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DETECTION OF INTRON 1 INVERSION MUTATION IN SUDANESE PATIENTS WITH HEMOPHILIA A

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ABSTRACT: *Hemophilia A is an X-linked bleeding disorder resulting from heterogeneous mutations in the factor VIII (FVIII) gene which is lead to absence or decreased function of coagulation factor VIII. About 72 patients regularly come to hemophilia center of Khartoum we find all study group are male two patients are intron 1 inverted mutation (2.7%) , 59 patients (81.9%) factor VIII activity is less than 1%, 13 patients (19.1%) factor VIII activity from more than 1 and less than 5%. About 8 patients (11.1%) are positive factor VIII inhibitor.*

KEYWORDS: Detection of Intron 1, inversion mutation, Sudanese patients, Hemophilia A

INTRODUCTION

Hemophilia A (factor VIII deficiency) is the most common hereditary disorder of blood coagulation. It is due to the absence or decreased function of coagulation factor VIII, resulting from mutations in the factor VIII gene. (Reinhold *et al*., 2007). The prevalence is 30-100 per million populations. The inheritance is sex-linked but up to 33% of the patients have no family history and result from spontaneous mutation. The factor VIII gene is situated near the tip of the long arm of the X chromosome (Xp2.6) and extremely large consisting of 26 exons. The factor VIII protein include triplicate regions A1, A2, and A3 with 30% homology with each other, a duplicated homology region C1and C2 and a heavy glycosylated B domain which is removed when factor VIII is activated by thrombin (Hoffbrand *et al*., 2001). Approximately half of the patients have missense, frame shift mutations or deletions in the factor VIII gene. In others a characteristic ‘flip-tip, inversion is seen in which the factor VIII gene is broken by an inversion at the end of the X chromosome. This lead to severe form of hemophilia A (Hoffbrand *et al*., (2001); (Hoffbrand *et al*., (2006)).

It was now recognized that inversion almost always forms during a male meiosis. It is believed that the presence of a large region of non – homology between the X and Y chromosomes during meiotic pairing may favor a misalignment and the presence of a second X chromosome with a complementary region may act as a stabilizing factor. An important clinical consequence of this observation is that when an apparently new and spontaneous case

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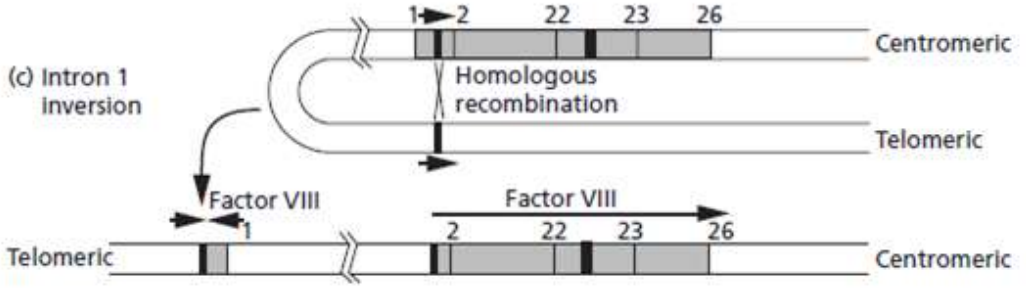
of hemophilia is diagnosed in which the gene inversion is identified, it is likely that the defect arose in the maternal grandfather's allele and thus the mother can generally be assumed to be a carrier and at risk of having another affected male child. The resulting truncated protein product is presumably unstable, resulting in severe hemophilia. The inversion is not found in individuals with mild forms of hemophilia.(Drew *et al.*, 2010).



Figure(1): Simplified representation of the gene inversion mechanisms resulting in severe hemophilia A. (Christine *et al*., 2005).

Four primers (9f, 9CR, 2F and 2R) were used for screening intron 1 inversion mutation for Intron 1 inversion PCR—Fragment 1 Int1h-1 we use primer mix 9F, 9cR and 2F and for Intron 1 inversion PCR – Fragment 2: Int1h-2 we use primer mix 2F, 2R and 9F. Performed each genomic DNA sample using conventional PCR method. Master mix was made containing long Taq polymerase deoxynucleotides sets, and buffer containing magnesium chloride (new England biolabs company) and four primers set (Applied bio systems).

Agrose gel electrophoreses:

Principle:
Agarose gel electrophoresis was the easiest and most popular way of separated and analyzed DNA. Here DNA molecules were separated on the basis of charge by applying an electric field to the electrophoresis apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process was called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide. (Tom *et al*., 2001)

Procedure:
Preparation of 0.6% agarose gel was done by 0.48g agarose in 80 ml of 0.6 x TBE was mixed and melted thoroughly in microwave, cooled to 60^{o}C. 0.5 µg/ml Ethidium bromide was added, poured in electrophoreses tank and allow setting. It was djusted according to the apparatus. Used a thin combs was improved band resolution. Then 5µl of 5x loading buffer was added to each 25 µl PCR product and 5 to10µl was added on the gel. Then 1kb ladder (from New England biolabs USA) was added, running of the gel at 90 volts for 5 hours and bands were visualized on gel using UV transilluminator and photograph through gel documentation

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systems.

Intron 1 inversion:
Four primers (9f, 9CR, 2F and 2R) were used for screening intron 1 inversion mutation, performed each genomic DNA sample using conventional PCR method. Master mix was made containing long Taq polymerase deoxynucleotides sets, and buffer containing magnesium chloride (new England biolabs company) and four primers set (Applied bio systems).

Reagent preparation:

Primer dried and preparation:
Primers set were shipped from applied biosystems company USA in concentration 10 nmol with distilled water, we made 100 µM and made aprimer mix (9 F, 9CR and 2F) and primer mix (2F, 9F and 2R) for intron 1 h1 and intron 1h-2 respectively each at 5 µM.

Procedure of intron 1 inversion:
- All reagents (New England Biolabs) were kept on ice before used.
- In sterile, clean and thin wall 0.2 ml eppindorf PCR tube volume were taken as follows:
- 5 µl of 5X Long Amp Taq Reaction Buffer was added.
- 0.75 µl of 10 mM dNTPs mix was added.
- 3 µl of 10 µM Primer mix was added.
- 1 µl of Template DNA was added.
- 1 µl of Long Amp Taq DNA Polymerase was added.
- Nuclease-free water was filled up to 25 µl.
- Mixture was spanned briefly and proceed to PCR step immediately and carried out PCR as followed:

Reaction setups:
All reaction components on ice and quickly transferring the reactions to a thermocycler preheated led to the denaturation temperature (94°C) (HAMSTeRS UK 2010).

Thermal cycle condition:
After initial denaturation for 2 minutes at 94^0C, 35 cycles of denaturation at 94^0C for 30 seconds, annealing at 55.5^{o}C for 30 seconds, and extension at 72^{o}C for 7 minutes were carried out, followed by a final extension step at 72^{o}C for 10 minutes. Polymerase chain reaction products were observed in 1% agarose gel electrophoresis.
(HAMSTeRS UK 2010).

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Table (1) intron 1 PCR primers 9 F, 9CR and 2F) and primer mix (2F, 9F and 2R)

Intron 1 inversion PCR—Fragment 1: Int1h-1	Intron 1 inversion PCR – Fragment 2: Int1h-2
9F 5'GTTGTTGGGAATGGTTACGG-3'	Int1h-2F 5'GGCAGGGATCTTGTTGGTAAA-3'
9cR 5'CTAGCTTGAGCTCCCTGTGG-3'	Int1h-2R 5'TGGGTGATATAAGCTGCTGAGCTA-3'
Int1h-2F 5'GGCAGGGATCTTGTTGGTAAA-3'	9F 5'GTTGTTGGGAATGGTTACGG-3'

(HAMSTeRS UK 2010)

RESULTS

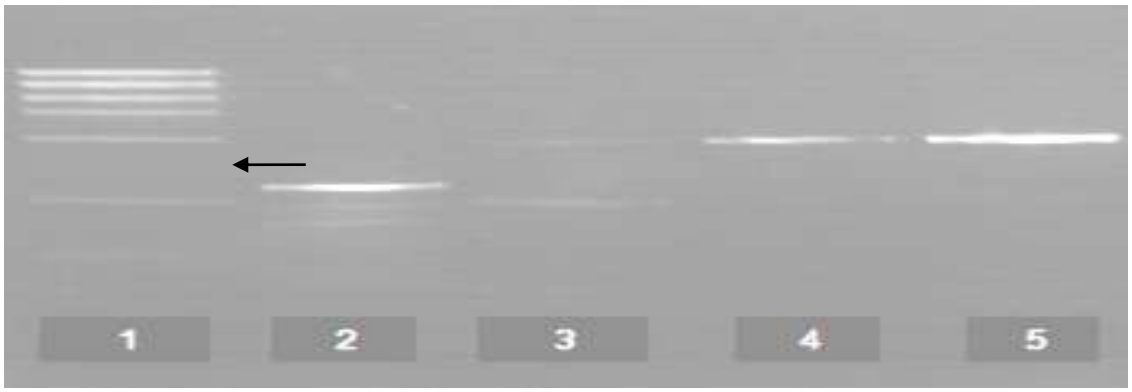

Figure (1): Polymerase chain reaction (PCR) amplification showing Intron 1 inversion in Cases with Hemophilia A , Lane 1 shows one kilo bp DNA ladder. PCR for Int1h-2 region; Lane2 wild-type control, lane 3: intron 1 inversion–positive Int1h- region 2 ; Lane 4 and 5 shows PCR for Int1h-2 region wild-type control. The arrow show 2000 bp marker.

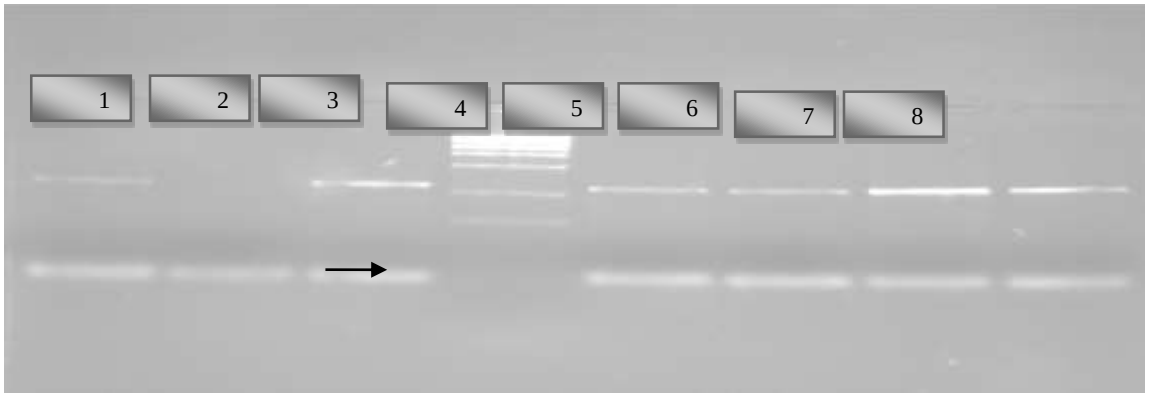

Figure (2): Polymerase chain reaction of Intron 1h1 region multiplex PCR int1h1 using primer mix (9f, 9CR and 2F) and long taq polymerase, lane 1, 3, 5, 6, 7, and 8 shows no

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inversion in int1h1, lane 2 sample without DNA and lane 4 was 1 kilo bp ladder. The arrow show 2000 bp marker.

Figure (3): polymerase chain reaction shows: lane 1 was1 kilo bp ladder, lane 2 was int1h2 region, lane 3and 4 int1h1 region, and lane 5 was mixed regions multiplex PCR with two primer set regions. The arrow show 1000 bp marker.

DISCUSSION:

The overall frequency of intron 1 inversion mutation in this study data were two severe patients 2.7%. All the remaining cases tested had the expected size of the normal bands in the first and second PCRs. This finding agreed study in Indian with two frequencies studies were reported, (Ghosh *et al.*, 2004) (Jayandharan *et al.*, 2005) detected (1.24%) three inversion positive mutations out of 241 severe cases of Hemophilia A and another study report three intron inversions out of 80 (3.7%) (Ahmed *et al.*, 2003). In the United Kingdom reported frequencies of Inv1 in FACTOR VIII gene vary from 1.8% to 4.8%, (Cumming, AM 2004). In the Czech Republic were 4.3%. (Habart *et al.*, 2003). In China 1.26% to 4.6% (Liang *et al.*, 2009) and (Feng *et al.*, 2010), Mexico, Venezuela, and Hungary the int 1 inv mutation was 0% in a certain studies while 1.5% in Argentina (1 out of 64), while the prevalence of the mutation, in Italy from 2 – 5.5% also in two different studies Table (4.8). (Clinical and Applied Thrombosis/Hemostasis 2012) (Feng *et al.*, 2010). The frequency reported in this study fall between these values, the difference between these values may be attributed to different sample size. The results of Inv1 in different series studied in 14 countries include 2963 individuals of which 69 showed Inv1, giving a worldwide mean of 2.3% (Clinical and Applied Thrombosis/Hemostasis 2012).

Recent study inversions in intron 1 of the factor VIII gene have been identified as a cause of severe hemophilia and this abnormality appears to be responsible for approximately 5% of all cases of severe hemophilia. Since approximately half of all cases of severe hemophilia are

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associated with these two inversions, it is usual practice to screen samples from new cases for these two abnormalities first.(Drew *et al*., 2010).

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REFERENCES:

1. Clinical and Applied Thrombosis/Hemostasis Nuzhat Jahan Faridi, Praveen Kumar and Nuzhat Husain Prevalence of Intron 1 Inversion of Cases With Hemophilia A in North Indian Population 2012 18: 599 originally published online 27 February 2012 DOI: 10.1177/1076029611435094.
2. Christine A. Lee, Erik E. Berntorp, W. Keith Hoots, Louis M. Aledort, Textbook of Hemophilia, chapter 4 Molecular basis of hemophilia A, 2005 p19-24 Blackwell Publishing Ltd.
3. Drew Provan, John G. Gribben- Molecular Hematology Chapter 18,the molecular basis of hemophilia p219-224 third edition(2010) by Blackwell Publishing Ltd.
4. Hoffbrand A.V, Pettit J.E, Moss P.A.H essential hematology, coagulation disorders 2001, chapter 20 p 261,262 fourth edition.
5. Feng Xue, Lei Zhang, Tao Sui, et al. Factor VIII gene mutations
profile in 148 Chinese hemophilia A subjects. Eur J Haematol. 2010;85(3):264-272.
6. Habart D, Kalabova D, Hrachovinova I, Vorlova Z. Significant prevalence of the intron 1 factor VIII gene inversion among patients with severe hemophilia A in the Czech republic. J Thromb Haemost. 2003;1(6):1323-1324.
7. Hoffbrand A.V, Pettit J.E, Moss P.A.H essential hematology, coagulation disorders, 2006, chapter 24 p 290,291 fifth edition.
8. Ghosh K, Shetty S, Mohanty D. Major disorganization of factor VIII gene as a cause of severe haemophilia A in Indian patients, Am J Haematol. 2004;76(1):96.
9. Jayandharan G, Shaji RV, Baidya S, Nair SC, Chandy M, Srivastava A. Identification of factor VIII gene mutations in 101 patients with haemophilia A: mutation analysis by inversion screening and multiplex PCR and CSGE and molecular modeling of 10 novel missense substitutions. Hemophilia. 2005; 11(5):481-491.
10. Liang Y, Yan ZY, Yan M, et al. Detection of factor VIII Intron 1 inversion in severe hemophilia A. Z honghua Yi Xue Yi Chuan Xue Za Zhi (Chinese Journal of Medical Genetics). 2009;26(3): 323-325.
11. The Hemophilia A Mutation, Structure, Test and Resource Site HAMSTeRS UK 2010.
12. Tom Maniatis, E. F. Fritsch, Joseph Sam brook, Molecular cloning-a laboratory manual, 7th edition, Cold Spring Harbor Laboratory, 1982 Primrose, S. et.al, Principles of Gene Manipulation. Oxford: Blackwell Science, 2001.
13. Reinhold M. Erhard H, Jonathan G, Ronald P, modern Hematology Biology and Clinical Management Second Edition 2007 Humana Press Inc. 999 Riverview Drive, Suite 208 Totowa, New Jersey 07512.

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