Original article

Molecular Analysis of Factor VIII (F8) Gene Mutations and Its Detection in Libyan Patients with Hemophilia A

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Abstract
Hemophilia A is an X linked recessive hemorrhagic disorder caused by mutations in the F8 gene that lead to qualitative and/or quantitative deficiencies of coagulation factor VIII (FVIII). Molecular diagnosis of hemophilia A is challenging because of the high number of different causative mutations that are distributed throughout the large F8 gene. Molecular studies of these mutations are essential in order to reinforce our understanding of their pathogenic effect responsible for this disorder. The present study aimed to obtain data on sequence variation in F8, a set of functionally validated control chromosomes of Libyan descent; we have performed for the first time molecular analysis of F8 in 43 Libyan hemophilia patients. This study included 63 cases 43 patient, 10 Carrier, and 10 Control, Amplifying of F8 by using PCR technique, and identification of mutations by using restriction enzyme Taq I for exon 23. Our results showed the presence of partial deletions in two brother patients in exon 23 this result had been concluded refer to areas that failed to be amplified by PCR. Our study may contribute to the knowledge on the risk factors of inhibitor development that allows libyan clinicians to assess whether an individual patient is at high risk.

Key words: Factor VIII, Hemophilia A, PCR, Restriction enzyme, Exons 23, 24, and 26 A+B, Molecular analysis

Introduction

The X-linked coagulation disorder hemophilia A (HA) is caused by mutations in the factor 8 (F8) gene, this gene encodes coagulation factor VIII (FVIII) and is located at Xq28, in the end of the long arm of the X chromosome, telomeric region (Alan et al.1987).The patients with severe hemophilia A have a factor VIII (FVIII) activity level of less than 0.01 IU/ mL. Severe hemophilia has changed from a debilitating disease to a condition with a good quality of life (Dorain *et al.*2003; Santacroce *et al.*2008). Studies show great advances in efficacy and safety of FVIII products and treatment strategies have made this possible, Factor VIII (FVIII) is an essential blood-clotting protein, also known as anti-hemophilic factor (AHF) (Plug *et al*.2008).The F8 gene mutation type is the most important predictors of the risk of inhibitor development in hemophilia A (Scwaab *et al.* 1995; Ter *et al*.2008).

Gouw *et al.* (2012) reported that the meta-analysis confirm earlier reports that the F8 genotype is an important determinant of inhibitor development in patients with severe hemophilia A. The inhibitor risk in large deletions and nonsense mutations was higher than in intron 22 inversions, the risk in intron 1 inversions and splice site mutations was equal and the risk in small deletions/insertions and missense mutations was lower.

Coagulation factor VIII is made chiefly by liver sinusoidal cells and endothelial cells throughout the body (Kumaran *et al*. 2005). This protein circulates in an inactive form in the bloodstream and bound to the other molecule called von Willebrand factor, until occurs damages in blood vessels. When injuries take place, coagulation factor VIII is activated and separates from von Willebrand factor (Sadler *et al*, 1998). The severity of hemophilia A is determined by the level of clotting activityof factor VIII in the blood (Millar *et al.* 1990).

In haemophilia A patients with the same mutation in the F8 gene show heterogenous bleeding phenotypes. Specific mutations in the A2 domain of factor VIII are associated with mild haemophilia and a higher risk of inhibitor development, so a double mutation in mild haemophilia A are rarely reported (Bakija *et al*.2005).

In general Hemophilia A is a single gene disorders, this group is determined primarily by a single mutant gene and follows the Mendelian pattern of inheritance. Hemophilia is caused by mutation in the F8 gene. Hundreds of mutations in this gene have been identified. These span a diverse range of mutation types, namely, missense, splice-site, deletions of single and multiple exons, inversions, etc. The gene for factor VIII was cloned in 1984. The factor VIII gene size is 186 kb and contains 26 exons and 25 introns. The exon length varies from 69 to 262 nucleotides except for exon 14 which is 3106 nucleotides long and exon 26 which has 1958 nucleotides. Also there are six long introns of more than 14 kb, such as intron 22 which is 32 kb long. (Antonarakis and Kazazian 1988; Antonarakis 1995).

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Hemophilia A is also known as classic Hemophilia (Alan and David 1987; Gad *et al.*1999; Lloyd and Samuel 1985). It is the most common type of this disease, affecting 1 from 10,000 males worldwide (Gad *et al.*1999; Mannucci and Tuddenham 2001). The severity of hemophilia A is determined by the level of clotting activity of factor VIII in the blood. There are levels of severity of hemophilia, mild from 5 to 30 % of normal, moderate from1 to 5% of normal, and severe less than 1% of normal (Taq 2007). Hemophilia usually affects only males, because the hemophilia gene is carried on the same chromosome that determines whether the person is male or female, The chromosomes that determine the person's sex are called X and Y. Males have an X and a Y chromosome and Females have two X chromosomes (Plug *et al.*2008).

Materials and methods

Blood samples collection

This study included 43 Patient (40 Male, 3 Female), 10 Carrier, and 10 Control (8 Male, 2 Female) the total is 63 cases. Blood samples were collected from Libyan patients (Department of Pediatric Oncology, Tripoli Medical Center). Five ml of blood were drawn from each patient and collected in tube containing EDTA and kept in freezer (-20ºC).

The blood samples were transported to DNA Laboratory, Human's Tissues Department and the Genetic Engineering Department , Biotechnology Research Center, Twesha – Libya, for DNA Studies Amplifying of F8 by using Polymerase Chain Reaction(PCR) technique, and identification of mutations by using restriction enzyme Taq I for exon 23 , exon 24 , and exon 26 A+B for researching for mutation in Exon 23, 2147 , CGA to TGA (Arg to Stop), Exon 24 , 2209, CGA to TGA (Arg to Stop), and Exon 26 A+B, 2307, CGA to TGA (Arg to stop).

Genomic DNA Extraction and Primers

DNA was extracted from frozen blood samples by QIAamp DNA Blood Mini Kit from Qiagen. Oligonucleotide primers used to amplify exons of factor VIII are listed in table (1).

Amplification of F8 gene by Polymerase Chain Reaction (PCR) condition**:** The PCR was performed in a final volume of 50µL containing 100ng genomic DNA, 125µM each dNTP, 200 nM of primers 1.5MM MgCl2 and 1 U of Taq DNA polymerase (Invitrogen). The 30 -40 cycle amplification program was carried out in a thermocycler T100 Thermal cycler BIO-RAD (Table1).The steps of PCR were as follows: denaturation, 30 sec at 95 ºC , annealing 30 sec at 50-60 ºC, extension 30 sec at 72 ºC and final extension step 6 min at 72 ºC (Saiki *et al.*1988; Saiki *et al.*1985).

Restriction enzyme analysis

The PCR products from the amplification were digested with Taq 1. PCR reaction mixture 10 µL (~0.1-0.5 µg of DNA),nuclease-free water 18 µL,10X Buffer TaqI 2 µL, TaqI 1-2 µL, Incubate under paraffin oil in a capped vial at 65°C for 1-16 hours. Stop the digestion reaction by adding 0.5 M EDTA, pH 8.0 (#R1021), to achieve a 20mM final concentration. Mix thoroughly, add an electrophoresis loading dye and load onto gel. The digests were electrophoresed on 2% gelelectrophoresis and the restriction enzyme pattern was visualized after staining the gel with ethidium bromide (Table2) (Taq1 BIORON GmbH).

Table 1: Oligonucleotide primers for PCR Analysis of Factor VIII.

Exon	5' Primer	3' Primer	Size of PCR fragment (bp)
23	5'- GAAGGAAGATATGATTGACAGA -3'	5'- AACTAGAACAGTTAGTCACC -3'	258
24	5'- ATAACTGAGGCTGAAGCATG -3'	5'- GTAGATCTGTTGCCTCTTACC -3'	242
26(A+B)	5'- GCTTTGCAGTGACCATTGTC -3'	5'- AGCTGAGGAGGGAGAGGTGA -3'	192

Table 2: Restriction Enzyme used in this study.

Name Restriction Enzyme	Source of Restriction Enzyme	Recognition Sequence	Number of Cuts in F8 Gene
Taq I	Thermus aquaticus bacterium BIORON GmbH	5'…T ^ CGA…3' 3'…AGC ^ T…5'	87 position

Results

Analysis of three Taq I sites in exons 23, 24 and 26 (A+B) in studied patients at Two patients with a moderate form of the disease demonstrated an abnormal electrophoretic pattern in exon 8 and sequencing by DNA Sequencing Capillary Electrophoresis from Applied Biosystem and analyzed by SeqScape Software, this software demonstrated missense mutations at codon 2147 for arginine within a thrombin activation site. These mutations were C to T transitions changing a CGA codon for arginine to a TGA termination codon, Results had been reached in this study showed the presence of tow partial deletions in two brother patients in exon 23 had been conclude refer to areas that failed to be amplified by PCR (Figure 1, 2 and 3). Previous studies showed that Partial deletion in

exons of FVIII gene refer to do not amplify by PCR (Table3).conclude refer to areas that failed to be amplified by PCR.

Table 3: Previous studies show that Partial deletion in exons of FVIII gene refer to do not amplify by PCR .

Exon	Severity	References
23	Moderate	J-M Lavergene et al. (1992)
24	Moderate	J-M Lavergene et al. (1992)
23-24	Moderate	J-M Lavergene et al. (1992)
23-26	Sever	J-M Lavergene et al. (1992)
26	Sever	J-M Lavergene et al. (1992)
24-25	-	Vidal et al. (2002)
25	-	Vidal et al. (2002)
25-26	-	Vidal et al. (2002)
23	mild	In this study

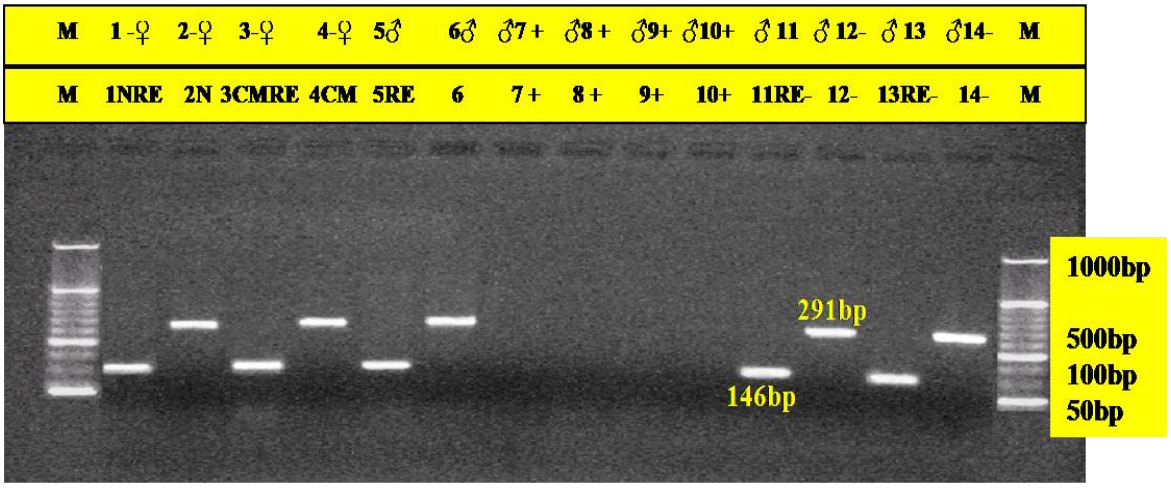

Figure 1: Detection of the Partial deletion [Exon 23, codon 2147, (Arg ⟶ Stop)] by Taq I digestion of the 291bp of amplified fragment to 145bp and 146bp this case is normal, but when does not cut is abnormal case. Lane M: DNA Marker. 7, 8,9,10 No PCR Product caused by Partial Deletion. Lanes: 1, 2,3,4,5,6,11,12,13,14 are normal for the nonsense mutation of codon 2147, (Arg ⟶ Stop). (PP=PCR Product; RE= Restriction Enzyme; N=Normal; CM=Carrier Mother; (+) With Mutation; (-) Without Mutation; ♀=Female; ♂=Male, *Taq* Icut innormal allele.

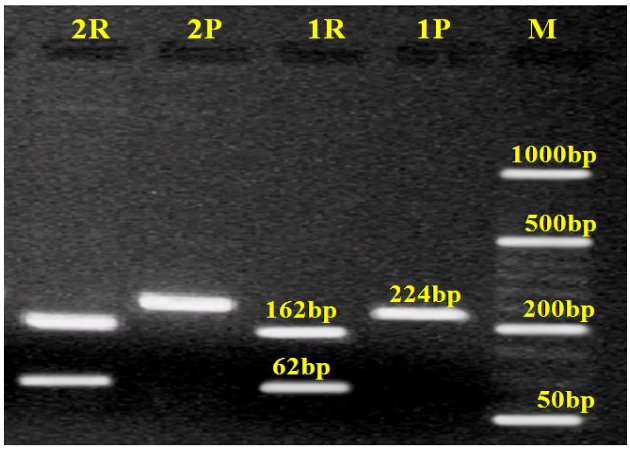

Figure 2: Gel electrophoresis of the amplicons of exon 24 For FVIII gene, size of PCR Productis 224 bp and Taq I restriction digestion gives two fragments. The size of the first fragment is 62 bp and the second is 162 bp. Lane M DNA Ladder (50-1000 bp); Lane 1P/N ♂; 1R/N-♂; 2P/H♂; 2R/H- ♂. P=PCR Product; R= Restriction Enzyme; N=Normal Case; H=Hemophiliacs; (-) Case Without Mutation;♂=Male; *Taq* Idigested innormal allele.

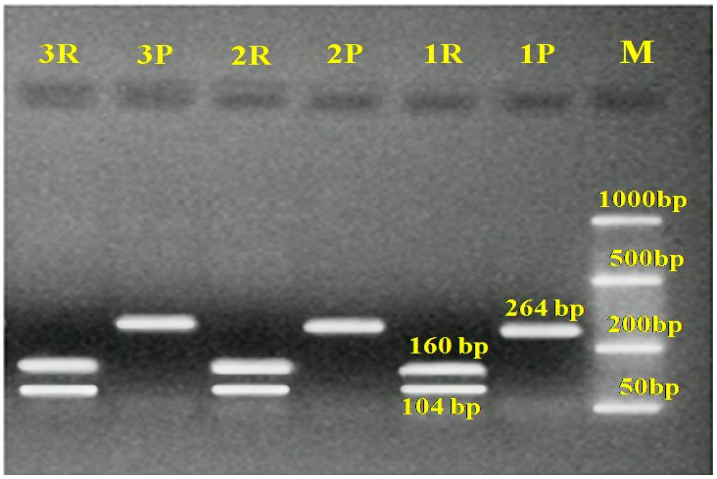

Figure 3: Gel electrophoresis of amplicon of exon 26 (A+B) of FVIII gene. Size of PCR Productis 264 bp and Taq I restriction digestion gives two fragments. The size of the first fragment is 104 bp and the second is 160 bp.(The DNA sequencing by Sequencing Capillary Electrophoresis from Applied Biosystem and analyzed by SeqScape Software, this software) Lane M DNA Ladder (50-1000 bp); Lane1P/N ♂; 1R/N-♂;2P/H♂; 2R/H- ♂; 3P/H ♂; 3R/H- ♂. P=PCR Product;R= Restriction Enzyme; N=Normal Case; H=Hemophiliacs; (-) Case Without Mutation; ♂=Male; *Taq* Idigested innormal allele.

The patients divided into three groups according to severity 19 Severe, 8 Moderate and 16 Mild (Table 4). People with hemophilia A often bleed longer than other people, bleeds can occur internally, into joints and muscles, or externally, from minor cuts, dental procedures or trauma. How frequently a person bleeds and the severity of those bleeds depends on how much FVIII is in the plasma, the straw-colored fluid portion of blood.

Normal plasma levels of FVIII range from 50% to 150%. Levels below 50%, or half of what is needed to form a clot, determine a person's symptoms. Mild hemophilia A 5% up to 30% of FVIII in the blood. People with mild hemophilia Agenerally experience bleeding only after serious injury, trauma or surgery. In many cases, mild hemophilia is not diagnosed until an injury, surgery or tooth extraction results in prolonged bleeding.

Moderate hemophilia A 1% up to 5% of FVIII in the blood. People with moderate hemophilia A tend to have bleeding episodes after injuries; bleeds that occur without obvious cause are called spontaneous bleeding episodes. Severe hemophilia A <1% of FVIII in the blood, people with severe hemophilia A experience bleeding following an injury and may have frequent spontaneous bleeding episodes, often into their joints and muscles (Pavlova and Oldenburg 2013).

Results had been reached in this study showing the presence of partial deletions in tow brothers patients in exon 23had been concluded refer to areas that failed to be amplified by PCR, the sequencieng of this exon confirm the presence of this tow mutions.

Discussion

The present study is concerned with screening of some Libyan studied patients with hemophilia A and the aim of the study was to identify CGA to TGA mutation by Taq I restriction enzyme in exons 23 , 24 , and 26 A+B for the factor VIII (F8) gene that causes hemophilia A among Libyan patients . Amplification of areas that contain restriction enzyme sites such as Taq I with CG dinucleotides in their recognition sequence are especially useful locations to begin a rapid screeing of hemophiliacs for mutations. Analysis of the five Taq I sites in exons 18, 22, 23, 24, and 26 shows that there are three nonsense mutations in patients HA579, HA360 and HA735 at codons 1941, 2116, and 2209, respectively (Lavergne *et al.*1992).

These mutations were C to T transitions changing a CGA codon for arginine to a TGA termination codon. The patients all suffered with severe hemophilia (Lavergne *et al.*1992). In this study no mutation of three Taq I sites in coding region of factor VIII gene have an in-frame CGA codon for arginine. In other studies it was found that partial deletions involving exons 23, 24, 23-26 and 26 are resulted in severe hemophilia A cases (Gitschier *et al.*1986; Youssoufian *et al.*1987; Lozier 2004). Exons 24-25, 25, 25-26, 23and 24 failed to amplify in patients. This is the first example of a moderate form of hemophilia A associated with a deletion involving two exons (Lavergne *et al.*1992) in this study partial deletions for mild hemophilia A in exon 23 of the factor VIII for two patients of 43 patients i.e approximately 4.65% of causative mutation in exon 23 failed to amplify in two brother patients.

These data may contribute to the knowledge on the risk factors of inhibitor development that allows libyan clinicians to assess whether an individual patient is at high risk. We performed study of various types of F8 mutations and we obtained more precise estimates of the relative risks according to the F8 genotypes in patients with hemophilia A.

Further investigation will be continue this study on patients from our Hemophilia Treatment Center in order to reinforce our understanding in the molecular defect of hemophilia A in Libya and to set up counseling genetic and the prenatal diagnosis for Libyan families with hemophilia A. Our study provides more precise estimates of the relative risks for different F8 genotypes

relative to exon 24 in libyan patients with Hemophilia A. In future research on other possible genetic and nongenetic risk factors, the predisposition related to the hemophilic genotype should be taken into account. The epigenetics study and gene gene interaction may open new approaches, will facilitate a study about implication of factor 8 gene in the ethiology of hemophilia. The design of antagonistic inhibitors of the factor VIII system (Lozier 2004; Fang *et al.*2007) could provide novel anticoagulants for the treatment of thrombotic disorders (Lavigne *et al.*2005).

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