairs (kb) of exon 14, while the biggest introns, intron 22 (*IVS22),* are 32.4 kb[9]. Exon 14 encodes the central B domain. All 26 exons produce 9kb of mature mRNA which is only from 5% of the whole gene and it also produces 2 additional mRNA which is expressed ubiquitously. In *IVS22*, there is a CpG island that promotes 2 additional coding genes *F8A* and *F8B*[10]. *F8A* is transcribed backwards from intronic sequence within *IVS22*. *F8A* has 2 additional repeat sequences placed nearer to *Xq* telomere (telomeric to *F8).* These 2 sites of *F8A* repeats are frequently involved in intra-chromosomal homologous recombination, resulting in recurrent inversion of *IVS22* (inv*IVS22*)[11]. Naylor et al, has defined the repeated region as 9.5 kb and termed as *inth22h-1*(intron 22 homologous region) and the other 2 copies, called *int22h-2* and *int22h-3,* are 300 kb and 400 kb, respectively, and situated 5' end or towards telomere of the *F8* gene (Figure 1C). Using chemical mismatch analysis, these 3 sites involved in inv *IVS 22* are matched 99.9%, hence inv *IVS 22* is one of the commonest *F8* mutations seen in HAs and severe HAs[12].

The exons 1-26 translate into 2,332 amino acids, forming a dimeric protein, consisting of light chain and heavy chain. The heavy chain domain structure A1-a1-A2-a2-B and the light chain with a3-A3-C1-C2. Sulfated tyrosines residues at a2 and a3 domain are the sites that act as co-factor of FVIII with von Willebrand factor (vWf). vWF protect and carried FVIII in the circulation. FVIII is activated by proteolysis at cleavage sites by thrombin and activated FX on C-terminal sites of arginine residues 372, 740 and 1689[12]. Upon activation FVIII leaves vWF to enter the tenase complex for thrombin generation in the coagulation pathway. Participation of FVIII in the tenase complex increases the efficiency of thrombin formation by 200,000-fold. This explain why severe FVIII deficiency profoundly reduces thrombin generation leading to spontaneous and prolonged bleeding.

Immunological response genes and development of inhibitors:

A systematic review on epidemiology of inhibitors in HAs revealed that inhibitors develop early in treatment to FVIII with an average of 10 to 15 days of exposure[13]. After 50-75 exposure days, the cumulative incidence of inhibitors reaches a plateau and beyond 75 days, incidences are not frequent[13]. There exist differences among epidemiology studies of HA inhibitor, especially in cohort studies as data captured are more of prevalence of HAs with inhibitors rather than true incidence rate.

Studies have been done to understand the pathophysiology of inhibitors development. Alloantibodies to FVIII are a mixed subclass of inhibitory immunoglobulin G (IgG) with IgG4 being the main contributor. Antigen presenting cells (APCs) such as dendritic cells (DC) internalized exogenous FVIII, cleaved into peptides, and presents via major histocompatibility / human leucocyte antigen complex class II (MHC/HLA Class II) to CD4+ T cell. Dasgupta et al (2007), has demonstrated that endocytosis of FVIII by human monocyte-derived DC is through the macrophage mannose receptor (MMR/CD206) that recognizes mannose-ending glycans on both heavy and light chains of FVIII[14]. The fragmented peptides of FVIII is presented via HLA Class II to CD4+T cells. The same study also shows that vWf prevents binding of FVIII to MMR/CD206 and blocked by dose-dependent manner the endocytosis of FVIII. This may explain why there is reduced inhibitors seen in HA patients treated with plasma derived FVIII (pdFVIII) concentrates which contain mixture of vWf[15]. Upon recognition of FVIII epitopes, T cells can undergo either one of these two transformations i.e. either to develop tolerance to FVIII or to help B-cell transform into plasma cells to produce antibodies against the epitopes recognised by the T cells.

A study by Hay CR et al (1997), on polymorphisms of HLA Class II genes in HA patients with and without inhibitors and has shown that risk of inhibitors is seen in 3 single-nucleotide polymorphisms (SNPs) HLA Class II which are HLADRB*1501, DQB1*0602 and

DQA1*0102, especially DQA1*0102 (OR 2.7; 1.2-5.9)[16]. Frequency of 3SNPs was reported to be high in patients with inhibitor and inv *IVS22* (OR 3.1; 1.0-10.1) for DQA1*0102[16]. However, this finding is not seen in a similar study by Oldenburg J et al (1997), on patients with inv *IVS22*[17].

In 2009, Pavlova et al performed another study on 260 severe HAs and correlate risk of inhibitors with HLA Class II and immune-regulatory genes. Their results revealed there were higher frequency of inhibitors occurrence in severe HAs with HLA Class II - DRB1*15 and DQB1*0602 alleles as well as of the haplotype DRB1*15/DQB1*0602[18]. Analysis on polymorphisms of the immune-regulatory genes showed alleles -308A in TNF-α, and -1082G allele in the IL-10 have a higher risk of developing inhibitors[18].

A recent study by David S et al (2019), on 447 Indian HAs patients, showed a prevalence of 19.5% inhibitors in this group, and results revealed there was a significant higher risk of inhibitor among HAs that have HLA DRB1*13 positive (RR=2.04; 95% CI 1.06-3.911; p=0.033)[19]. There is also a trend in decreased risk of inhibitor in HAs with HLA DRB1*07 and this achieved significance among high titre inhibitor positive (RR =0.24; 95% CI 0.055-1.05; p=0.047). Of the 14 polymorphisms of immuno-regulatory genes studied, it is found that there is a decreased risk of inhibitor noted with heterozygous IL4-590 C/T allele (residue 2243250) (RR = 0.22;95% CI 0.108-0.442: P-0.000)[17]. IL-4 plays a major role in differentiation of antigen-stimulated naïve T cells and immunoglobulin class switching of IgE and IgG4.

FVIII gene mutations and development of inhibitors:

Up to now there are ~ 2179 mutations of FVIII identified to cause haemophilia A and listed in databases Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), European Association for Haemophilia and Allied Disorders (EAHAD) FVIII gene variant database, previously called HAMSTeR (the Haemophilia A Mutation, Structures, Test and Resource (website; www.factorviii-db.org), CDC Haemophilia Mutation Project-CHAMP for FVIII mutations, and Hemobase: Haemophilia A mutation registry.

Mutations of FVIII gene are divided into 2 main groups, null mutations where no FVIII protein is being produced and the second group where dysfunctional FVIII protein is being produced. The null mutation consists of large single or multi-domain deletions, nonsense mutations, and inv*IVS22* and more than 30% of HAs in this group has develop inhibitors[19]. The second group consists of small deletions, missense and splice site mutations and less than 10% of HAs with these mutations develop inhibitors[20]. Oldenburg J et al (2002), evaluated more genetic data based on type, size and location of FVIII mutations, and include all severity of HAs and concluded that large deletions affecting more than 1 domain have a high risk of (~88%) developing inhibitors than a single domain deletion (~25%) [21]. 40% of HAs with nonsense mutations in the light chain developed inhibitors while 17% of HAs with nonsense mutations in the heavy chain developed inhibitors. Inv *IVS22* and inv *IVS1*demonstrated an intermediate inhibitor prevalence of 21% and 17%, respectively. Mutations of the poly-(A) tail, either a small deletions or insertions were associated with only 3% inhibitor risk while mutations located outside the poly-(A) region has 21% of HA developed inhibitors. Splice site mutations contributed to 17% prevalence in inhibitor where this mutation produced a corrupted mRNA which formed a different FVIII protein to the FVIII concentrates being used as treatment. Missense mutations in the light chain leads to structural and functional changes and this differences carried a higher risk (10%) of developing inhibitors than those in the heavy chain (3%) [12]. It can be generalised that any light chain mutations have a higher risk of developing inhibitors than any heavy chain mutations[21].

In Malaysia, Zahari M et al (2018), from National Blood Centre, Kuala Lumpur performed a study on 100 unrelated HAs, which consisted of 83 (83%) with severe HA, 9 (9%) moderate and 8 (8%) mild HA[22]. This is the first study in Malaysia to comprehensively analyse FVIII gene mutations in 100 HA patients, and the data may

not be representative with other centres in Malaysia. In UK, Green et al, 2008 reported there were 42.7% of severe HA, 16.3% moderate and 41.0% mild HA after they screened 842 families with HA[23]. Hence, the 83% of severe HA in Zahari M et al study was probably due to the fact that, severe HAs were the most frequently seen seeking treatment in hospitals. In this study, there are 14 (14%) HAs with inhibitors. Within the severe HA, 44 (53%) has inv *IVS22*, and 3 (3.6%) have inv *IVS1*[22]. Zahari M et al has also identified 22 novel mutations, including one involving intron 22 with 2 donor splice sites mutation. Of the 44 patients with inv *IVS22*, 5 (11.4%) have inhibitors, and among 3 patients with large deletions, 2 (66.6%) has inhibitors[22]. There should be more studies on HA to include HLA Class II polymorphisms, immune-regulatory genes with FVIII mutations and correlate with risk of inhibitor development. 6.4% of HA with inhibitors in Malaysia[2] is probably under-reporting and should be substantiated further in collaboration with other centres treating haemophilia.

A study by the Italian AICE (Italian Association of Haemophilia Centres) study group on genetics of haemophilia A has analysed 1,296 unrelated HAs and identified *F8* mutations in only 89% of these HAs[24]. They characterised 380 mutations including inv *IVS22* and inv *IVS1*. Inv *IVS22* was found in 52% of patients with severe HA, while inversion *IVS1* was present in 2% of them. Null mutations which includes inv *IVS22*, inv *IVS1*, large deletions, insertions and nonsense mutations are seen in 80% of severe HA, 15% of moderate HAs, and < 1% in mild HAs. About 1/4th of point mutations identified are seen in the coding region of exon 14. After excluding inv *IVS22* and *IVS1*, most of the mutations leading to null phenotype allele are seen in exon 14 which comprises of small deletions (53%), small insertions (74%) and nonsense mutations (33%)[24]. Gouw SC et al, 2012 performed a meta-analysis of 5,383 severe HA and estimated the relative risk of inhibitor development using inv *IVS22* as the reference. Inhibitor risk with large deletions and nonsense mutations was higher than inv *IVS22* (pooled OR= 3.6, 95% CI, 2.3-5.7, and OR = 1.4, 95% CI, 1.1-1.8, respectively[25]. Inhibitor risk with inv *IVS1* and splice-site mutations are equal with inv *IVS22* (pooled OR = 0.9; 9 5%CI, 0.6-1.5 and OR = 1.0; 95% CI, 0.6-1.5), and the risk in small deletions/insertions and missense mutations was lower (pooled OR = 0.5; 95% CI, 0.4-0.6 and OR = 0.3; 95%CI, 0.2-0.4, respectively) [25].

Inv *IVS22* is a null mutation but it poses as an intermediate risk to inhibitor development. It is hypothesized that there are 2 separate FVIII protein which includes exons 1-22 and exons 23-26, expressed intracellularly (refer to Figure 1D). Hence, inv *IVS 22* has endogenous FVIII protein which is non-functional but may provide some degree of tolerance toward exogenous FVIII concentrates used as replacement therapy and this lead to a reduced risk of inhibitor development[25]. Similar hypothesis may explain why risk inhibitor in mutations involving light chain is higher than heavy chain mutations in both missense and nonsense mutations. It is the expression of second mRNA, *F8B* which runs from within the CpG island in *IVS22* through exon 26 at the end of the F8 gene[10]. that is expressed intracellularly that may give the partial tolerance to exogenous FVIII. Any mutations outside *F8B* gene, will have partial tolerance, hence reduced likelihood of developing inhibitor while mutations that also involve *F8B* gene will have increased risk of developing inhibitor[26].

Environmental risk factors:

Inhibitor development has also been studied with clinical parameters which includes age of HA when the first treatment was started with FVIII concentrates, intensity of treatment and type of FVIII concentrates used either pdFVIII concentrates and recombinant FVIII products (rFVIII). Lorenzo et al (2001) reported that infusing FVIII concentrates before 6 months of age is associated with higher risk of inhibitor formation[27].

Intensity of treatment with continuous infusion and large doses given has been reported to induce inhibitor formation[28]. Gouw SC et al (2013) reported the findings under the Research of Determinants of Inhibitor development (RODIN) Study Group that high-dose intensive FVIII

treatment used to control haemostasis in surgery was associated with increased risk for inhibitor development[28]. The same study also revealed that, between prophylactic treatment and on-demand treatment, there is a decreased risk of inhibitor development in patients given prophylactic FVIII concentrates. This a surprising finding because the study also discovered that within the first 20 exposure days, patients receiving prophylaxis had exactly the same inhibitor risks as the patients treated on demand[28]. Hence, there is no clear conclusion which patients will benefit from the protective effect of prophylactic treatment on inhibitor development.

Rosendaal et al (2017) reported that in previously untreated haemophilia A patients (PUPs), those randomised to pdFVIII concentrates have shown no development of inhibitors in PUPs with low risk FVIII mutations, whereas high risk of FVIII mutations PUPs had a cumulative incidence of 31%[29]. The risk was similar among low risk FVIII mutations and high risk FVIII mutations PUPs when they were treated with rFVIII (43% and 47%, respectively). This implied that there was a 43% risk increment for patients with low risk FVIII mutations when they were exposed to treatment with rFVIII. Number needed to harm with rFVIII, was 6.3 for patients with high risk FVIII mutation and only 2.3 for patients with low risk FVIII mutations. This study illustrated the need to stratify patients based on their genetic mutations to allocate appropriate FVIII products for their replacement therapy[29].

PdFVIII concentrates contains vWf and transforming growth factor beta (TGF-β) [28]. vWF bind to FVIII and protects it from proteolysis and stabilizes in circulations. As pdFVIII products are manufactured from plasma of numerous donors, heterogeneity in FVIII protein sequence and other minor plasma proteins exposed to HA patient, may have an immunomodulatory effect or a role in reducing the immune response relative to rFVIII concentrates[30]. Presence of TGF-β reduces the inflammatory response and stimulation to recognise the exogenous FVIII as foreign antigen. Recombinant FVIII has second generation full length rFVIII products utilising albumin and sucrose as a stabilizer, whereas the third generation does not have these stabilizers. Studies has not proven any of these stabilizers to influence immune response[30].

Conclusion:

Studies on mutations of FVIII, family history of inhibitors among family members afflicted with HA, MHC class II and polymorphism in immune-regulatory genes were undertaken to identify risk factors for development of inhibitors. Some studies were not able to replicate results shown by others but mutations of FVIII gene has been a consistent finding as a strong risk factor to develop inhibitor.

The mutations that lead to absence of circulating FVIII and including absence of endogenous intracellular expression of FVIII protein has the highest rate of inhibitor development > 80%. Inv *IVS22* is a null mutation that has no circulating FVIII glycoprotein but if the disrupted exon 1-22 and exon 23-26 able to produce 2 fragmented FVIII glycoproteins, this may induce partial tolerance to exogenous FVIII and reduce the development of inhibitors. Profiling the genetic risks also involve profiling the polymorphisms exhibit by HLA Class II genes and immuno-regulatory gene variants. Although results on these 2 genetic risks studies are not conclusive, it is probably the polymorphisms are dependent on ethnicity or races of the population being studied and some of the studies are actually underpowered due to rarity of the disease.

On the environmental risks, inhibitors are seen in HAs who is exposed at earlier age and with high dose exposure of the first treatment. On products used for replacement therapy rFVIII product has a higher risk that pdFVIII in development of inhibitors. Most of these studies are using second generation rFVIII product. There are better rFVIII designed product with third generation FVIII without albumin, B domain deleted rFVIII product and extended half-life FVIII product in the market. There is a need to design a more robust study on risk factors that incorporates genetic risks namely, FVIII gene mutations, polymorphisms in HLA Class III, immuno-regulatory gene polymorphisms and, although it is not being discussed in this paper, FVIII gene

haplotype polymorphisms together with the environmental risk factors. Using this data, predictive algorithm can be constructed and a personalised treatment strategy can be planned to a newly diagnosed HA patient to reduce the probability of developing inhibitors. New bypassing agents that do not require FVIII exposure can be given to reduce early exposure for exogenous FVIII concentrates in large deletion mutations.

From this review, it is highly recommended that Malaysia needs a national HA database registry that incorporate genetics, laboratory and clinical data so that a predictive strategy can be used to decide on how best to treat our HAs in order to reduce their incidence of developing inhibitors. Any HA child diagnosed with large deletion, inv *IVS22* and inv *IVS1* should not be given large doses of FVIII concentrates in their first exposure. Bypassing agents may be a better choice for these HAs.

Studies on FVIII inhibitors has been carried out for 3 decades and a lot has been discovered but not totally conclusive. There remain a lot more to be discovered and understood, and inhibitor development need to be solved in order for an effective treatment strategy that actually reduces the healthcare costs of treating haemophilia A.

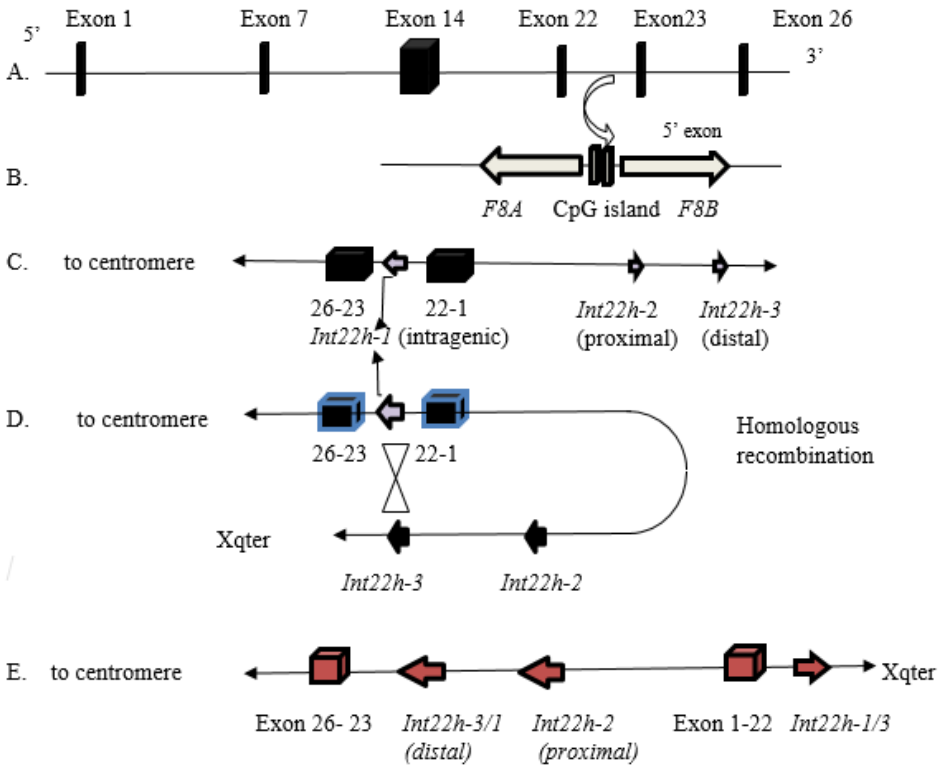

Figure 1: A. Organization of human Factor VIII with 26 exons span over184kb DNA. B. FVIII gene with 3 ORF (open reading frame) and transcribe 3 FVIII mRNA - 9kb FVIII transcript, F8A with backwards transcription and F8B with 5'exon taken from intron 22 and spliced to join the exons 23 o 26[11,12]. C: Location of the Int22h homologues. D. In homologous recombination of chromosome X, the intragenic inth22h homologue will align with either the distal inth22h-3 or the proximal inth22h-2 and produce a disrupted FVIII gene in E with Exons 1-22 are displaced towards the telomere[11,12]. This result in non-continuity from exon 1-22 to exon 26-23 of FVIII gene and mRNA transcription is disrupted leading to absence of FVIII protein in circulation [10,12].

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