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INVESTIGATION OF THE INHERITED MUTATIONS RELATED TO INVERSION PROCESS OF INTRON 22 IN A SAMPLE OF IRAQI PATIENTS WITH HEMOPHILIA A

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ABSTRACT

Hemophilia (also Haemophilia) is an X- linked recessive bleeding disorder, it is caused due to the deficiency of the coagulation factor eight (FVIII) causing Hemophilia A, or coagulation factor nine (FIX) causing Hemophilia B. The first type (Hemophilia A) is more frequent than the second type (Hemophilia B), representing 80 % of the total of cases of hemophiliacs. About 45% of Hemophilia A caused by inversion in intron 22 of FVIII gene. Two-third of Hemophilia casesare due to inherited mutations, therefore, patients with a family history of this disease. The aim of this study was to detection of mutations of FVIII gene in 18 families ((18 Carriers (patients' mother) and 18 patients) of hemophilia A patients. Polymerase chain reaction (PCR) and direct sequencing was performed for specific regions in intron 22 of the FVIII gene.

Keywords: Hemophilia A, Mutation, Inversion, PCR

INTRODUCTION

Hemophilia A is the most common a bleeding disorders in the men, it caused by mutations in (FVIII) gene, of which inversion mutations for about 45 % of the cases (Lakich *et al*., 1993). Coagulation FVIII gene is large gene, spans 186 kb of the DNA, and located in X chromosome (Xq28) (Husain, 2009). Factor 8 is synthesis in the liver and endothelial cells outside the liver (Kaufman, 1991). The Hemophilia A causing mutations are unique and dispersed all over the entire length of FVIII gene (Berg *et al*., 1990). In the meiosis of spermatogenesis, the recombination occurs, leading to a large inversion in exons 1-22 are translocted away from the exons (Vehar *at al*., 1984; Hay, 2010). Inversion mutations in intron 22 of FVIII gene (Naylor *et al*., 1993), account for disease in 20% of all patients, and result always sever disease, it result for homologous recombination between reputed DNA sequence (Lakich *et al*., 1993). Hemophilia A (HA) carriers identified through analyses of family pedigree, and verified by family history (Paroskie *et al*., 2015).

MATERIALS AND METHODS

This study was carried out at the Institute of Genetic Engineering and Biotechnology, University of Baghdad. A total of 36 samples [(18 Carriers (patients' mother) and 18 patients] were collected from patients with hemophilia A, in addition to 30 control samples from genetic diseases center in Thi-Qar province, south of Iraq, during the period from April, 2016 to April, 2017. The ages ranged from (4 -63) years, to detect the mutations in hemophilia A.

The data were collected, according to a questionnaire forma. Venous blood samples (5 ml) were collected from each patient and healthy control. Each blood sample was collected in to EDTA tubes for Molecular studies. PCR technique was done for all samples by using specific primers (table 1). PCR products were sequenced at Macrogen Company (South Korea).

Genomic DNA extraction: Genomic DNA was extracted using gsync DNA mini kit 100 prep (Geneaid, Taiwan). PCR was performed using AccuPower® PCR pre-Mix (Bioneer, South Korea). The extracted DNA puritywas measured by ND-2000 spectro-photometer (Thermo Scientific Inc., USA). The extracted DNA was stored at

Table 1: Sequences of primers used in this study

Set	Name of primers	Sequence of primers	Annealing Temp.	PCR Product size
1 a	**F** primer 1 (***F8***) in 22 **R** primer 1 (***F8***) in 22	5′- TTCCGCAGGAACCGCTTCTT -3' 5′- CGCTGGTTCTGAGCAAGT CA-3'	**54 ºC**	**730 bp**
2 b	**F** primer 2 (***F8***) in 22 **R** primer 2 (***F8***) in 22	5'- GCAACACTGCAGTCATGGTC -3' 5′- TTCAGTGAGCCAGGTGGAGC-3'	**57 ºC**	**723 bp**
3 a	**F** primer 3 (***F8***) in 22 **R** primer 3 (***F8***) in 22	5'- GGC CGT CAG GTA CTC AAT AA -3' 5′- GTTGCCAGGGACTATCGGGA-3'	**57 ºC**	**801 bp**
4 c	**F** primer 4 (***F8***) in 22 **R** primer 4 (***F8***) in 22	5'- CCTATAAGCAGTCACTTGCC -3' 5′- TGGTACTGCCATCGTGATCG-3'	**66 ºC**	**1662 bp**

a: Specific primers designed in this study; b,c: Specific primers used according to Naylor *et al.* (1995).

PCR Procedure: PCR was carried in Veriti™ thermo-cycler (Applied Biosystem) using the standard cycle procedure. Predenaturation at 95 °C for 5-minutes, then 30 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 66°C, 57°C and 54°C depending on the primers (table 1), 30 seconds extension at 72 °C and 5 min for final extension at 72 °C. PCR products were then analyzed by sequencing.

PCR products sequencing: The PCR products were sent to Macrogene Company (South Korea) for sequencing. Sequences were analysed by using snap gene program to detect the mutations.

Results and discussion: The study of int22h-1 was covered by fourteen overlapping PCR assays (Naylor *et al*,1995). In this study, selected middle region of Int22h-1, approximately 3595 bp, using four primers (1, 2, 3 and 4) to amplify this region was coducted (Figure 1).

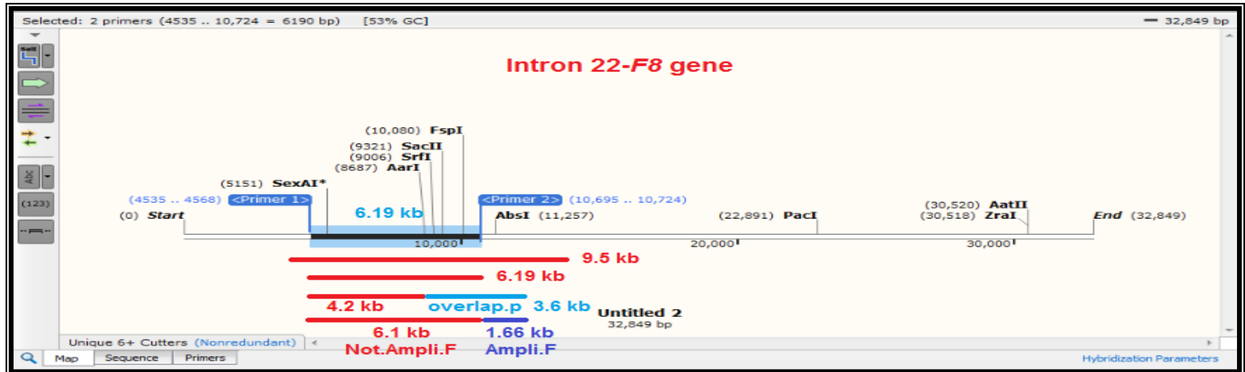

Figure 1: Intron 22 of F8 gene with region (3595 bp) overlapping primers, primer 1, 2, 3, and 4 tables (1)

The primers (1, 2 and 3) gave a positive result with the control group while no results obtained with the patients group as shown in Figure 2.

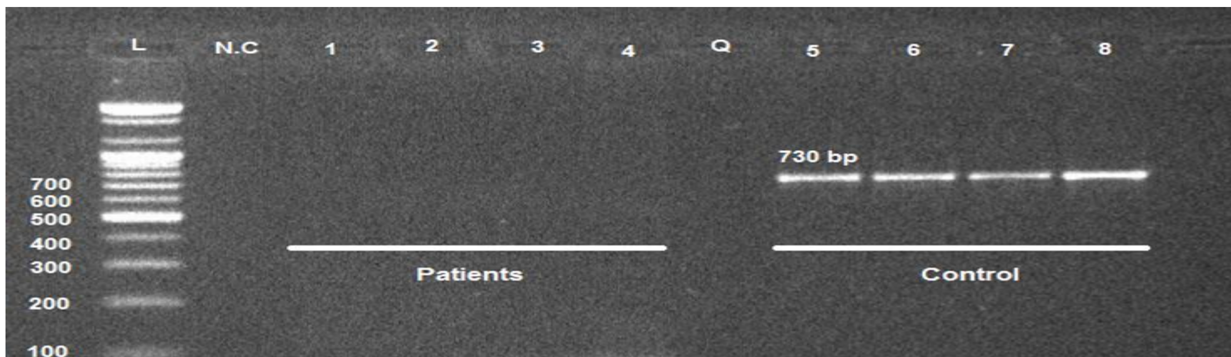

Figure 2: PCR product (730 bp). The electrophoresis was done on 2% agarose gel at 70 V for 75 min; L= DNA ladder =100bp; N.C=negative control. Lane (1-4) patients. Lane (5-8) control.

A successful amplification of the fourth primer was obtained with the control and the patients groups. The amplified bands were 1662 bp (from 138, 163 bp to 139, 825 bp) within 9.5 kb of int22h (F8 gene) as shown in Figure 3.

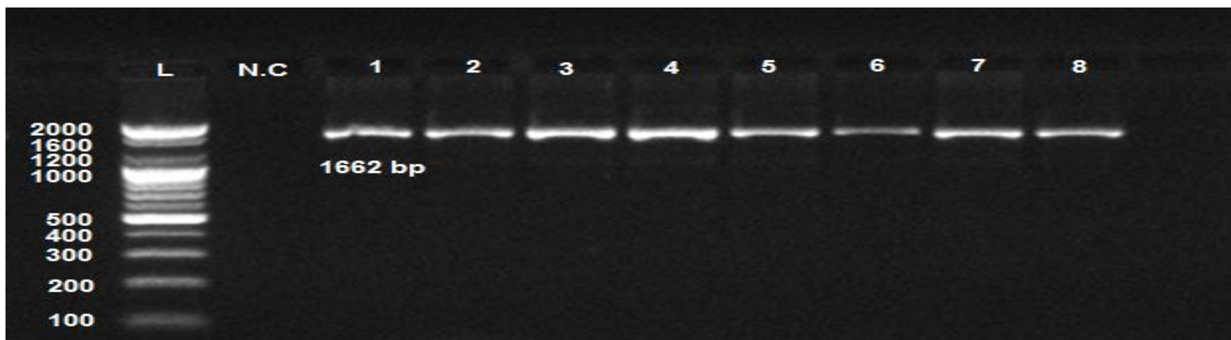

Figure 3: PCR product (1662 bp). The electrophoresis was on 2% agarose gel at 70V for 75 min; L= DNA ladder =100bp; N.C=negative control. Lane (1-8) patients

Mutations in F8 gene lead to produce truncated protein that leads to the disruption of F8 function and suppress the normal protein interaction with another proteins involved in the coagulation cascade. The results showed the deletion, insertion and substitution mutation occurred in intron 22 of HA gene for all patients. These results indicate that mutations may constitute a risk of developing hemophilia A disease (Figure 4).

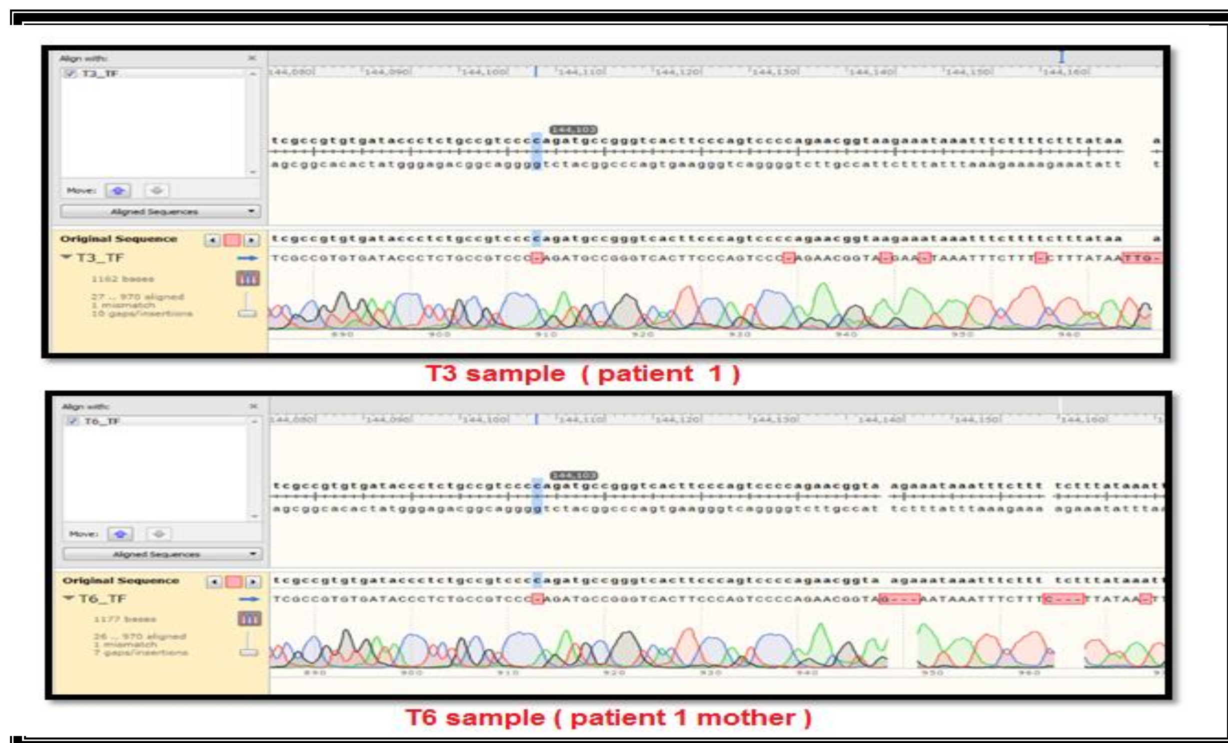

Figure 4: Sequence analysis for mutation identification in patient 1 and his mother (carrier).

Bowen (2002) explained that the factor 8 mutations can occur at diverse sites in a variety of types such as structural variation (inversion of intron 22) and sequence variation (insertion, deletion and substitution, latter variation) leads to the nonsense, missene and frameshift mutation. A large deletions and insertions are among F8 defects, the major cause of frame-shifts is associated with severe Hemophilia A, Sukarova *et al.,* (2001). In this study, thirty-six mutations were detected in intron 22 (with 9.5 kb). These mutations were substitution, deletion and insertion (Table 2).

Table 2: Location, Numbers, Frequency and Percentage of mutations in F8 gene

Mutations	Locatin	No. of Mutation	frequency	Percentage of frequency
g.143276 G > A g.144365 T > A	Intron 22	2	14	38.8 %
g.144369 T > G	Intron 22	1	13	36.1 %
g.144426 G > A	Intron 22	1	12	33.3 %
g.144406 G > A	Intron 22	1	10	27.7 %
g.144363 G > A	Intron 22	1	9	25.0 %
g.144404 T > A g.144408 G > A	Intron 22	2	8	22.2 %
g.144368 G > T g.144437 *ins* T g.144440 T > G	Intron 22	3	7	19.4 %
g.144295 *ins* C g.144304 T > G	Intron 22	2	6	16.6 %
g.144304 T > A g.144338 T > A g.144352 G > A	Intron 22	3	5	13.8 %
g.144335 A > C g.144431 A > C g.144432 C > A	Intron 22	5	4	11.1 %

g.144448 A > C g.144453 *ins* T				
g.143988 *del* G g.144103 *del* A g.144350 T > A g.144362 A > C g.144403 *ins* G g.144433 A > T g.144442 T > G g.143577 T > A g.143595 T > A g.143598 C > T g.143625 C > T g.143630 C > T g.143648 T > G g.144376 *ins* G g.144380 T > G	Intron 22	15	3	8.3 %
Chi-Square	---	---	---	9.416 **
** (P<0.01).				

According NCBI, all mutations listed in table (3) were novel mutations. Most studies about this intron, were done by using a long-PCR assay, while this study was done by using overlapping PCR assay, for detecting mutations in this region by using more than one primer to amplify a long fragment up to three thousand of nitrogen bases.

Some of mutations occurred in high frequency, such as g.143276 G> A, g.144365 T > A, g.144369 T > G, g.144426 G > A, g.144406 G > A. A large number of mutations within a relatively low region may indicate the presence of fragile fragments of DNA in this region, which explains the occurrence of inversion in this homologues region. This leads to the occurrence of hemophilia A. The statistical analysis showed a high significant difference (P<-0.01) between these mutations. Two of the samples (T9 and T13) showed a mutation at site g.144380. Because of this mutation, the base T was substituted by G base (ATGT changed to AGGT). The sequence AGGT represents a cutting site of splicing process between the exon and intron that leads to separate the exon from intron (remove all introns for mRNA synthesis). This type of unregulated splicing process is called (Retained intron). This may lead to a fusion in Part of intron 22 with the next exon 23. This mutation was inherited from the patient to his daughter at the same site.

Matching between the patients and their mothers was done by NCBI. The results showed several mutations, most of which were identical between patients and their mothers (carriers) and with most of the patients. Thirteen of 18 carrier's samples were identical in some mutations with their sons, within the studied region (Table 3).

Table 3: Mutations that passed from patients mothers (carrier) to their sons (patients)

No.	Mother sample	Trier son sample	No. Hereditary mutation	Percentage of frequency
1	T 3	T 6	3	16.6 %
2	T 4	T 7	0	0.0 %
3	T 5	T 8	0	0.0 %
4	T 10	T 11	8	44.4 %
5	T 12	T 13	6	33.3 %
6	T 14	T 15	3	16.6 %
7	T 16	T 17	7	38.8 %
8	T 18	T 19	2	11.1 %
9	T 20	T 21	4	22.2 %
10	T 22	T 23	5	27.7 %
11	T 24	T 25	0	0.0 %
12	T 26	T 27	4	22.2 %
13	T 28	T 29	0	0.0 %
14	T 30	T 31	4	22.2 %
15	T 32	T 33	3	16.6 %
16	T 34	T 35	3	16.6 %
17	T 36	T 37	4	22.2 %
18	T 39	T 39	0	0.0 %

Rossiter *et al.,* (1994) indicated that although most patients with hemophilia have family history of the disorder, about one in three cases is acquired through spontaneous genetic mutation. Therefore, the proportion 72.2% of hemophilia was inherited from the mother (inherited mutation, with family history) while 27.8% were new (spontaneous) genetic mutations.

CONCLUSION

The result showed that mutations were identified in 13 out of 18 patients' mothers (carriers) with their children, the proportion 72.2% of hemophilia was inherited from the mothers (inherited mutation, with family history), while 27.8% were new

(spontaneous) genetic mutations and this agreed with many international studies. These mutations may be caused by the fragile fragments that lead to inversion presses that are responsible for about 45% of hemophilia A causes.

REFERENCE

Berg, L., Wieland, K. and D. Millar, Detection of a novel point mutation causing haemophilia A by PCR/direct sequencing of ectopically-transcribed factor VIII mRNA. Hum. Genet. 85: 655-658 (1990).

Bowen, D. Haemophilia A and B. Haemophilia, Molecular insights. J. Clin. Pathol. 55: 1–18 (2002).

Hay, C., National Hemophilia Databases. Textbook of Hemophilia, Second Edition Pp. 415-9 (2010).

Husain, N., Carrier analysis for hemophilia A: ideal versus acceptable. Expert Review of Molecular Diagnostics 9(3): 203–7 (2009).

Kaufman, R.J., Insight into the structure, function, and biosynthesis of factor VIII through recombinant DNA technology. Annals of Hematology 63(3): 155 165 (1991).

Lakich, D., Kazazian, H., Antonarakis, S. and J. Gitschier, Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. Nature Genetics 5(3): 236-41 (1993).

Naylor, J., Brinke, A., Hassock, S. et al., Characteristic mRNAabnormality found in half the patients with severe haemophilia A is due tolarge DNA inversions. Hum. Mol. Genet. 2: 1773-1778 (1993).

Naylor, J., Buck, D. and P. Green, Investigation of the factor VIII intron 22 repeated region (int-22h) and the associated inversion junctions. Hum. Mol. Genet. 4: 1217-1224 (1995).

Paroskie, A., Gailani, D., Michael, R., Baun, D. and R. Sidonio, A Cross Sectional Study of Bleeding Phenotype in Haemophilia A Carriers. Br. J. Haematol. 170(2): 223–228 (2015). doi:10.1111/bjh.13423. (2015).

Rossiter, J.P., Young, M., kimberland, M.L., Hutter, P., Ketterling, R., Gitschier, J., Horst, J., Morris, M., Schaid, D., Sommer, S., Kazazian, H.H. and S. Antonarakis, Factor VIII gene inversions causing severe hemophilia A to originate almost exclusively in male germ cells. Hum. Mol. Genet. 3(7): 1035–1039 (1994).

Sukarova E., Dimovski A.J., Tchacarova P., Petkov G.H. and G.D. Efremov, An Alu insert as the cause of a severe form of hemophilia A. Acta haematologica 106(3):126-129 (2001).

Vehar, G., Keyt, B., Eaton, D., Rodriguez. H., O'Brien, D., Rotblat, F., Oppermann, H., Keck, R., Wood, W., Harkins, R. and E. Tuddenham, Structure of human factor VIII. Nature 312: 337-342 (1984).