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Identification of factor VIII gene mutations in patients with haemophilia A

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ABSTRACT

This study aimed to identify common FVIII gene mutations in Iraqi patients by molecular analysis and to investigate the correlations between mutations and disease severity. This study included 25 Iraqi patients with haemophilia A, 18 of whom were male haemophilia patients and 7 of whom were carrier mothers, from seven unrelated families. After determining the purity and concentration of the extracted DNA, selected areas of the FVIII gene, including exons 18, 22, 23, and 24 and intron 22, were amplified. Sequencing of all exons and intron 22 was conducted in all patients and controls. Sequencing analysis revealed that many mutations were distributed among the exons (18, 22, 23, and 24) and intron 22; most of the identified mutations were point mutations in exons, predominantly in exon 24. Exon 18 mutations were detected in one male and one mother as a carrier. Exon 22 mutations were detected in four patients, and exon 23 mutations were detected in two males and two carrier mothers. Exon 24 mutations were detected in 12 haemophilia patients and two carrier mothers. Additionally, seven inversion mutations were detected in haemophilia patients. The results also revealed positive correlations between disease severity and mutations in intron 22 and exon 24. We therefore concluded that exon 24 mutations are the most frequent type of mutation that occurs in haemophilia patients in Iraq, followed by intron 22 mutations, and that almost all severe haemophilia cases have mutations located in these two gene segments.

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INTRODUCTION

Haemophilia A (antihaemophilic globulin (AHG), factor VIII deficiency) is an X-linked recessive genetic bleeding disease that clinically occurs in males. The chance of a female having two defective copies of the gene is very rare, with such females being almost exclusively asymptomatic carriers of the disorder (Loomans *et al.,* 2014). Haemophilia A is the most common inherited human bleeding disorder (Peyvandi, 2005). The estimated incidence of haemophilia A is between 1:5,000 and 1:10,000 live male births (Rodriguez-Merchan, 2002). The prevalence of haemophilia A in Iraq is 3.6 individuals (per 100,000 males) (UNDESA, 2012).

Haemophilia patients, approximately 80-85% have haemophilia A and 10-15% have haemophilia B. The diagnosis of haemophilia in children with a positive family history is not made shortly after birth in some countries due to poor counselling regarding the role of family history and heredity (Hazewinkel *et al.,* 2003). Additionally, approximately 30% of haemophilia cases are caused by spontaneous mutations and are not associated with a family history (Abshire, 2005). However, acquired forms of haemophilia do exist, mostly in older patients, due to the actions of inhibitors (au-

toantibodies) against FVIII or haematological malignancy, but the majority of cases are inherited; the incidence of acquired haemophilia is approximately 1:1,000,000 patients (Franchini and Lippi, 2008) and is associated with neutralization of infused clotting factor concentrates. Increased morbidity in patients with haemophilia is sometimes associated with immune system disorders, cancer, or allergic reactions to certain drugs (Fischer *et al.,* 2015; Srivastava *et al.,* 2013). Haemophilia A is a good model for the study of mutations because it is a relatively widespread disease with a variable phenotype (inclusion allelic heterogeneity). Additionally, there is a high ratio of new mutations in FVIII (Goodeve, 2015), a component of the intrinsic blood clotting pathway, resulting in aberrant FVIII.

The FVIII gene is composed of 26 exons and 25 introns, spanning 186 kb and encoding 2332 amino acids, with a high GC content that results in a high mutation rate (2.5–4.2 × 10−5) (Ilić *et al.,* 2013). Depending on the level of FVIII activity, patients with haemophilia may present with easy bruising, insufficient clotting from traumatic injury or, in the case of severe haemophilia, spontaneous haemorrhage or obstructive clotting (thrombosis) (Kashiwakura *et al.,* 2012). To the best of our knowledge, this study is the first to aim to identify common mutations of the FVIII gene in the Iraqi population by molecular analysis and investigate the correlation between mutations and disease severity.

PATIENTS AND METHODS

patients

Informed consent was obtained from all patients and the project was approved by the Faculty of Medicine, University of Al-Qadisiyah Ethics Committee. Procedures for obtaining oral informed consent and protecting individuals were approved and monitored for compliance by the Institutional Review Boards (IRBs) of the organizations coordinating the survey. Permission for the study was granted by the IRBs of the Iraq Ministry of Health, the Iraq Central Organization for Statistics and Information Technology (COSIT), and the Faculty of Medicine at University of Al-Qadisiyah. Standardized descriptions of the goals and procedures of the study, data use and protection, and the rights of respondents were provided in both written and verbal form to all predesignated respondents before obtaining verbal informed consent for participation in the survey The study included 25 haemophilic Iraqi patients (18 haemophilic male patients and 7 mothers as haemophilic carriers) from unrelated families who ranged in age at disease onset from 1 to 35 years, and 16 healthy subjects (age- and sex-matched) served as controls. Patient information from January to June 2015 was collected from Al-Sadder Medical City in Najaf province/Iraq.

These patients were previously diagnosed based on standard symptoms and confirmed by laboratory findings, namely, prolonged partial thromboplastin time (PTT) and FVIII activity assays that showed factor levels below 1% of normal values (normal value between 50% and 150%). This disease activity level is considered severe, while moderate levels are between 1% and 5% and mild levels are between 5% and 40%. Furthermore, patients always show normal prothrombin time (PT), thrombin time (TT), and bleeding time (BT) (Xie *et al.,* 2009).

Blood samples

Venous blood samples (5-10 mL) were collected from both haemophilic patients and healthy subjects. Each blood sample was collected in EDTA tubes for molecular studies and stored at 4°C until use.

Genomic DNA extraction

Genomic DNA was extracted from fresh blood collected in EDTA anticoagulant tubes for molecular assessments conducted using an *AccuPrep*® Genomic DNA Extraction Kit (Bioneer, South Korea). DNA isolation was based on the method by Keijzer *et al.,* (2010)

Agarose gel electrophoresis

Agarose gel electrophoresis was performed following genomic DNA extraction to determine the concentration and purity of the extracted DNA (Sambrook *et al.,* 1989).

DNA concentration and purity estimation

The concentration and purity of all extracted DNA samples were determined using a spectrophotometer. One microliter of extracted DNA was added to the spectrophotometer to estimate the concentration in ng/µL, which ranged between 11-14 ng/µL. The purity was calculated by observing the optical density (OD) ratio at 260/280 to determine whether there was any contamination of the samples with protein. The 260/280 ratio for pure DNA was between 1.7 and 1.9 (Sambrook and W Russell, 2001).

DNA amplification

For mutation screening, DNA extracted from both patients and controls was amplified using polymerase chain reaction (PCR)-specific primers for exons 18, 22, 23, and 24 and intron 22. PCR products were checked for specificity by electrophoresis and then electrophoretically purified using the DNA gel extraction method to highly purify the DNA and to avoid any non-specific amplification or

DNA fragmentation. The PCR products were sequenced using the primer extension method by Macrogen (Biotechnology Company, South Korea).

HA gene alterations

The four exons and intron 22 of the HA gene were screened by direct sequencing of samples from 25 haemophiliac male patients and haemophiliac carriers and 16 controls. The outcomes were compared with the human reference mRNA-HA sequence (HTTP: National Center for Biotechnology Information (NCBI) Reference Sequence: NM_000547.4) via a software sequencer (ChromasPro, type: 1.5) available on the web.

DNA sequence analysis

DNA sequencing is used to determine the nucleotide sequence (adenine, guanine, cytosine, and thymine) of a DNA molecule. The investigation of nucleotide sequencing was completed using a basic local alignment search tool (BLAST) program. Nucleotide sequences were also translated into amino acid sequences using BLAST, which aligned all exons and intron 22 sequences with the corresponding reference FVIII gene sequence (FVIII mRNA reference for exons and reference genomic DNA for intron 22) to check for any differences. The same sequence was then aligned using Mutation Surveyor software to check the normal variation and to identify any amino acid changes.

FVIII gene mutation detection

Mutation selection

The sequences of primers used in this study for the detection of the most common FVIII mutations in exons 18, 22, 23, and 24 and intron 22 were previously reported by Chetta *et al.,* (2008).

Primers

All primers were supplied by Bioneer Company (South Korea) and were lyophilized at different concentrations. The lyophilized primers were dissolved in DNase/RNase-free water as recommended by the manufacturer and left in a water bath to dissolve completely. This protocol yielded a final concentration of 100 pmol/µL as a stock solution. Each primer was diluted to a concentration of 10 pmol/µL as aliquots and kept frozen at -20°C until use as a working solution.

PCR programs

PCR optimization was achieved using *in silico* PCR software to calculate the annealing temperature of most primers. After numerous trials to obtain the best PCR outcomes, an optimized PCR was obtained.

Purification and sequencing of PCR products

Sequencing of PCR products

The PCR products of the examined FVIII gene regions and primers were sent to Macrogen Company (http://dna.macrogen.com; South Korea) for sequencing.

Statistical analysis

Statistical Package for Social Sciences version 16 (SPSS v16) was used for data input and examination. Continuous variables are presented as the mean and discrete variables are presented as numbers and percentages. The chi-square test for goodness of fit was used to test the significance of the observed distributions. The P values are asymptotic and two-tailed, and values below 0.05 indicate significance.

RESULTS

Distribution of HA patients

The majority of patients were male (18 cases; 72%), and 7 mothers were carriers (28%). A family history of haemophilia was reported for 14 patients (78%), while 4 patients had no family history of haemophilia (22%). Twelve haemophilic patients had positive consanguineous marriages (67%), and six patients had non-consanguineous marriages (33%). The severity of haemophilia classified according to factor VIII activity was as follows: 13 male patients had FVIII activity <1% (severe type) and 5 male patients had factor VIII activity between 1% and 5% (moderate severity).

DNA isolation

The genomic DNA extracted from the blood of haemophilia A patients showed a clear single band when fractionated by gel electrophoresis (Fig. 1) and was then checked for purity using a spectrophotometer device (Avans Biotechnology, Taiwan).

All nucleotide sequences of the *Homo sapiens* (Human) mRNA-HA sequence (bases 1 to 191041) were downloaded from GenBank (http://www.ncbi.nih.gov/nuccora/NM_000547.4) and aligned using the ClustalW method in the MEGA4 program (this software was used for multiple alignments with a reference sequence) as shown in Fig. 2, and the mRNA-HA sequence was modified to cDNA by converting uracil into thymine. Intron 22 was compared with the genomic DNA sequence to check for inversion mutations, which were analysed using virtual restriction-enzyme targeting with NTI vector software (this software was used to check for inversion mutations in intron 22 by comparing it with the genomic DNA sequence and determining the presence of the Sab I restriction site).

Table 1: Detection of Gene Mutations in Male Haemophilia Patients and Haemophilic Carriers (mothers) with Normal Variation and Severity

Sample No.	Male patient/ Carrier (mother)	Gene segment	Mutation/ Genome	Mutation type	Normal variation	Sever-ity
			exon 18			
1	Carrier	Exon 18	5826delT	Frameshift		-
11	Patient	Exon 18	5836C>T	Point mutation		Severe
			exon 22			
2	Patient	Exon 22	6342delA	Frameshift		Severe
4	Patient	Exon 22	6315G>T	Point mutation		Severe
19	Patient	Exon 22	6313A>T	Point mutation		Severe
23	Patient	Exon 22	6408 G>A	Point mutation		Mod-erate
			exon 23			
2	Patient	Exon 23	6533A>T, 6539G>A, 6546C>A	Point mutation = =		Severe
5	Carrier	Exon 23	6509G>A	Point mutation	GAT>AAT	-
6	Patient	Exon 23	6581del T	Frameshift		Severe
8	Carrier	Exon 23	6668G>A	Point mutation	CGA>CAA	-
Sample No. 22 in Exon 23; Male had no mutation with normal variation 6959 GG/CA						Severe
			exon 24			
1	Carrier	Exon 24	165658delA	Frame shift		-
2	Patient	Exon 24	165658delA	Frame shift		Severe
3	Patient	Exon 24	165825A>C	Point mutation		Severe
4	Patient	Exon 24	165897delG	Frameshift		Severe
6	Patient	Exon 24	165707T>TC	Insertion		Severe
7	Patient	Exon 24	165897delG	Frameshift		Mod-erate
8	Carrier	Exon 24	165897delG	Frameshift		-
9	Patient	Exon 24	165825A>C	Point mutation		Severe
11	Patient	Exon 24	165898C>CA	Insertion		Severe
12	Patient	Exon 24	165897G>GC	Insertion		Severe
14	Patient	Exon 24	165897G>GC	Insertion		Severe
15	Patient	Exon 24	165897G>GC	Insertion		Severe
18	Patient	Exon 24	165900G>GA	Insertion		Severe
22	Patient	Exon 24	6590delG, 6592A>T 6701A>C	Frameshift Point mutation Point mutation	TGC>TCG, CAC>CCC	Severe
			intron 22			
2	Patient	Intron 22	Inth22	Inversion		Severe
4	Patient	Intron 22	Inth22	Inversion		Severe
6	Patient	Intron 22	Inth22	Inversion		Severe
9	Patient	Intron 22	Inth22	Inversion		Severe
11	Patient	Intron 22	Inth22/	Inversion		Severe
14	Patient	Intron 22	Inth22	Inversion		Severe
19	Patient	Intron 22	Inth22	Inversion		Severe

Mutation screening

The mutation screening conducted throughout the study showed that most mutations were located in intron 22 and exon 24; mutations in these locations exhibit the greatest effect on disease severity. The mutations located in exon coding regions were point mutations, deletions of a single nucleotide, or insertions. Most point mutations result in changes in single amino acids. For example, 2230H>P in exon 24 (sample number 21, a male patient) indicates that the amino acid histidine was changed to proline at codon 2230 of the FVIII gene nucleotide sequence. Additionally, in exon 22, H>L and K>N in

sample 19 and R>Q indicate that large structural influences may be involved if the protein structure largely depends on amino acid properties and their locations in the polypeptide chain. Other point mutations exist but are just silent mutations, which are not mentioned in our study; these mutations may indicate suspected genomic instability in patients. Other changes in exons that were also neglected in our study include normal variations, which represent normal changes in genomes. There are approximately four variants in the FVIII gene, all of which have been included in this study. The exon DNA sequences involved were aligned and compared with all of the possible normal variants. Table 1 shows the detected gene mutations that caused haemophilia in 18 haemophilia patients and 7 haemophilic carriers (mothers) with normal variation and severity. No gene abnormalities were detected in any of the 16 control samples. Two mutations in exon 18 were detected in two patient samples and mothers. Regarding exon 22 and 23 defects, four mutations in four patients were detected in exon 22 and six mutations in four patients and mothers were detected in exon 23. Sixteen mutations were found in fourteen samples, including patients and mothers. The first ATG that appeared in the aligned exons was regarded as the first codon (Fig. 3 shows the aligned sequence of E18_EF).

Mutation detection and haemophilia A severity

Exon 18 mutations were detected in one male and one carrier mother. Table 2 shows the relationship between haemophilia severity and exon 18 mutations. One of the patients with severe disease (7.69%) had a mutation with a P value of 0.523.

Table 2: Relationship between Exon 18 Mutations and Haemophilia A Severity

Mutation	Severity		Total
	Moderate	Severe	
No	5	12	14
Yes	-	1	4
Total	5	13	18

* The value of Pearson's chi-square statistic is 0.407 based on 1 degree of freedom (d.f.).
* The level of probability (null hypothesis) at p = 0.523 is not significant**.**

Table 3: Relationship between Exon 22 Mutations and Haemophilia A Severity

Mutation	Severity		Total
	Moderate	Severe	
No	4	10	14
Yes	1	3	4
Total	5	13	18

* The value of Pearson's chi-square statistic is 0.02 based on 1 d.f.
* The level of probability (null hypothesis) at p = 0.888 is not significant

Mutations in exon 22 were detected in four patients (two sets of brothers). Table 3 shows the relationship between haemophilia severity and exon 22 mutations. Most of the patients with severe disease (23.07%) had a mutation with a P value of 0.888.

Exon 23 mutations were detected in two patients and their mothers. Table 4 shows the relationship between haemophilia severity and exon 23 mutations. Two of the patients with severe disease (15.38%) had a mutation with a P value of 0.352.

Table 4: Relationship between Exon 23 Mutations and Haemophilia A Severity

Mutation	Severity		Total
	Moderate	Severe	
No	5	11	14
Yes	-	2	4
Total	5	13	18

* The value of Pearson's chi-square statistic is 0.065 based on 1 d.f.
* The level of probability (null hypothesis) at p = 0.352 is not significant.

Exon 24 mutations were detected in 12 haemophilic patients and two of the mothers that were carriers. Table 5 shows the relationship between haemophilia severity and exon 24 mutations. Most of the patients with severe disease (84.6%) had a mutation with a P value of 0.009.

Table 5: Relationship between Exon 24 Mutations and Haemophilia A Severity

Mutation	Severity		Total
	Moderate	Severe	
No	4	2	6
Yes	1	11	12
Total	5	13	18

* The value of Pearson's chi-square statistic is 6.785 based on 1 d.f.
* The level of probability (null hypothesis) at p = 0.009 is significant.

Table 6: Relationship between Intron 22 Mutations and Haemophilia A Severity

Mutation	Severity		Total
	Moderate	Severe	
No	5	6	11
Yes	-	7	7
Total	5	13	18

* The value of Pearson's chi-square statistic is 4.406 based on 1 d.f.
* The level of probability (null hypothesis) at p = 0.036 is significant.

These results demonstrate that this mutation was present in seven of the thirteen patients with severe haemophilia A (53.84%). Table 6 shows the

Table 7: Gene Mutation Frequency Identified in Male Haemophilia Patients

Patient sample No.	Gene segment	Mutation/genome	Mutation type	Severity
2	Exon 22	6342delA	Frameshift	Severe
	Exon 23	6533A>T, 6539G>A, 6546C>A	Point mutation = =	
	Exon 24	165658delA	Frameshift	
	Intron 22	Inth22	Inversion	
3	Exon 24	165825A>C	Point mutation	Severe
4	Exon 22	6315G>T	Point mutation	Severe
	Exon 24	165897delG	Frameshift	
	Intron 22	Inth22	Inversion	
6	Exon 23	6581del T	Frameshift	Severe
	Exon 24	165707T>TC	Insertion	
	Intron 22	Inth22	Inversion	
7	Exon 24	165897delG	Frameshift	Moderate
9	Exon 24	165825A>C	Point mutation	Severe
	Intron 22	Inth22	Inversion	
11	Exon 18	5836C>T	Point mutation	Severe
	Exon 24	165898C>CA	Insertion	
	Intron 22	Inth22	Inversion	
12	Exon 24	165897G>GC	Insertion	Severe
14	Exon 24	165897G>GC	Insertion	Severe
	Intron 22	Inth22	Inversion	
15	Exon 24	165897G>GC	Insertion	Severe
18	Exon 24	165900G>GA	Insertion	Severe
19	Exon 22	6313A>T	Point mutation	Severe
	Intron 22	Inth22	Inversion	
22	Exon 24	6590delG, 6592A>T 6701A>C	Frameshift Point mutation Point mutation	Severe
23	Exon 22	6408 G>A	Point mutation	Moderate

Table 8: Gene Mutation Detection in Haemophilic Carriers

Carrier Sample No.	Gene Segment	Mutation/genome	Mutation type
1	Exon 18	5826delT	Frameshift
	Exon 24	165658delA	Frameshift
5	Exon 23	6509G>A	Point mutation
8	Exon 23	6668G>A	Point mutation
	Exon 24	165897delG	Frameshift

relationship between disease severity and intron 22 mutations.

Mutation frequency

In this study, four different mutations were detected in 18 Iraqi haemophilia A patients from seven unrelated families. Mutation detection methods led to the identification of different mutations, including 11 point mutations (10 of which were associated with the severe disease phenotype), 7 inversions, 6 frameshifts and 6 insertion mutations, one of which was associated with a moderate phenotype. Of the 35 mutations identified, there were 6 different mutations among the different exons examined (exons 22, 23, and 24) and intron 22 in patient sample 2. There were also multiple different mutations (three) in patient samples 4, 11, and 6. Two different mutations were detected in two different exons in patient samples 9, 14, and 19, and three different mutations were detected in exon 24 of sample 22, as detailed in Table 7.

Additionally, two frameshift mutations in two different exons were detected in carrier sample 1, and two different mutations in two different exons were identified in carrier sample 8, as shown in Table 8.

DISCUSSION

The severity of haemophilia was classified according to FVIII activity as follows: 13 male patients

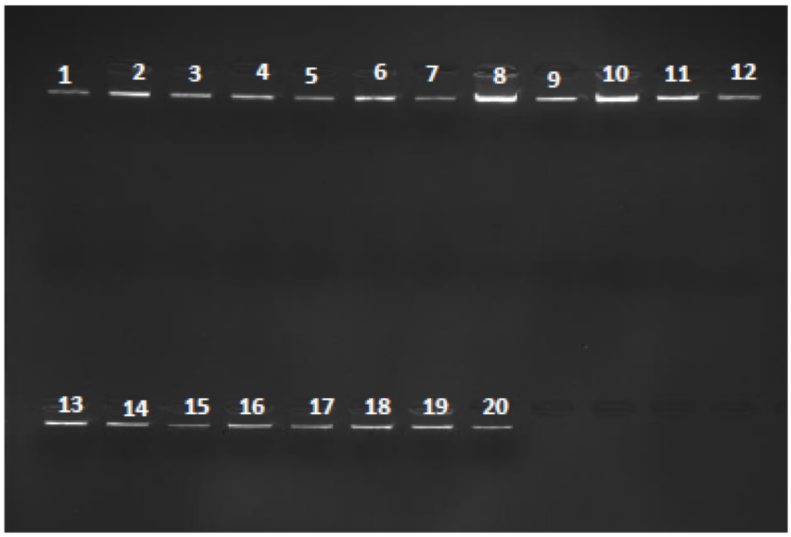

Figure 1: Chromosomal DNA Electrophoresis Banding on a 2% Agarose Gel after 1 Hour.

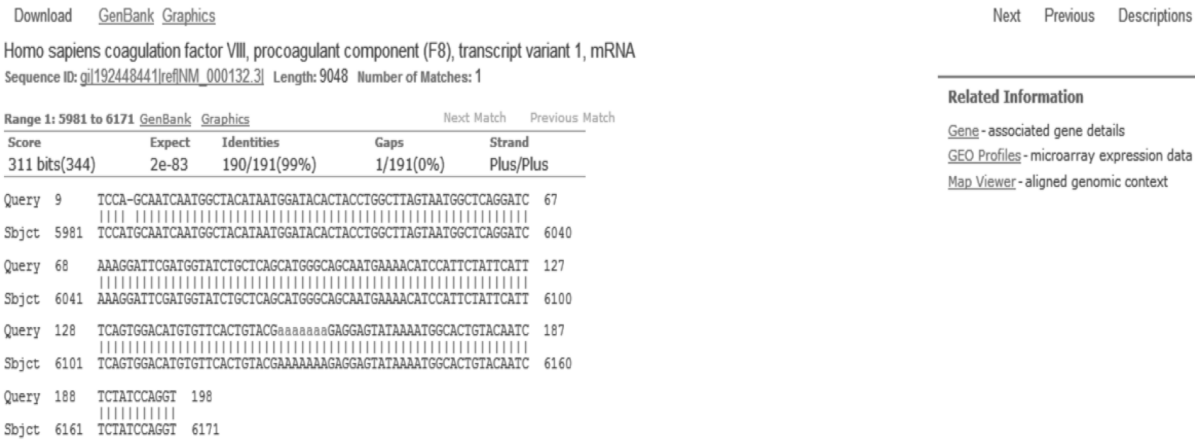

Figure 2: Alignment of a Haemophilia Patient's Exon 18 Sequence with the Reference Gene

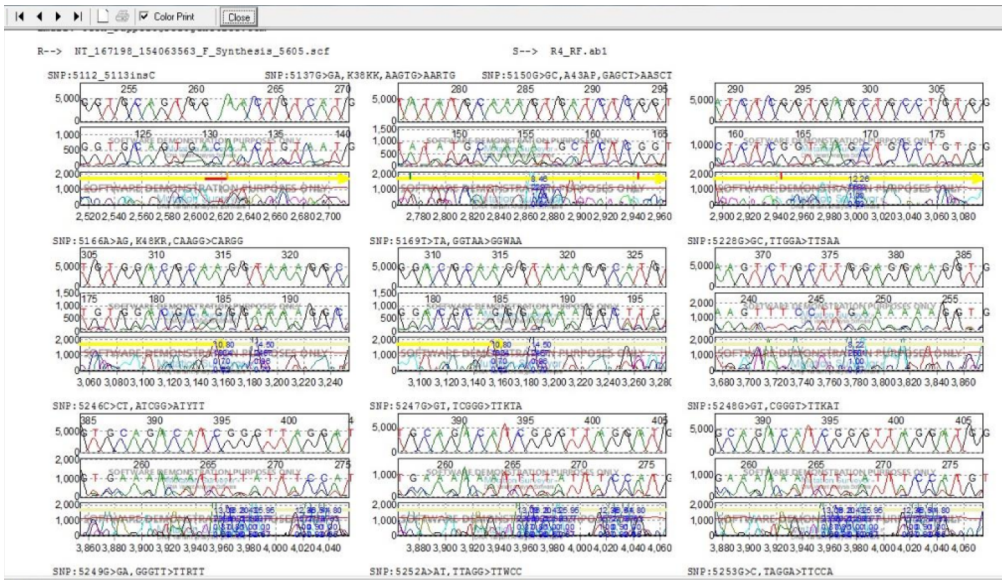

Figure 3: Alignment of Different Exon Sequences with the Reference Gene in Haemophilia Patients and Controls

had 1% FVIII activity (severe type) and 5 male patients had 1-5% FVIII activity (moderate severity). While females are only carriers, they may also harbour genetic defects; therefore, genetic defects affecting males are identified by FVIII activity, while the plasma and clinical evaluations of female carriers do not yield obvious diagnoses because of the lyonization phenomenon (Sun *et al.,* 2015). The lyonization phenomenon permits the expression of only one allele of the genes situated on the dynamic

X chromosome. Consequently, women express alleles from both their mothers and fathers, and each X chromosome contributes to approximately 50% of gene expression (Amos-Landgraf *et al.,* 2006), which provides sufficient protection against the disease. Exon 18 mutations were detected in one male and one carrier mother. Three out of 18 analysed Italian patients had this mutation, according to a study by Bicocchi *et al.,* (2005). Youssoufian *et al.,* (1986)identified one patient out of 83 who harboured exon 18 mutations.

In this study, exon 23 mutations were detected in two patients and their mothers. Exon 23 mutations were detected in one of 240 patients in North Carolina in a study by Youssoufian *et al.,* (1988), and exon 24 mutations were identified in four patients. Additionally, three out of 281 analysed British haemophilic patients harboured exon 24 mutations in a study by Green *et al.,*(2008).

Table 6 shows the relationship between disease severity and intron 22 mutations. Specifically, seven (53.84%) of thirteen patients had severe haemophilia; this rate is high compared with that reported by other researchers studying severe haemophilia A. Intron 22 mutations are the most common type occurring in haemophiliacs worldwide. Specifically, inversion mutations in intron 22 are the most common HA-causative mutations present in 40–45% of severe haemophiliacs, and 2–5% of severe HA cases are caused by intron 1 inversions (Nair *et al.,* 2010). These inversions originate almost exclusively in male germ cells because pairing Xq with its homologous chromosome in female meiosis would most likely inhibit the proposed intrachromosomal recombination (De Brasi *et al.,* 2000). It is well known that introns do not encode proteins but are simply removed from the DNA molecule during transcription by the action of spliceosomes (Chorev and Carmel, 2012).

All exons examined in this study are located in the light chain of the mature FVIII protein, which is composed of A3, C1, and C2 domains (Kane and Davie, 1986), and intron 22, which is located in the non-coding region between exons 22 and 23. Mutations in these exons and introns lead to defects at the transcriptional or translational levels or to changes in single amino acids in the FVIII protein that result in FVIII deficiency (Jacquemin, 2009). The risk of inhibitor development is higher when the mutations are located in the light chain than when they are located outside of the light chain (i.e., in the A1, A2, or B domain) of the FVIII protein (Levinson *et al.,* 1992) .

Studies of families have revealed that most mutations in haemophilia A patients originate in male germ cells, based on the high prevalence of point mutations (Leuer *et al.,* 2001). In this study, 2 different mutations, including 2 point mutations and 3 frameshift mutations, were identified in 7 female carriers.

Analyses of families with numerous mutations have frequently reported conflicting phenotypic severity; consequently, the presence of several mutations shows the importance of additional DNA analyses in patients with identified mutations who have uncommon phenotypes or additional mysterious clinical symptoms because these multiple mutations have direct implications for genetic diagnosis and genetic counselling (Shetty *et al.,* 2011).

The results of this study showed that all double mutations occurred only in patients with a severe phenotype and that the most common FVIII gene mutations in patients were point mutations, followed (in order) by inversion mutations, frameshift mutations and insertion mutations. The existence of two mutations has a somewhat synergistic effect on FVIII activity, while patients with a single mutation exhibit the highest FVIII activity (Bayele *et al.,* 2010). In genotyping, the coexistence of two mutations should never be excluded, especially in cases with discordant clinical presentation.

CONCLUSION

Exon 24 mutations are the most frequent types of mutations that occur in haemophilia patients in the Najaf Province, followed by intron 22 mutations, and almost all severe haemophilia cases have mutations located in these two gene segments. The results of this study also revealed that the most frequent mutations in the FVIII gene in patients were point mutations, followed (in order) by inversion mutations, frameshift mutations and insertion mutations. Further, this study showed strong correlations between exon 24 and intron 22 mutations and bleeding severity.

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