Factor VIII Intron 22 Inversion Mutation in Samples of Iraqi Patients with Hemophilia A

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Abstract

Background: Hemophilia A is an X-linked recessive inherited bleeding illness characterized by a lack of procoagulant factor VIII; the factor VIII gene has more than 3000 different mutations, and the most frequent molecular changes in severe hemophilia A are intron 22 and intron 1 inversions (Inv 22 and Inv 1). **Objectives:** To detect intron 22 inversion mutation in samples of Iraqi patients with hemophilia A and reveal its role in inhibitor production. **Materials and Methods:** Eighty patients with hemophilia A were enrolled in this study from two Iraqi centers. The specialist centers for hemophilia in Babil Teaching Hospital for Maternity and Children and Welfare Teaching Hospital. Genetic analysis of the Inv 22 mutation was done by real-time thermal cyclic quantitative PCR (qPCR). Mixing study and Bethesda assay were used for the detection of inhibitor development. **Results:** Eighty patients with hemophilia A were partitioned into 22 (27.5%) with inhibitors and 58 (72.5%) without inhibitors. Most patients (48.8%) were diagnosed at age 6–9 months; according to the disease severity, patients were divided into severe hemophilia 76.25%, moderate hemophilia 16.25%, and mild hemophilia 7.5%. Among all patients, positive Inv22 mutation was detected in 83.7%. **Conclusion:** the results of inversion 22 are consistent with overall reports, being a significant major genetic transformation in severe hemophilia A. q-PCR is a basic, fast method for the detection of inversion 22. The mutation is detected in 74.6% of severe cases and is considered an important risk factor for inhibitor production.

Keywords: Factor VIII, hemophilia A, inversion 22

Introduction

Hemophilia A is the most prevalent congenital bleeding disorder caused by a lack of the clotting protein factor VIII (FVIII).[1] Factor VIII deficiency is an X-linked recessive condition. Regardless of race, one out of every 5000 male births has factor VIII deficiency.[2]

The disorder is clinically diverse and categorized according to the residual factor VIII clotting activity to severe when FVIII:C 1%, moderate when FVIII:C 5%, and mild when FVIII:C 5%–40%. The World Federation of Hemophilia estimates that Iraq has a male hemophilia prevalence of 3.6/100,000.[3]

Hemophilia A originated from an expansive range of heterogeneous transformations that influence FVIII quality, promoting quantitative and subjective irregularities in factor activity, which is a fundamental component in the factor X activation complex.[4]

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The mutations documented in the FVIII gene are exceedingly varied, with gene rearrangements in addition to point mutations, large deletions, and insertions are the most prevalent.[5]

Inversion 22 is brought about by recombination between a sequence inside intron 22 of the FVIII gene and one of the two homologous regions telomeric to the gene, and intron 1 inversions are the most popular molecular changes found in severe hemophilia A.[6]

There are two types of intron 22 inversion: type 1 (Inv22-1) and type 2 (Inv22-2) in hemophilia A, Inv22-1 has a higher

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frequency than Inv22-2. Three amplicons of 333,457 and 584 base pair for Inv22-1 and three amplicons 385,405 and 584 base pair for Inv22-2.[7]

Inversion of intron 22 is the most common causative mutation of severe hemophilia A, which is detected by the inverse shifting-PCR method; this method is cost-effective, saves time, and improves the molecular detection of inversion. Also used for carrier detection and prenatal diagnosis of hemophilia A disease.[8]

Intrachromosomal recombination between intron 22h-1 within the FVIII gene (9.5 kilobase) and its duplicons intron 22h-2 and intron 22h-3, which lie 500 kilobase and 600 kilobase distal to FVIII gene, respectively, causes FVIII gene inversions.

Inv22h-2 is involved in the proximal type (type 2) of inversion, and Inv22h-3 is involved in dis(type 1) of the inversion.[9]

For the purpose of detecting mutations in hemophilia A, many techniques have been used, like linkage analysis, mutation screening methods that detect regions of the gene as a target region, sequencing, and direct sequencing of the full gene because it is expensive, direct sequencing is still not widely used.[10,11]

This paper aims to detect the frequency of this specific mutation among hemophilic patients, its relation with disease severity and inhibitor production, accompanied by the potential diagnostic utility of the mutation.

Materials and Methods

Eighty patients were enrolled in this cross-section study from two Iraqi centers: the specialist centers for hemophilia in Babil Teaching Hospital for Maternity and Children and Welfare Teaching Hospital, during the period from October 2021 to April 2022. All patients are male. The clinical diagnosis was based on detailed family history, age at diagnosis, age of evaluation, number of exposure to VIII, and a diminished FVIII level. Blood samples of four milliliters were taken from each patient, 2 mL of which was kept in an EDTA tube at 4°C and used later for extraction of DNA; 2 ml was transferred into a tube with sodium citrate (final concentration of 3.8%) with a 9:1 ratio volume. Coagulation tests were performed using Sysmex CA 500 equipment,[12] APTT mixing study, and the Bethesda method was used to recognize inhibitors' presence.

DNA purification: Human genomic DNA was taken out from whole blood samples of patients using the protocol for DNA separation from whole blood samples of patients. Human genomic DNA was taken out from entire blood samples of patients' individuals using the Wizard® Genomic DNA Purification Kit.

Primers preparations: This study uses (4) primers to detect genes of intron 22 inversions (P, Q, A, and B). The primers were supplied by Macogen (Global Market for Genetic Analysis, Geumcheon, Seoul). The NCBI Primer-BLAST program was used to examine the specificity of the primer sequences.

Primers P and Q generate a 12-kilobase (kb) region in the DNA and are highly specific to the sequences flanking int22h1. Primers A and B create a 10-kilobase region that is novel to arrangement for flanking Int22h2 and Int22h3, respectively, as shown in Table 1. Because every P and B and A and Q generates an 11 kilobase segment in the DNA of patients, four distinct two-primer combinations, A and B, P and Q, A and Q, P, and B, were performed on each sample in order to identify inversion 22 in the FVIII gene. This was done because Inv22 can happen because of homologous recombination among Int22h1 and either Int22h2 or Int22h3.

All primer pairs used in this study were spin down before opening the cap of the primer tube. According to the instructions provided by the primer manufacturer, a desired amount of nuclease-free water was added to each primer to produce a 100 pmol/µL concentration of the primer stock solution. The primer stock solution was suspended by transferring 10 µL to a 1.5 mL Eppendorf tube containing 90 µL of free nuclease water to yield 10 pmol/µL used in PCR amplification, and the primer stock was stored at -20°C, as shown in Table 2.

Table 1: The primers of intron 22 inversion

Type of primer	Sequences	Gene Bank accession number
P	F:(h1)GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC	AF062514
Q	R:(h1)GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA	X86012
A	F:(h2 and h3) CAC AAG GGG GAA GAG TGT GAG GGT GTG GGA TAA GAA	AF062515
B	R:(h2 and h3)CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT A	AF062516

PCR mixture & cycle

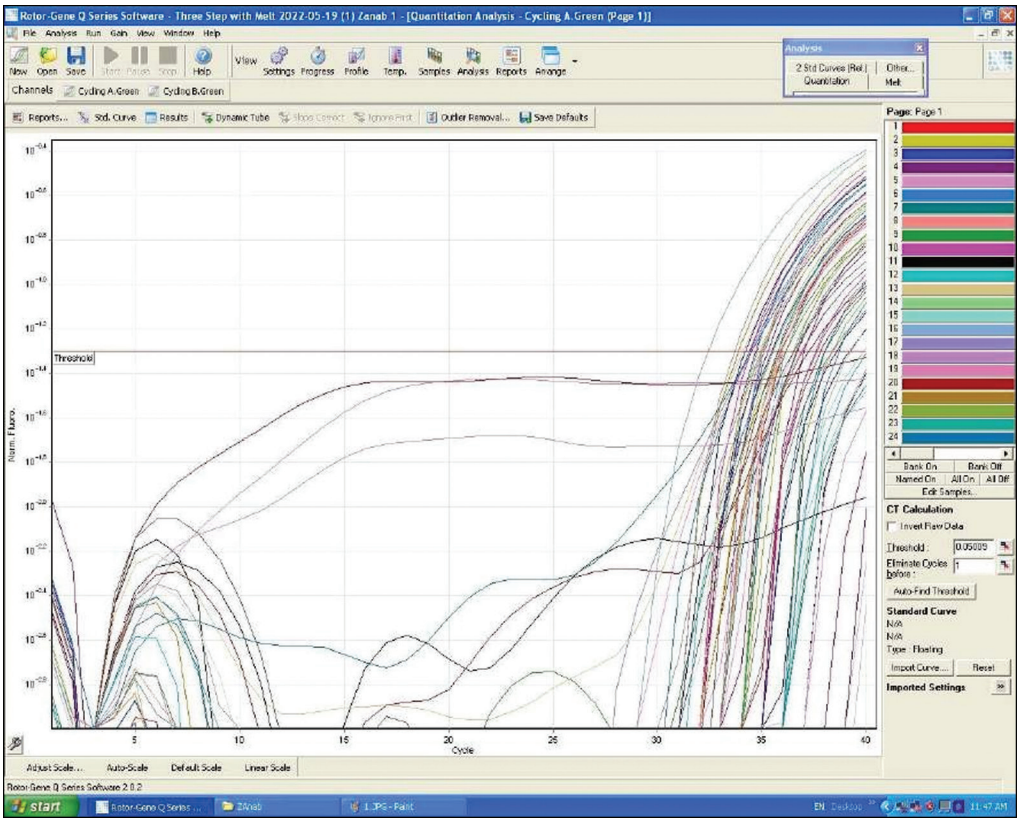

Table 2: Patient distribution based on age of diagnosis (*N* = 80)

Study variables	Number (*N* = 80)	%
Age of diagnosis (months)		
1–3	3	3.8%
3–6	16	20.0%
6–9	39	48.8%
9–12	5	6.2%
12–24	17	21.2%

Run of PCR cycle

SPSS version 27 was used for statistical analysis, and frequencies and percentages were used to represent categorical variables (means SD) was used to represent continuous variables. The Student *t*-test was utilized to compare the means between the two groups. Mann and Whitney, when the variable under study was not normally distributed, employed the *U* test to compare two groups. The Fisher-Exact test was performed to determine the relationship between categorical variables. A *P*-value of 0.05 was regarded as significant.

Approval for the study was acquired from the local institutional committee at the College of Medicine in Babylon, and all patients provided written permission.

Results

Eighty patients with hemophilia A were enrolled in this study, subdivided into 22 (27.5%) with inhibitors and 58 (72.5%) without inhibitors. Most of them (*N* = 39, 48.8%) were diagnosed at age 6–9 months. The mean age of diagnosis was (8.95 ± 11.74) months, as shown in Table 2.

Patients with a positive family history represent 86.3% (*N* = 69), while patients with a negative family history represent 13.7% (*N* = 11) of patients, as shown in Figure 1.

According to the site of bleeding (skin, nose, knee joint, elbow joint, and ankle joint), the majority of patients 45% presented with elbow joint bleeding, 38.8% of patients presented with knee joint bleeding, seven patients (8.8%) of patients presented with cutaneous bleeding, three patients (3.7%) presented with ankle joint bleeding and the same number presented with nasal bleeding, as shown in Figure 2.

According to disease severity, patients are divided into severe hemophilia 76.25%, moderate hemophilia 16.25%, and mild hemophilia 7.5%, as shown in Table 3.

The patients were divided according to inhibitor status (positive and negative). Positive inhibitor status represents

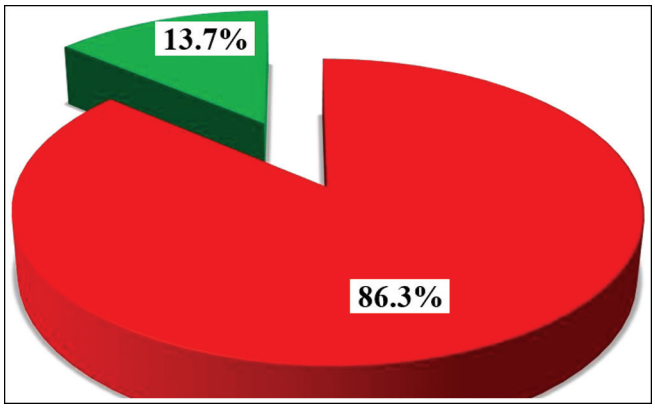

Figure 1: The distribution of patients according to family history of hemophilia (N = 80)

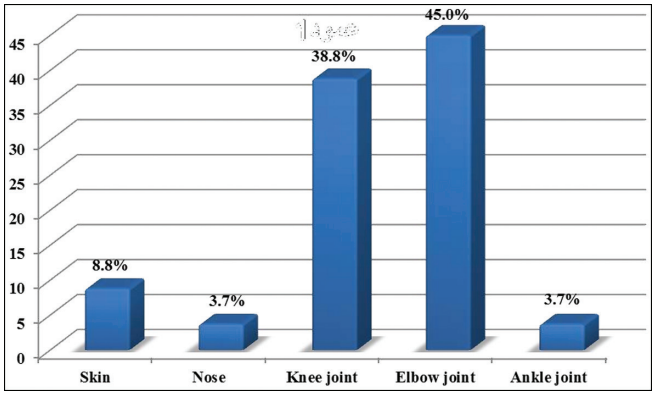

Figure 2: Distribution of patients according to the site of bleeding (N = 80)

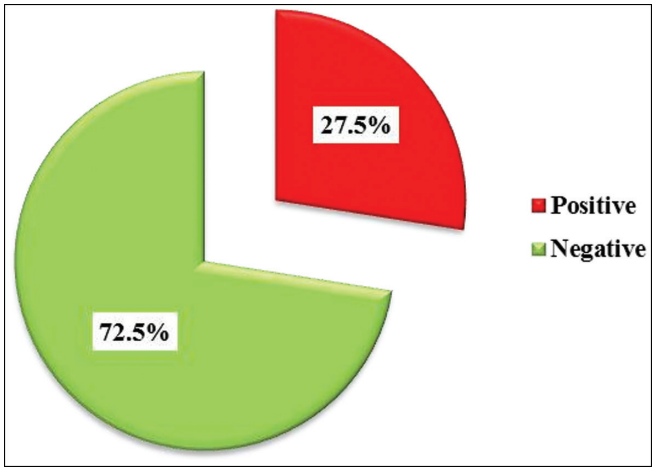

Figure 3: Patient distribution based on inhibitor status (N = 80)

(N = 22/80, 27.5%) patients, while those with negative inhibitor status represent (N = 58/80, 72.5%) of patients, as shown in Figure 3.

Patients are divided according to intron 22 inversion (positive and negative). Positive intron 22 inversion represents (N = 67, 83.7%) patients, while those with negative intron 22 inversion represent (N = 13, 16.3%) of patients, as shown in Figure 4.

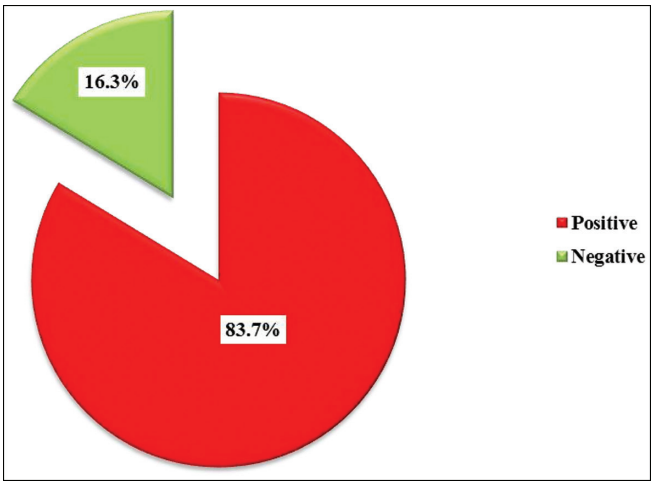

Figure 4: Patient distribution based on mutation detection (N = 80)

Table 3: Distribution of patients according to the severity

Severity	No.	%
Sever	61	76.25
Moderate	13	16.25
Mild	6	7.5
Total	80	100

The association between intron 22 inversion (positive and negative) and study variables (severity of disease and inhibitor status). There was a significant association between intron 22 inversion and inhibitor status, as shown in Table 4.

Discussion

Over 900 mutations have been found in the FVIII coding and nontranslated segments; most cases of severe hemophilia A result from nonsense, missense, splice site, frameshift, and extensive (>50 bp) deletion variations. Detection of molecular abnormalities is considered an important tool in hemophilia for predicting the clinical course and administering family with safe genetic counseling.[13]

In the present study, patients with positive intron 22 inversions were 67/80 (83.75%); this frequency is higher than that reported by Abdulqader *et al.*[3], in Iraqi Kurdish patients, which was (36.3 %) in Egypt by Sherief *et al.*[14] (23%) and other neighboring countries like Saudi Arabia (50%), Palestine (36.6%), Lebanon (29%), Jordan(52%), and Iran (40%).[11,13,15-17] Variations in reported prevalence rates between nations due to a variable number of analyzed cases, ethnic heterogeneity, involvement of persons with moderate and mild hemophilia, and different techniques used for genetic detection.

Table 4: Association between intron 22 inversion and study variables

Study variables	Intron 22 inversions (80)		Total	*P*-value
	Positive (67)	Negative (13)		
Severity of disease				
Mild	6 (9.0)	0 (0.0)	6 (7.5)	
Moderate	11 (16.4)	2 (15.4)	13 (16.3)	0.866
Severe	50 (74.6)	11 (84.6)	61 (76.2)	
Inhibitor status				
Positive	13 (19.4)	9 (69.2)	22 (27.5)	<0.001*
Negative	54 (80.6)	4 (30.8)	58 (72.5)	
Total	67 (100.0)	13 (100.0)	80 (100.0)	

*P value ≤ 0.05 was significant. Fisher's exact test

The increased occurrence of this mutation in the male may be clarified by unpaired X chromosome throughout spermatozoa meiosis, and this stimulates the long arm's tip flipping; however, the occurrence of Inv22 in individuals with hemophilia A varies somewhat between studies.[3]

In the present study, positive intron 22 inversion in severe cases was 50/67 (74.6%), in moderate cases 11/67 (16.4%), and in mild cases was 6/67 (9.0%), which is consistent with studies worldwide, this association between intron 22 inversion and severity is interpreted by its interference with full-length mRNA transcription lead to inefficient factor VIII protein production and thus severe hemophilia.[14]

According to the available data, 19.4% (13/67) of the cases who possessed mutation had inhibitor development, and other studies reveal that the percentage of individuals with inversion 22 who developed inhibitors is variable, 5%–51%.[18-23]

People with Inv22 are capable of synthesizing a nonfunctional polypeptide, as well as an unmodified FVIII protein. Patients may develop immunological tolerance to therapeutic-FVIII as a result of these two polypeptides, each of which has the entire sequence for a full-length FVIII protein are present intracellularly. This may also explain the moderate risk for inhibitor development observed in inversion 22-positive individuals.[24]

In conclusion, inversion 22 intron mutation of FVIII is regarded as a significant genetic variant causing severe hemophilia A in Iraqi patients, and our findings are higher than those reported from neighboring nations. The mutation was found in 74.6% of severe cases and is an important risk factor for inhibitor production.

The need for larger studies and exploration of other genetic variants (inversion in intron 1) is highlighted.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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