Prevalence of Intron 22 Inversions in Pakistani Hemophilic Patients

Muhammad Salman Khan¹, Muhammad Rizwan², Rubina Ghani³*, Saara Ahmad⁴, Nazia Jameel⁵

¹&²Assistant Professors, Department of Pathology, ³Associate Professor, ⁴Assistant Professor, Department of Biochemistry, ⁵Assistant Professor, Department of Community Medicine, Baqai Medical University, Karachi, Pakistan.

ABSTRACT

Background and Objectives: Hemophilia A is characterized by deficiency in factor VIII clotting activity that results in prolonged bleeding after injuries, tooth extractions, or surgery, and delayed or recurrent bleeding prior to complete wound healing. The age of diagnosis and frequency of bleeding episodes are related to the level of factor VIII clotting activity. It is caused by the mutations in factor VIII (F8) gene. In severe HA, intron 22 inversions (IVS-22) of the F8 gene is the most prevalent mutation accounting up to 50 % of all mutations. The knowledge of the pathogenetic mutation is important for i) the basis of carrier detection and ii) for the risk estimation of inhibitor formation. Material and Methods: We studied 50 unrelated patients suffering from severe HA reporting to Fatimid Hemophilia Center, Karachi. Proforma for each patient was filled. Subcycling Polymerase Chain Reaction (SPCR) was performed to detect IVS-22. Results: The bands were observed at 12 kb on the upper level and 10 kb band at lower level in which IVS-22 was absent. Whereas bands at 11 kb and 10 kb position signifies IVS-22. The number of patients with IVS-22 accounted to 44%. Conclusion: IVS-22 is the major genetic mutation for severe hemophilia A. The results were consistent with the concept that IVS-22 will always result in severe deficiency of F8. Rapid detection of this common mutation can helpfully guide the direction of molecular study in genetic counseling.

KEYWORDS: Hemophilia A, intron 22, Subcycling Polymerase Chain Reaction.

INTRODUCTION

Hemophilia A (HA) is the most common type of hemophilia (85%). It occurs in all racial groups and can be found worldwide with a typical incidence rate of 1: 5,000-7,000 live births. HA is characterized by deficiency in factor VIII (F8) clotting activity that results in prolonged bleeding after injuries, tooth extractions, or surgery, and delayed or recurrent bleeding prior to complete wound healing. The age of diagnosis and frequency of bleeding episodes are related to the level of F8 clotting activity [1].

In severe hemophilia A, spontaneous joint or deep-muscle bleeding is the most frequent symptom. Individuals with severe hemophilia A are usually diagnosed during the first two years of life; without prophylactic treatment, they may average up to two to five spontaneous bleeding episodes each month. Individuals with moderate hemophilia A seldom have spontaneous bleeding; however, they do have prolonged or delayed bleeding after relatively minor trauma and are usually diagnosed before age five to six years; the frequency of bleeding episodes varies, usually from once a month to once a year. Individuals with mild hemophilia A do not have spontaneous bleeding episodes; however, without pre- and postoperative treatment, abnormal bleeding occurs with surgery or tooth extractions; the frequency of bleeding episodes varies widely, typically from once a year to once every ten years. Individuals with mild hemophilia A were often not diagnosed until later in life [1].

In any individual with HA, bleeding episodes may be more frequent in childhood and adolescence than in adulthood. Approximately 10% of carrier females are at risk for bleeding (even if the affected family member is mildly affected) and are thus symptomatic carriers, although symptoms are usually mild. After major trauma or invasive...
procedures, prolonged or excessive bleeding usually occurs, regardless of the severity [2].

Hemophilia A and B are clinically indistinguishable from each other. Diagnosis must be confirmed by specific factor assay [3].

Objective:
The main aim for carrying out this study was to assess the frequency of IVS-22 in severe Hemophilia A patients in Karachi, Pakistan

MATERIALS AND METHODS
In our study we screened 50 patients with severe HA having F8 levels was less than 1%. All the samples were collected from Fatimid Hemophilia Centre. Informed/written consent was taken from each patient before sampling. 3 ml of whole blood samples were taken in each EDTA and Citrate Vacutainers® for DNA extraction and coagulation test.

The hemophilia A was diagnosed first from the factor VIII clotting activity. Subcycling Polymerase chain reaction (SPCR), with amplification of type specific primers allowed the rapid detection of intron 22 inversion, was performed with some modifications as described by Liu and Sommer [4].

DNA extraction
The genomic DNA was extracted from whole blood collected in EDTA tubes according to the Epicenter DNA Purification Kit (Cat No.MCD85201) procedure. The multiplex PCR was performed in a single tube using mutation specific primers.

The PCR reaction was first prepared by mixing KAPA HiFi enzyme1U/ml, GC-Buffer (5x) and 1.2 mM MgCl₂. The second mixture consisted of 0.25 mM of 7-deaza-dGTP, 0.25 mM of dGTP, 0.5 mM of other dNTPs, 7.5% DMSO, P/Q primer 0.8µM, A/B primer 0.25 µM and water, to make the volume up to 25 µl was used. The DNA sample (150 ng) was mixed with second mixture and then it was added with first mixture and the reaction cups were transferred to thermocycler quickly to prevent the premature activation of enzyme.

The thermal cycling condition consisted of initial denaturation at 94°C for 2 minutes continuing to 10 cycles of 94°C for 12 s, four subcycles per main cycle consisting of: primer annealing at 60°C for 2 minutes and extension at 65°C for 2 minutes. Remaining 16 cycles 94°C for 12 s, and four subcycles per main cycle consisting of 60°C for 2 minutes plus an additional 3 s added per cycle and 65°C for 2 minutes plus an additional 3 s added per cycle. The final extension was 72°C for 7 minutes.

Electrophoresis
Fifteen micro liters of the PCR products were removed and mixed with 3 µL of a loading buffer and then loaded on 0.6% agarose gel. The gel was set at 85volts for 5 hour and then stained with ethidium bromide. After staining, the bands were visualized when observed under UV light.

RESULTS
In this study, 50 unrelated patients were included who were suffering from severe hemophilia A. Upon mutational analysis using Subcycling PCR technique, 22 (44%) of the patients were found to have IVS-22 (table 1). The samples of the patients who have IVS-22 typically showed an 11 kb band and 10 kb band. Those patients who are negative for IVS-22 showed 12 kb band and 10 kb band which signifies the absence of inversion 22. The banding patterns are shown in figures 1-4.

| Table: 1 Frequency of intron 22 inversions identified hemophiliac patients |
|-----------------------------|---------------|----------------|
| IVS-22 n=50                | Frequency     | Percentage (%) |
| Yes                        | 22            | 44.0           |
| NO                         | 28            | 56.0           |
| Total                      | 50            | 100.0          |
Figure: 1 The 0.6 % agarose gel shows the IVS-22 detected in lane 1-3, 6, 12, 13 and 16. Lane M is 1 Kb marker

Figure: 2 The 0.6 % agarose gel shows the IVS-22 detected in lane 1-5, 10, 11 and 14-16. Lane M is 1 Kb marker

Figure: 3 The 0.6 % agarose gel shows the IVS-22 detected in lane 6, 7 and 16. Lane M is 1 Kb marker
DISCUSSION
The primary objective was to detect IVS-22 in Pakistanis suffering from Hemophilia A. When DNA analysis was performed, typical two bands at 10 Kb and 11 Kb level were observed which was specific for intron 22 inversion and these were 44% present out of 50 patients. 56% patient showed normal pattern having band position at 10 Kb and 12. In our study the occurrence of intron 22 inversions in Pakistani hemophilics patients has been found to be comparable to that reported in other countries [5, 6, 7, 8, 9, 10, 11, 12 and 13].

Pan, Tzu-Yu et.al in 2014 also reported that intron 22 inversion of factor VIII gene (F8) causes 40–50% of severe bleeding disorder of Hemophilia A, in all human populations. Consequently, identification of the disease-causing mutations is becoming increasingly important for accurate genetic counseling and prenatal diagnosis [14]. In his study, the key steps of inverse-shifting polymerase chain reaction (IS-PCR) and of short-end injection capillary electrophoresis were used for more specific and rapid genotyping of intron 22 inversion of F8.

Molecular genetic testing of F8, the gene encoding factor VIII, identifies pathogenic variants in as many as 98% of individuals with hemophilia A [15].

CONCLUSION
Our study revealed that IVS-22 is the major genetic mutation responsible for severe hemophilia A patients in Karachi, Pakistan. There is a high probability of de novo mutation of this genetic change. We found 44% of severe hemophiliacs have IVS-22. Our study suggests that SPCR is a robust and valuable procedure to detect IVS-22 in severe hemophilia A patients. It provides information about IVS-22 in 24 hours and is less laborious as compared to Southern Blotting Technique which takes about one week, laborious and requires handling of radioactive chemicals. The only disadvantage of this procedure is that it does not discriminate between Type I and Type II of IVS-22.

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REFERENCES
7. Reitter S.;Sturn R.;Horvath B.;Freitag R.; Male C;Muntean W.; Streif W.;Pabinger I.; Mannhalter C;


*Corresponding author: Dr Rubina Ghani
E-Mail: rg.musavvir33@hotmail.com