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Identification of Factor VIII Gene Mutations in Patients with Haemophilia A

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ABSTRACT

The study is aimed to detect the primary mutations of the FVIII gene among Iraqi patients using molecular analysis and to identify the relationships between these mutations and the severity of the disease. The study involved twenty-five patients with haemophilia type A, eighteen of those patients were males, and seven of them were mothers with disease carriers, from seven families but un-related. The extracted DNA determined by the concentration and purity and then after the amplification of selected parts of the FVIII gene involving intron 22 and exons 18, 22, 23, 24 were done. Sequencing of these exons and introns were done for all patients and control subjects. Sequencing analysis showed that the majority of mutations were "point mutations" in exons, mostly in exon 24. In contrast, the mutations in exon 18 were identified in one male in addition to one carrier mother. The mutations in exon 22 were identified in four patients, and the mutation in exon 23 was identified in two males in addition to two carrier mothers. The mutations in exon 24 were identified in twelve patients with haemophilia in addition to two carrier mothers. Moreover, the numbers of identified inversion mutations in the study were seven in hemophilic patients. The study showed there was a direct relationship between the severity of disease and the mutations in exon 24 and intron 22. So, the ultimate conclusion of this study showed that the most common mutations in Iraqi hemophilic patients were exon 24 mutations and followed by intron 22 mutations. The majority of these mutations occurred in severe cases of haemophilia.

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INTRODUCTION

Haemophilia A also considered as antihaemophilic globulin (AHG) having a deficiency of factor VIII is termed as X-linked recessive disease of genetic bleeding which clinically happens in males. However, in females, the probability of comprising two defective gene copies exceptionally uncommon. Thus, being asymptomatic, these females are deliberated as the carriers of such disorders (Loomans *et al.*, 2014) .

The haemophilia A occurrence per 100,000 male in Iraq considered is 3.6 individuals (UNDESA, 2012).

Among the patients of Haemophilia, around 80 to 85% patients are haemophilic with type A while 10 to 15% is haemophilic B. The haemophilia identification among children having a family with a positive blood test is not made abruptly after birth because of the counselling concerning the heredity and family history in various countries (Hazewinkel

et al., 2003). Furthermore, roughly thirty per cent of the cases of haemophilia occur due to the spontaneous mutation rather than an association with the history of the family (Abshire, 2005). Though, there also subsist the acquired forms of haemophilia typically in elderly patients because of the inhibitor's actions (autoantibodies).

The Haemophilia A is termed as an utmost communal inherited disorder of bleeding in humans (Peyvandi, 2005). Its anticipated prevalence in live births of males is amongst the 1:5,000 & 1:10,000 (Rodriguez-Merchan, 2002).

In opposition to the haematological malignancy or FVIII, however, majorly these are inherited. Thus, the acquired occurrence rate of the haemophilia is an approximation of 1:1,000,000 in sick individuals (Franchini and Lippi, 2008) which is further linked to the infused concentrate clotting factors' neutralization. The escalation of morbidity in haemophilic patients is often related to the certain drugs allergic reactions, cancer or disorders of the immune system (Lassila *et al.*, 2015; Srivastava *et al.*, 2013). Hence, the Haemophilia A can be thought to be an excellent study model for mutations as it is comparatively prevalent illness having variable phenotypes, including allelic heterogeneity. Besides, according to Goodeve (2015) a novel mutation ratio has been observed in FVIII, with which a component of the pathway of intrinsic blood clotting of results in irregular FVIII formation.

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 ($2.5–4.2 \times 10-5$) (Ilic *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.

MATERIALS AND METHODS

Patients

Informed consent was obtained from all patients, and the project was approved by the Faculties of Medicine and Science at the University of Wasit 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 Faculties of Medicine and Science at the University of Wasit. 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 predestinated 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. Sick individual information was gathered from January 2015 to June 2015 from Al-Karama hospital in Wasit province/Iraq.

The diagnosis of all these patients formerly relied upon the standard symptoms which inveterate the findings of laboratory known as PTT (Prolonged partial thromboplastin) & Activity assays of FVIII which indicated the levels of factors below the average values of 1% where the typical values were observed to be amongst the 50-150%. The activity level of this ailment is thought to be rigorous. The moderate levels are observed to be 1% & 5%, yet, mild levels are found to be 5% to 40%. Moreover, patients constantly demonstrate normal bleeding time (BT), thrombin time (TT) and prothrombin time (PT) (Xie *et al.*, 2009).

Samples of Blood

The samples of (5-10ml) venous blood were gathered from both healthy subjects and haemophilic patients as well. For molecular studies, each of the blood samples was collected in EDTA tubes and were stored at 4°C until further usage.

Extraction of Genomic DNA

The extraction of genomic DNA was done from freshly collected blood in the anticoagulant (EDTA) tubes.

Further, the molecular assessments were conducted by the usage of *AccuPrep*® Genomic DNA Extraction Kit (Bioneer, South Korea).

DNA isolation was carried out based on the Keijzer *et al.* (2010) method.

Electrophoresis of Agarose gel

For the determination of extracted DNA's purity and concentration, the Agarose gel electrophoresis was done after the genomic DNA extraction (Sambrook

et al., 1989).

Purity estimation and concentration of DNA

To find out the purity & concentration of isolated DNA samples, a spectrophotometer was used. For the estimation of concentration in ng/μL, one microliter extracted DNA was put into the spectrophotometer having a range of amongst 11-14ng/μL. Furthermore, the purity was deliberated through the observations of optical density (OD) ratio at 260/280. This was to verify any sort of contamination in the sample containing proteins. Thus, the pure DNA's 260/280 ratio was observed to be in between 1.7 & 1.9 value (Sambrook and Russell, 2001).

Amplification of DNA

To screen the mutation, the amplification of both controls and patients' extracted DNA was amplified with the assistance of polymerase chain reaction (PCR) having specified primers for intron 24 and exons 24, 23, 22 and 18.

The specificity of PCR products was checked through electrophoresis. Further, these were electrophoretically purified with the usage of DNA gel extraction procedure to keep away any DNA fragmentation, non-specific amplification and to purify the DNA highly.

The sequencing of these PCR products was carried out by a method known as the Macrogen primer extension (Biotechnology Company, South Korea).

Alterations of HA gene

The screening of the HA gene's intron 22 and four exons was carried out through sample's direct sequencing taken from the 16 controls, haemophiliac carriers and haemophiliac male patients. All the results were evaluated with the sequence of human reference mRNA-HA with the assistance of software sequence (Chromas Pro, type: 1.5) available on the web (HTTP: National Center for Biotechnology Information (NCBI) Reference Sequence: NM_000547.4)

DNA sequence analysis

DNA molecules' nucleotides sequence such as thymine, cytosine, guanine and adenine is determined by DNA sequencing. BLAST program (Basic local alignment search tool) was used to accomplish the examination of the nucleotide sequencing. Moreover, the translation of the nucleotide sequences into the amino acid sequences was done with BLAST, in which inton 22 and all exons were aligned as a corresponding reference FVIII gene sequences to observe any differences. Thus, the reference genomic DNA was used to find out the difference in intron 22, while reference FVIII mRNA was used for exons. Mutation Surveyor software was utilized with the alignment of the same sequence to find out the alterations in amino acids and to ensure the normal variations.

Detection of FVIII gene mutation

Selecting of Mutation

The primer sequences in this research utilized to detect the utmost communal (Chetta *et al.*, 2008). They reported FVIII mutations in exons 24, 23, 22 and 18 as well as in intron 22 beforehand.

Primers

The supply of all primers and being lyophilized at various concentrations was done by Bioneer Company (South Korea). All lyophilized primers were further dissolved in DNase/RNase free water as per the recommendation of the manufacturer, and then these were left in water for complete dissolution. Finally, this protocol produced a final 100 pmol/μL concentration as a stock solution. Further, each primer was diluted to a 10pmol/μL concentration in the form of aliquots and reserved as frozen at -20°C till their usage as a working solution.

PCR programs

The most primer's annealing temperature was calculated by utilizing *in silico* PCR software and also to achieve the PCR optimization. To attain the best PCR outcome, after several trials, an optimized PCR was acquired.

PCR products' sequencing and purification

PCR products' sequencing

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

Statistical analysis

SPSS "16 (Statistical Package for Social Sciences version 16) was utilized for further examination and data input. Thus, the discrete variables are shown as percentages and numbers, whereas continuous variables are given as mean. To analyze the observed distribution's significance, the Chi-square test of goodness fit was used. Therefore, being two-tailed and asymptotic, P values presents the significance of the values below 0.05.

RESULTS

Distribution of HA patients

The majority of the patients comprised of 28% carriers (7 mothers) while 72% of males (18 cases). Family history for this disease was reported in fourteen

patients (78%) whereas; four patients (22%) had no family history. Moreover, six haemophilic patients (33%) had non-consanguineous marriages, while twelve patients (67%) had positive consanguineous marriages. The classification of the haemophilia based on the severity of the factor VIII activity was as followed: 5 male patients have amongst 1% & 5% of moderate severity of factor VIII while 13 male patients had 1% FVIII severe type activity.

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

DNA isolation

The extracted genomic DNA from the haemophilia A patient's blood illustrated a single band after the fraction by gel electrophoresis (Figure 1). Purity was further checked with the help of a spectrophotometer device (Avans Biotechnology, Taiwan).

The humans' (*Homo sapiens*) nucleotide sequences, i.e. mRNA-HA gene sequence of bases1 to 191041 was downloaded from GenBank (http://www.ncbi.nih.gov/nuccora/NM_000547.4.). These were further aligned with the help of the MEGA4 program's ClustalW method. This program is utilized for the multiple alignments according to the reference sequence given in Figure 2. Further, the modification of the sequence of mRNA-HA into the cDNA was done by converting the Uracil to Thymine. Inversion mutations were checked by comparing the intron 22 to the genomic DNA sequence. These were analyzed utilizing virtual restriction enzyme aiming the software of NTI vector which is used to find out the inversion mutations in intron 22 through its comparison to the genomic DNA sequence and determination of the SabI restriction site's presence.

Mutation screening

The screening mutation all through the research indicated the location of most of the mutations in exon 24 and intron 22; mutations in these locations exhibit the most significant effect on disease severity. In the coding regions of an exon, mutations observed were the point mutations, insertions or single nucleotide's deletion. Majority of the point mutations occur due to the alterations in single amino acids. For instance, sample # 21 (male patient) with 2230H>P in exon 24 exhibited that histidine amino acid was altered to the proline at 2230 codon of the nucleotide sequence of the FVIII gene. Besides, K>N & H>L of the exon 22 of sample 19, as well as R>Q, signified the involvement of vast structural influences in case of the

Huge dependence of protein structure to the properties of amino acids and on the polypeptide chain locations. There is an existence of other point mutations rather merely silent mutations that are stated in our research; such mutations might designate the suspected instability of genome in patients—other neglected changes of exons in our study consist of normal variations that represented the normal genome changes. Thus, the approximate FVIII gene's four variants were also integrated into this study. Hence, the implicated DNA sequences of exon were compared & aligned with all other normal possible variants. The detected mutations in the gene caused the haemophilia in 7 mothers (carriers) and 18 haemophilic patients having severity and normal variations, as shown in Table 1. All the 16 control samples indicated no gene Abnormality.

Moreover, two mothers and patient samples contained two mutations in the exon 18. Concerning the 23 and 22 exon's defects, four mutations were detected in exon 22 of four patients while six mutations were observed in exon 23 of the four patients and mothers. However, 14 samples showed 16 mutations in mothers and patients as well. Thus, the appearance of the first ATG was termed as the first codon in aligned exons (Figure 3 presents the E18_EF aligned sequence).

Mutation detection and haemophilia a severity

The mutations in exon 18 were observed in 1 carrier mother and one male. The relation amongst the severity of haemophilia and mutations in exon 18 has been illustrated in Table 2. The severe disease (7.69%) in one of the patients had a mutation with 0.523 P-value.

Four patients with two sets of brothers showed exon 22 mutation. The association amongst the mutations in exon 22 and severity of haemophilia have been given in Table 3. Majority of patients that had severe (23.07%) ailment showed a mutation of P-value 0.888.

Mutations in exon 23 were noticed in 2 patients, along with their mothers. Thus, Table 4 indicates the correlation between the severity of haemophilia &

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	city
1	Carrier	Exon 18	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- rate
			exon 23			
2	Patient	Exon 23	6533A>T,	Point mutation		Severe
5	Carrier	Exon 23	6539G>A,	=	GAT>AAT	-
			6546C>A	=		
			6509G>A	Point mutation		
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	Frameshift		-
2	Patient	Exon 24	165658delA	Frameshift		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- rate
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,	Frameshift	TGC>TCG,	Severe
			6592A>T	Point mutation	CAC>CCC	
			6701A>C	Point mutation		
2	Patient	Intron 22	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

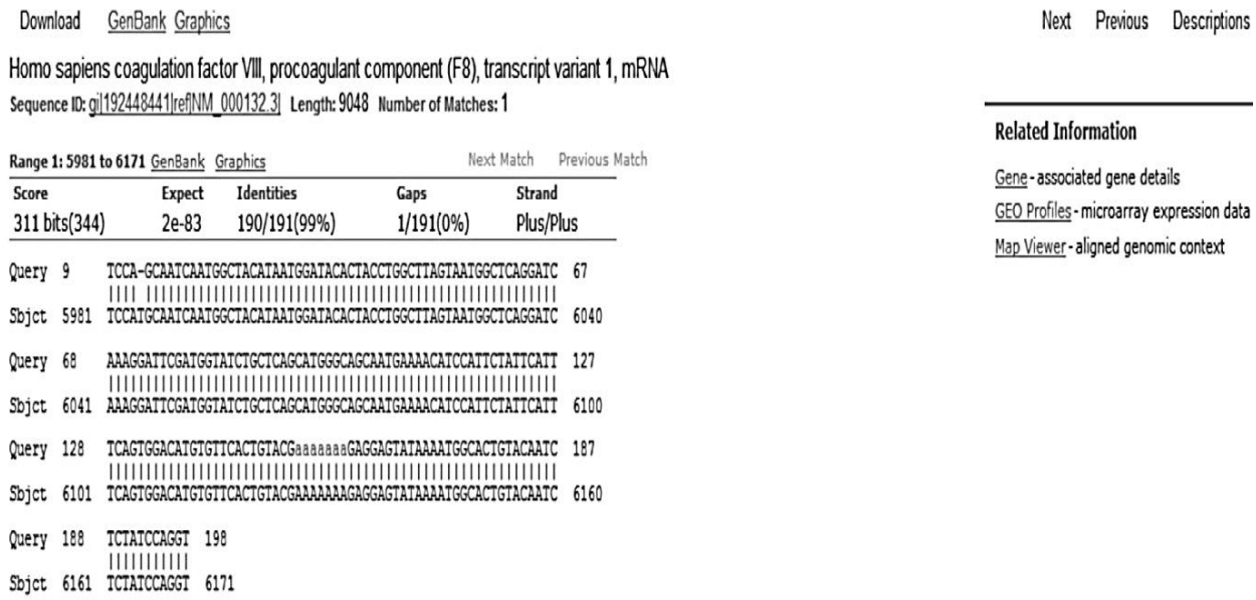

Figure 2: Alignment of a Haemophilia Patient's Exon 18 Sequence with the Reference Gene

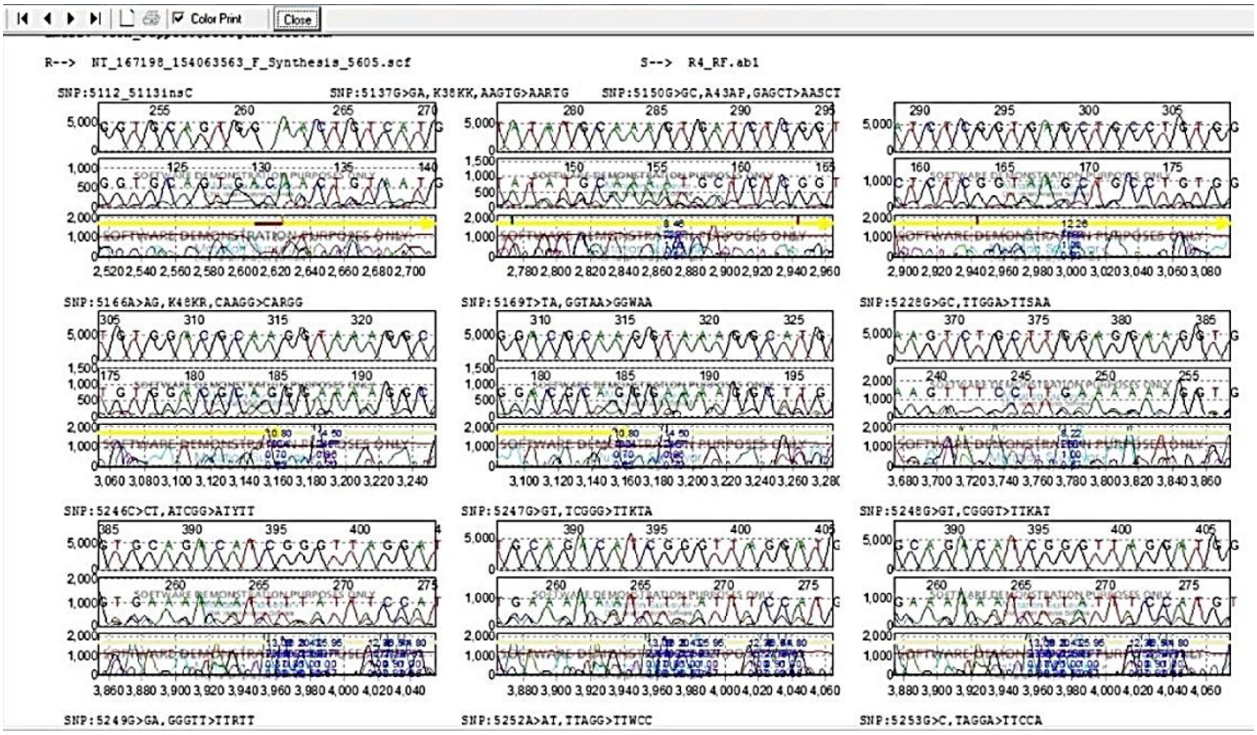

Figure 3: Alignment of Different Exon Sequences with the Reference Gene in Haemophilia Patients and Controls

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

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

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

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

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

*Depending on d.f 1, the valuable statistic of Pearson's chi-square is 0.065.
*The null hypothesis (probability level) showed no significance at p=0.352.

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 Pearson' chi-square statistic value is 6.785 based on d.f 1.
*The probability level (nullhypothesis) is significant at 0.009.

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

*Relying on the d.f.1, the value of Pearson's chi-square statistics is 4.406.
*The null hypothesis probability level is significant at P= 0.36.

mutations in exon 23. The 2 patients of severe sickness (15.38%) had a 0.352 P-value mutation.

The mutations in exon 24 were observed in twelve haemophilic patients with 2 mother carriers.

Table 5 demonstrates the linkage between mutations in exon 24 and haemophilia severity. Mostly patients of severe illness (84.6%) indicated a mutation of 0.009 P-value, represents the relation among mutation in intron 22.

Mutation frequency

In this research work, 4 different mutations were identified in the 18 Iraqi patients of haemophilia A from seven discrete families.

Various mutations were identified through mutation detection method comprising 7 inversions, 11 point mutations in which 10 were linked to the severe disease phenotype, 6 insertion mutations in which one was related to the moderate phenotype and 6 frame shift mutations.

Out of the 35 recognized mutations, 6 various mutations were observed between exons inspected such as 24, 23 and 24 and intron 22 as well in sample 2 patient.

In three samples of patients (4, 6 and 11), multiple different mutations were found. While in two different exons of a sample of patients 19, 14 and 9 showed two various mutations and three mutations were detected in sample 22's 24 exons as given in Table 7.

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	Insertion	
12	Exon 24	165897G> GC	Insertion	Severe
14	Exon 24	165897G>GC	Insertion	Severe
	Intron 22	Inth22	Insertion	
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	Frameshift	Severe
		6592A>T	Point mutation	
		6701A>C	Point mutation	
23	Exon 22	6408 G>A		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	Point mutation
5	Exon 23	6509G>A	
8	Exon 23	6668G>A	Point mutation
	Exon 24	165897delG	Frameshift

Besides, the detection of two frame shift mutations in two different exons was observed in the sample carrier, whereas, two various exons showed two different mutations in sample 8 carrier, as indicated in Table 8.

DISCUSSION

The haemophilia severity was classified, rendering the FVIII activity as following: thirteen male patients. Showed 1% severe type FVIII activity whereas five male patients indicated moderate 1-5% of FVIII activity. However, females are merely carriers and might contain genetic defects. Thus, FVIII activity can identify the genetic defects that impact males. Due to the lyonization phenomenon, the clinical and plasma estimations of female carriers do not produce apparent diagnosis (Sun *et al.*, 2015). The authorization of lionization phenomenon for the expression of only one allele of genes located at dynamic X chromosome.

As a result, women articulate alleles from both their fathers and mothers and every X chromosome expresses about 50% of the genes (Amos-Landgraf *et al.*, 2006), it also offers adequate security against the ailment. The mutations were identified in exon 18 in one carrier mother and one male. The investigated 3 out of the 18 Italian patients demonstrated this mutation through the study of Bicocchi *et al.* (2005). The 1 out of the eighty-three patients had eighteen mutations recognized by Youssoufian *et al.* (1986).

The current study designated the mutations in exon 23 in two patients and their mothers. 1 of 240 patients expressed mutations in exon 23 in North Carolina in a research conducted by Youssoufian *et al.* (1988) while, 4 patients showed mutations in 24 exons. Moreover, 3 of the 281 evaluated haemophilic British patients contained twenty-four mutations in a study carried out through Green *et al.* (2008).

The association among the severity of ailment and mutations in intron 22 has been presented in Table 6. Distinctively, 53.84% which represented the 7 out of 13 patients having severe haemophilia; this is the highest reported rate when related to the other researches of the severe haemophilia A. The most widespread type taking place in the worldwide haemophiliacs is the mutations in intron 22. In particular, the inversion mutations of intron 22 are commonly the HA-causative mutations observed with 40%-45% of severe haemophiliacs whereas, only 2-5% of the cases if severe HA are occurred because of the inversions of intron 1 (Nair *et al.*, 2004). All such inversions instigate in the germ cells of males due to the pairing of Xq with its homologous chromosome. During female meiosis, these would most probably restrain the intrachromosomal recombination (De Brasi *et al.*, 2000). Thus, it is acknowledgeable that introns do not encode the proteins rather are detached from the molecule of DNA in the transcription through the spliceosomes' actions (Chorev and Carmel, 2012).

The inspection of all exons in recent study specified their location in the mature protein FVIII light chain, that is composed of domains C1, C2 7 A3 (Kane and Davie, 1986), as well as the intron 22, is situated among the non-coding regions of exons 23 & 22. All these exons' mutations direct to the flaws at the levels of translation or transcription or alterations in the FVIII proteins' single amino acids resulting in the deficiency of the FVIII (Jacquemin, 2009). The development of inhibitor is at higher risk as the mutations are to be found in the light chain rather than they are positioned outside of the FVIII proteins' light chain for instance in the domain B, A2 or A1 (Levinson *et al.*, 1992).

The analysis of the family studies has highlighted utmost mutations in the patients of haemophilia A instigate in the germ cells of males based on the significant occurrence of point mutations (Leuer *et al.*, 2001). In the current study, two various mutations comprising 3 frameshift and 2 point mutations were detected in seven female carriers.

The investigation of families harbouring several mutations has repeatedly stated while disagreeing the severity of phenotypes. As a result, the incidence of the quite a few mutations exhibits the significance of extra DNA examination in patients with detected mutations having strange clinical indications and infrequent phenotypes. Due to such problems, multiple mutations have direct inference for the genetic counselling and genetic diagnosis (Shetty *et al.*, 2011).

The outcomes of this research confirmed that all of the double mutations happened merely in severe phenotype patients. Moreover, the utmost communal mutations of the FVIII gene were followed in order through inversion mutations, frameshift mutations & insertion mutations. Thus, the presence of the two mutations has a collaborative impact on FVIII activity instead. However, the single mutation patients demonstrated uppermost activity of the FVIII (Bayele *et al.*, 2010). Further, in genotyping, the two mutation's coexistence should never be barred, specifically in the dissonant clinical presentation cases.

CONCLUSIONS

The mutations in exon 24 are mainly recurrent types of mutations which takes place in the patients of haemophilia at waist Province trailed by the mutations in intron 22. Roughly all cases of severe haemophilia have mutations sited in this segment of two genes. The conclusions of this study also expressed that utmost repeated mutations FVIII gene patients were point mutations which were followed by inversion mutations, frameshift mutations & insertion mutations." Moreover, this research deliberated a strong association among the mutations of intron 22 and exon 24 & bleeding severity as well.

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Conflict of interest

The authors declare that they have no conflict of interest for this study.

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