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Identification of Factor VIII Gene Mutations in Iraqi Patient with Hemophilia A

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ABSTRACT

Aims: Identification of factor VIII gene mutations in Iraqi patients with hemophilia A which is an X linked recessive disease, clinically occur only in male and rare in females. Unfortunately in Iraq, no studies were done previously regarding hemophilia gene detection. Present study was conducted for detection of mutations in the FVIII gene as well as to find a relationship between mutations and the severity of disease and identify common mutations in the study population.

Methods: 25 Iraqi patients with haemophilia A and 16 healthy members as control were included. All samples collected for study were processed in medicine, science colleges and of AL-Karama Teaching Hospital laboratories from January 2015 to June 2015. These patients were previously diagnosed based on standard symptoms and confirmed by PTT and the level of FVIII activity.

Results: Results showed 14 from 18 patients (78%) had family history of hemophilia and 67% of patients had positive consanguinity marriage. Mutation distributed in all exons: 18, 22, 23, 24 and intron22. Most mutations detected were point mutations then inversion mutations followed by frame shift mutations. Result showed relation between severity and exon 24 mutations with P-value 0.009 and 0,036 in intron 22, P-value P≤0.05were considered significant.

Conclusions: Results indicate the bad effect of positive family history and the consanguinity marriage. Most mutation located in exon 24 and intron 22 and there was relation between severity of the disease and exon 24 mutations, as well as intron 22. All double mutation occurs in patients with severe phenotype and causes more severe phenotype.

KEYWORDS: F8 gene Mutations, Hemophilia A, Symptoms severity.

INTRODUCTION

HEMOPHILIA A is the most common inherited human bleeding disorder[1], the estimated incidence of haemophilia A is between 1:5,000 and 1:10,000 in live male births and rarely in females who mostly act as carriers which inherit the defective gene from either their mother or father, or it may be a new mutation.[2-4]

Hemophilia A is a good model disease for the study of mutation and the high proportion of new mutations is seen due to the lowered probability of survival of disease alleles.[5]

Hemophilia A is caused by defect in factor VIII, a component in the intrinsic pathway of blood coagulation. The X-chromosome-linked disease almost certainly stems from a heterogeneous collection of genetic lesions.[6] FVIII gene is one of the largest, spans 186 kb of the DNA and maps to the distal band of the long arm of the X chromosome (Xq28)[7]. It contains 26 exons and 25 introns. The mature FVIII protein encode 2332 amino acids arranged in the order A1 – a1 – A2 – a2 – B – a3 – A3 – C1 – C2 which have three homologous A domains, two homologous C domains and the unique B domain[8]. These domains play an important role in the FVIII function as each domain includes particular binding sites for different components of the coagulation cascade[9]. Genetic disorder can impact these interaction sites and result in Hemophilia A[10]. However, it is likely that there are additional unknown factors, both genetic and environmental, FVIII deficiency can also be seen in von Willebrand disease[11], especially the type 2N VWD. Mutations in VWF that affect the F8-binding site (encoded by VWF exons 18–20) have been identified in patients with von Willebrand disease phenotype that can be described as 'pseudohaemophilia'.[12] The circulating FVIII heterodimer associates primarily with VWD factor

through the interactions with acidic region a3 at the light chain and with C2 domain[13]. FVIII circulates in plasma complexed non-covalently with von Willebrand factor (VWF) which acts as a plasma transporter, that appears to be a defence for FVIII from proteolysis and fast clearance subsequently, serves to stabilize FVIII in the circulation and when thrombin cleavage sites to activation FVIII to FVIIIa, the active form of FVIII is released from VWF factor[14]. FVIII gene produces two additional alternatively spliced transcripts. Transcript Variant 1 composed of 26 exons and encodes the full-length isoform (a), a large glycoprotein which circulates in plasma and associates with von Willebrand factor in a noncovalent complex. This protein undergoes numerous division events. Transcript Variant 2 encodes a putative small protein isoform (b), which consists firstly of the phospholipid binding domain of factor VIIIc. This binding domain is essential for coagulant activity and contains a unique 5' exon located within intron 22 of transcript variant 1. This exon codes for eight amino acids and is spliced to exons 23-26 maintaining the reading frame. Isoform (b) is very shorter contrast to isoform (a). Defects in this gene results in hemophilia A and also known F8B; F8C[15]. Gene defects associated with haemophilia A can be divided into gross gene reorganisations (an inversion) results in approximately 50% of all severe disease cases worldwide while Intron 1 inversion results in about 2 – 5% of severe haemophilia A cases. Insertions or deletions of genetic sequence of a size changing from one base pair up to the entire gene and single DNA base substitutions resulting in either amino acid replacement (missense), premature peptide chain end (nonsense or stop mutations) or mRNA splicing defects.[16,17]

Intron22 separating exons 22 and 23 (IVS22) comprises a CpG island which contains these two nested genes. F8B is transcribed in the same direction the F8 gene while F8A is transcribed in the reverse direction to the F8 gene. Furthermore, two additional copies of F8A were positioned telomeric to the F8 gene so, the large majority of the "missing" cases of severe haemophilia were clarified by homologous recombination between the 9.5 kb intragenic (termed int22h - 1) and one of the two extragenic homologs of this sequence (int22h - 2 and int22h- 3).[18] During the meiotic division of spermatogenesis, this recombination resulting in a large inversion and translocation of the gene sequence including exons 1 – 22 away from exons 23 –26. Of these two common types of intron 22 inversion, the distal homolog is responsible for the majority of the severe haemophilia A inversion cases, while crossover with the proximal copy results in a further minority of cases[19]. Female carriers are predictable to have a plasma concentration of factor VIII corresponding to half the concentration found in healthy individuals, which is generally normal for normal hemostasis,[20] because one of their two X chromosome undergo random X-chromosome inactivation (lionization) which happen in the early embryonic life[21]. Hemophilia is classified according to clinical severity as severe, moderate and mild when FVIII activity level is less than 1%, 1-5% and more than 5% respectively[22]. Patients with haemophilia A had higher cancer incidence than the age and sex matched patients, especially for the elderly in which haemostasis is associated with the development and spread of cancer[23].

AIMS OF STUDY

Detection of mutations in the gene-coding FVIII by polymerase chain reaction (PCR) using different primers with different base pair product as show in figures 1, 2, 3. As well as to find a relationship between mutations and the severity of symptoms of the disease and Identify common mutations in the community.

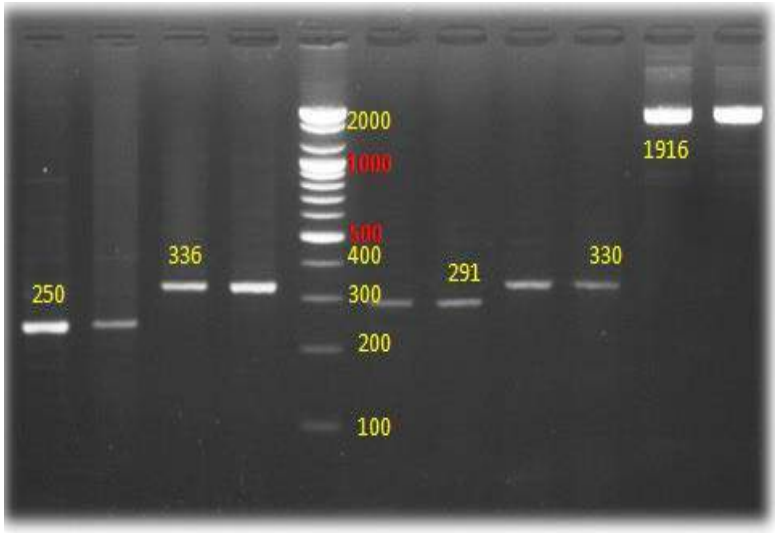

Fig 1: PCR products of FVIII gene on 2% agarose gel at 70 voltages for one hour. lane 1 – exon 24- patient sample no.\37,control sample no.\ 13,- exon 22-patient\26,control\2-DNA ladder, exon23-control-10,patient\32-exon18-patient\40,control\11-\intron22-patient\33,control\10from control and patient with HA (Ethidium bromide staining).

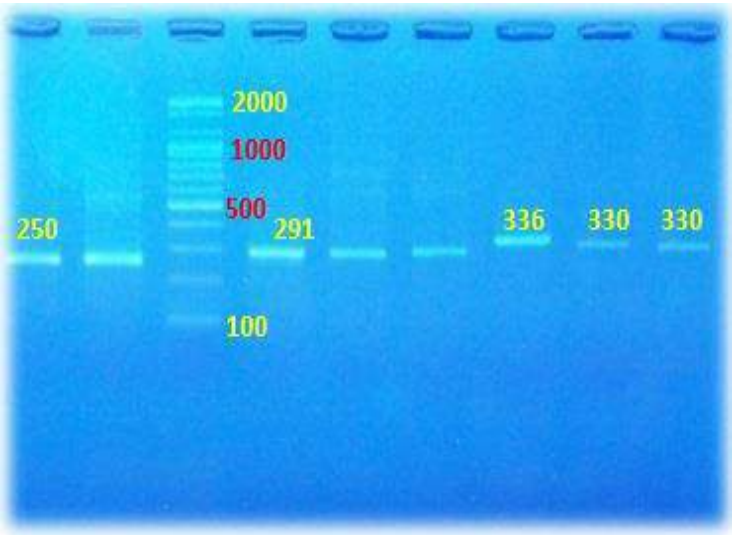

Fig 2: PCR products of FVIII gene on 1% agarose gel at 70 voltage for one hour.
Lane-1exon-24, patient no.\5,control,6, DNA ladder,exon23,control\13,patient,18,31,exon22-patient\24,exon18-patient\37,control-12 from control and patients with HA. SYBER green staining.

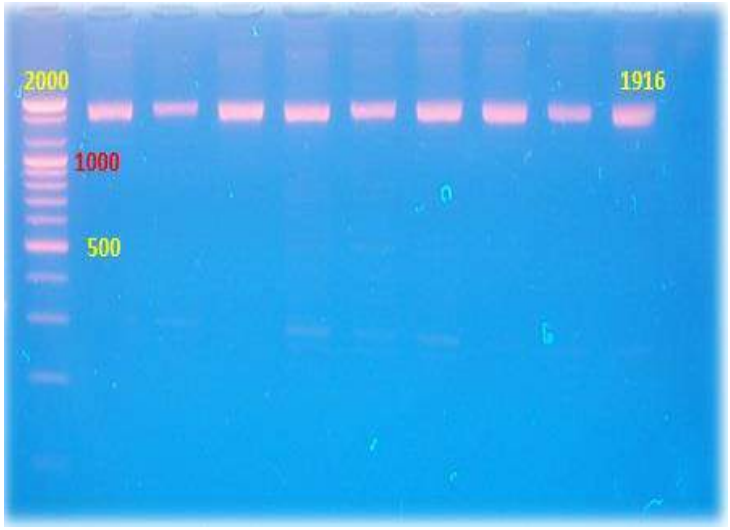

Fig 3: PCR products of FVIII gene on 1% agarose gel at 70 voltage for one hour.
DNA ladder, intron22\control sample no. 5, patient sample no., 22\30,29,27,26,20,18,37 from control and patients with HA. (Ethidium bromide staining)

Table 1: Percentage of Hemophiliac patients and carriers group data

Gender		Family history		Consanguinity state		Hemophilia severity	
male	female	yes	no	positive	negative	sever	moderate
72%	28%	78%	22%	33%	67%	72%	28%

MATERIALS AND METHODS

Collection of samples

25Iraqi patients with haemophilia A from unrelated families and 16 healthy members as control from Al-Karama teaching hospital, in Wasit province-kut-city, were included to participate in present study. The age of patients were ranged from 1.1 to 35 years.

Methods

All samples collected for study were processed in medicine, science colleges and of AL - Karama Teaching Hospital laboratories. These patients were previously diagnosed based on standard symptoms and confirmed by PTT and the level of FVIII activity. Disease is considered to be severe when factor levels are below 1% of normal values (normal value between 50-150), moderate when they are between 1 -5% and mild when levels range between 5- 40%. Some basic demographic information such as age, sex, relative state, family history was collected. After checking the extracted DNA for it purity and concentration, its being subjected to amplification to selected area of FVIII, which includes Exon 18, 22, 23, 24 and intron 22, then sequencing has been conducted for all the exons and intron 22 for all patients and controls for molecular analysis for detection of mutations of most common segment of FVIII.

RESULTS

The purpose of this research was to identification of factor VIII gene mutations in Iraqi Patients with Hemophilia A and the relation between these mutations and bleeding severity of hemophilia A with 18 hemophiliac patient male and 7 hemophiliac female as carrier from 4 un related families.

DNA sequence analysis

Exons sequences were done by aligned with the corresponding DNA reference FVIII mRNA sequence using NCBI/ BLAST. First ATG appears in the exons aligned was regarded as first codon of the geneofexon18. The study was done for 18 hemophiliac patients (male), 7 hemophilic carriers (mothers) and 16 control samples, to detect gene abnormalities which responsible for hemophilia disease. All control samples were obtained from male gender. Percentage of Hemophiliac patients and carriers group data.is depicted in Table1.

Table 2: Gene mutation detection in hemophiliacs with normal variation and severity.

sample no.	Patient Male \Carrier (Mother)	Gene segment	Mutation\ genome	Mutation type	Normal variation	Severity
			EXON 18			
17	Carrier	Exon 18	5826delT	Frameshift		-
27	Patient	Exon 18	5836C>T	Point mutation		**severe**
			EXON 22			
18	Patient	Exon 22	6342delA	Frameshift		**severe**
20	Patient	Exon 22	6315G>T	Point mutation		**severe**
35	Patient	Exon 22	6313A>T	Point mutation		**severe**
39	Patient	Exon 22	6408 G>A	Point mutation		**moderate**
			EXON 23			
18	Patient	Exon 23	6533A>T, 6539G>A, 6546C>A	Point mutation = =		**severe**
21	Carrier	Exon 23	6509G>A	Point mutation	GAT>AAT	-
22	Patient	Exon 23	6581del T	fram shift		**severe**
24	Carrier	Exon 23	6668G>A	Point mutation	CGA>CAA	-
	Sample no.38 in exon23/ male had no mutation with normal variation 6959GG/CA					**severe**
			EXON 24			
17	Carrier	Exon 24	165658delA	Frame shift		-
18	patient	Exon 24	165658delA	Frame shift		**Severe**
19	patient	Exon 24	165825A>C	Point mutation		**Severe**
20	patient	Exon 24	165897delG	Frame shift		**Severe**
22	patient	Exon 24	165707T>TC	Point mutation		**severe**
23	patient	Exon 24	165897delG	Frame shift		**moderate**
24	Carrier	Exon 24	165897delG	Frame shift		-
25	patient	Exon 24	165825A>C	Point mutation		**severe**
27	patient	Exon 24	165898C>CA	Point mutation		**severe**
28	patient	Exon 24	165897G>GC	Point mutation		**severe**
30	patient	Exon 24	165897G>GC	Point mutation		**severe**
31	patient	Exon 24	165897G>GC	Point mutation		**severe**
34	patient	Exon 24	165900G>GA	Point mutation		**severe**
38	patient	Exon 24	6590delG, 6592A>T 6701A>C	Frame shift Point mutation Point mutation	TGC>TCG,C AC>CCC	**severe**
			INTRON 22			
18	patient	INTRON 22	Inth22	inversion		**severe**
20	patient	INTRON 22	Inth22	inversion		**severe**
22	patient	INTRON 22	Inth22	inversion		**severe**
25	patient	INTRON 22	Inth22	inversion		**severe**
27	patient	INTRON 22	Inth22	inversion		**severe**
30	patient	INTRON 22	Inth22	inversion		**severe**
35	patient	INTRON 22	Inth22	inversion		**severe**

Mutations screening conducted throughout the study shows that most mutations were located in intron 22 & exon 24 which also has more effect upon the severity of hemophilia patients. Mutations that are located in the coding regions, the exons, were point mutations or deletions of single nucleotide. Most point mutations result in changing in single amino acid-like for example (2230H>P) in exon 24 (sample number 37 in patient male) that means amino acid histidine change to proline at codon 2230 of F8 gene nucleotide sequence also H>L and K>N, R>Q in exon 22 (sample number 35, 39 respectively in male patients) which means large structural influences may be involved, if we know the protein structure depend to a large extents on amino acids properties and their location in the polypeptides chain. Other point mutations were also found but they were just silent mutations, which never mentioned in our study even these mutations may reflect suspected genome instability of the patients. Other changes in exons which were also neglected from our study, were the normal variations, which represent normal changes in genomes. There were about four variants in F VIII gene. All the variants have been included in this study; all exons involved have been aligned and compared the all possible variants. Mutations results were shown in Table 2.

Table 3: Mutation detection and their relation with patient male bleeding severity

Gene segment	Mutation detection samples no.		Relation between bleeding Severity and exons mutations in patient (males).		P.value Significance	Total
	HA males.	HA carriers				
Exon18	1 sample	1 sample	Moderate-0	Sever-1	o.523	2
	percentage from 25 HA males and females		7.69% from 13 sever patient		no significant	
	4%	4%				
Exon22	4 samples	No sample	1	3	0.888	4
	percentage from 25 HA males and females		23.07% from 13 sever patient		no significant	
	16%	0				
Exon23	2 samples	2 samples	0	2	o.352	4
	percentage from 25 HA males and females		15.38% from 13 sever patient		no significant	
	8%	8%				
Exon24	12 samples	2 samples	1	11	0.009	14
	percentage from 25 HA males and females		84.6% from 13 sever patient		significant	
	48	8%				
Intron22	7 samples	No sample	0	7	0.036	7
	percentage from 25 HA males and females		53.84% from 13 sever patient		significant	
	28%	0				
Total	26	5	2	24		31

In order to understand the severity of the hemophilia disease it is important to detect the gene structure and its domains as severity of hemophilia disease depends solely on where mutation takes place, on which domain they affect, so the more protein deformation occurs the more deficiency produced[25]. Mutation detection percentage in exons 18,22,23,24 and intron 22 samples and their relation with bleeding severity is shown in table 3. Results shows and according to the chi square ($P \leq 0.05$) analysis which asymptotic significant 0.036 less than 0.05, there is significant and influential relation between intron 22 mutations and severity and according to the chi square ($P \leq 0.05$) analysis which significant 0.009 less than 0.05, there is significant and influential relation between exon 24 mutations and severity.

DISCUSSION

In this study, 3 different mutations were detected in 18 Iraqi hemophilia A patients from four unrelated families. Mutations detection methods led to identification of different mutations which included 17 point mutations, 7 inversion and 6 frame shift. During this work, 2 different mutations were identified in 7 female as carriers distributed as: 2 - point mutations, 3 - frameshift mutations. In total cases studied (patients males and carriers) there are 19 point mutations, 7 inversion and 9 frameshift were identified as shown in Fig 4.

From the 35 mutations obtained, there are 6different mutations in different exons detected (exons 22,23,24 and intron 22) are located in patient sample no.18 also there are multiple different mutations (three) inpatient

samples no.20, 22,27 .Two different mutation in two different exons in patient samples no. 25,30,35 and three different mutation in exon 24 of the sample no. 38 as detailed in table (4) below.

Table 4: Gene mutation frequency identified in hemophilic patients males

Patient no.	Gene segment	Mutation\genome	Mutation type	severity
18	Exon 22	6342delA	Frame shift	**Severe**
	Exon 23	6533A>T, 6539G>A, 6546C>A	Point mutation = =	
	Exon 24	165658delA	Frame shift	
	Intron22	Inth22	inversion	
19	Exon 24	165825A>C	Point mutation	**severe**
20	Exon 22	6315G>T	Point mutation	**severe**
	Exon 24	165897delG	Frame shift	
	Intron 22	Inth22	inversion	
22	Exon 23	6581del T	frame shift	**severe**
	Exon 24	165707T>TC	Point mutation	
	Intron22	Inth22	inversion	
23	Exon 24	165897delG	Frame shift	**moderate**
25	Exon 24	165825A>C	Point mutation	**severe**
	Intron 22	Inth22	inversion	
27	Exon 18	5836C>T	Point mutation	**severe**
	Exon 24	165898C>CA	Point mutation	
	Intron 22	Inth22	inversion	
28	Exon 24	165897G>GC	Point mutation	**severe**
30	Exon 24	165897G>GC	Point mutation	**severe**
	Intron22	Inth22	inversion	
31	Exon 24	165897G>GC	Point mutation	**severe**
34	Exon 24	165900G>GA	Point mutation	**Severe**
35	Exon 22	6313A>T	Point mutation	**severe**
	Intron 22	Inth22	inversion	
38	Exon 24	6590delG, 6592A>T 6701A>C	Frame shift Point mutation Point mutation	**severe**
39	Exon 22	6408 G>A	Point mutation	**moderate**

Table 5: Gene mutation detection in hemophiliac carriers

Carrier no.	Gene segment	Mutation\genome	Mutation type
17	Exon 18	5826delT	**Frameshift**
	Exon 24	165658delA	**Frame shift**
21	Exon 23	6509G>A	**Point mutation**
24	Exon 23	6668G>A	**Point mutation**

Also two frameshift mutations in two different exons detected in carrier sample no.17 and two different mutations in two different exons identified in carrier sample no. 24 as shown in table 5.

The results showed by some cases in which double or multiple different mutations were exist, may be reflection of a kind of genomic instability, structure or protein function disorder in those patients and so they may suffer from another possible diseases or may be due to inheritance of different mutated copies from the parents. Double mutations are being increasingly reported and raise many questions with regard to the specificity of the mutations in causing the disease. In a proportion of families with multiple mutations, discordant phenotypic severity is often observed among the affected members[27]. Another possible cause of multiple mutations is an indirect result of impaired DNA repair system[28], so the presence of multiple mutations confirms the importance of additional DNA testing in patients with known mutations who have unusual phenotypes or additional unexplained clinical problems because these multiple mutation has direct effect on genetic diagnosis as well as genetic counseling[27]. Double mutations in the same gene can have synergistic, equal or opposing effects on the clinical phenotype[29] and can cause a more severe phenotype[30]. Result in present study

showed all double mutation occurs just in patients with severe phenotype and there is relation between exon 24, intron 22 mutations and bleeding severity. The most common mutations in FVIII gene of patients is the point mutation then inversion mutations followed by frameshift mutations. The existence of two mutations has a slightly synergistic effect on FVIII activity while the patient with a single mutation had the highest FVIII activity[31]. In genotyping, the coexistence of double mutations should never be excluded, especially in cases of discordant clinical presentation[30].

CONCLUSION

Hence present study indicated that most of hemophilia A patients were males and almost all severe Hemophilia cases have mutations located in exon 24 and intron 22. Intron 22 mutations are most common type of mutations that occurs in hemophiliacs patients in Wasit city as well as exon 24 mutations. Intron 22 occurs in hemophiliac patient male with severe type and exon 24 mutations occur in patient with severe and moderate phenotype and also in carriers. Most of cases are with a hemophilia history (78%) represent a major factor for genetic predisposition lead to defective FVIII gene and about 67% of patients under study with consanguinity marriage result in concentrated the bad gene copy so this is highly suggestive that hemophilia disease is not uncommon in Wasit province. Relation between exon 24 and intron 22 mutations with the severity of bleeding were seen. Result showed all Double mutation occurs in patients with severe phenotype and the most common mutations in FVIII gene of patients is a point mutation then inversion mutations followed by frameshift mutations. Detection of FVIII mutation is important in identifying female with genetic defects for subsequent further genetic counseling. There is an obvious public ignorance about the role of heredity in many disorders.

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