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Molecular genetic study of hemophilia B in an Algerian population

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Hemophilia B is inherited as x-linked recessive disorder, carried by females, where males are affected. Rare cases of females affected with hemophilia B are known. This is also known as factor IX (FIX) deficiency, or "Christmas disease", originally named after Stephen Christmas; the first patient was described with this disease in 1952. It is characterized by spontaneous or prolonged hemorrhages due to factor IX deficiency. Factor IX mutations have not been previously reported in Algerian patients. To understand the molecular basis of hemophilia B in Algeria, polymerase chain reaction (PCR) and direct sequencing have been applied to be the important regions of the factor IX gene from 11 patients; we identified 2 point mutations. Mutations identified in our patients was linked with disease severity. Complications are problems that develop during treatment of the disease. Inhibitor (alloantibodies to exogenous factor XI) development is currently the most significant treatment complication. In this study, we evaluated the relationship between inhibitor development and FIX gene mutation types. In summary, our preliminary results will be used to build an Algerian mutation database which would facilitate genetic counseling.

Key words: Hemophilia B, factor IX gene, mutation, inhibitors.

INTRODUCTION

Hemophilia B (Christmas disease) (HB) is a hereditary x-linked recessive bleeding disorder of coagulation caused by mutation in factor IX (FIX) gene which result in FIX deficiency. The FIX gene spans about 34 kilo bases (Kb) of genomic DNA and is located on the long arm of the X chromosome at Xq27.1 (Yoshitake et al., 1985; Bowen, 2002). This gene contains eight exons. FIX mRNA

comprises of 2.8 kb (Anson et al., 1984; Yoshitake et al., 1985).

Mutations in this gene has been mostly expressed in males. Low activity of plasma FIX is necessary to confirm a diagnosis of HB. Based on the plasma levels of FIX, HB is classified as severe (<1%), moderate (1 to 5%) and mild (>5 to 30%) and the severity of the disease

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is in close correlation with the type and position of the mutation in FIX gene (Lillicrap,1998). Currently, 1095 mutations in the FIX gene have been identified worldwide, among which 73% are point mutations, 16.3% are deletions and the rest are insertions, duplications, or combinations of insertions and deletions (Rallapalli et al., 2013).

The main treatment for hemophilia is called replacement therapy. Inhibitor (alloantibodies) development is currently the most challenging complication of therapy. The nature of the mutation in the FIX gene is linked to a genetic predisposition of developing inhibitors.

The objective of this study were, to identify the mutations that produce different forms of HB disease among Algerian patients, to characterise mutations of the FIX gene and to develop our knowledge about the molecular basis of this disease.

MATERIALS AND METHODS

Four families including 11 patients (9 patients with severe HB and 2 with the moderate form) of Algerian origin were enrolled in this study. Informed consent was obtained from each family and all the patients were male. The participants were from Batna region, cared from the association of hemophilia of the University Hospital, Batna.

The blood sample was taken from venipuncture into evacuated tubes containing trisodium citrate (0,109 M) as an anticoagulant. The tests performed activated partial thromboplastin time (aPTT), FIX activity and FVIII activity. The severity of the disease was classified based on FIX activity. Clotting factor inhibitor screening was performed by activated partial thromboplastin time mixing studies using normal pool plasma.

DNA extraction

About 5 to 10 ml of peripheral blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) ; the DNA was extracted by salting out method (Miller et al., 1988) and stored at 4°C. This method comprises the following steps: lyses cells with detergents that will disperse the lipid bilayers of membranes and denature proteins, especially those associated with DNA in chromatin. Elimination of proteins are done using a supersaturated NaCl solution. The denatured proteins form a precipitate, while the DNA remains in solution in the aqueous phase which is recovered after centrifugation. Concentration of DNA by precipitation with alcohol (isopropanol) are added to the aqueous phase, dried and redissolved in TE⁻⁴ (Tris-EDTA) buffer.

Quality and quantity of DNA are estimated by spectrophotometry. The method of spectrophotometry is used for precise quantification of DNA. Thus, the DNA concentration are determined by, measuring the optical density (OD) in spectrophotometer at the wavelength 260 nm knowing that the DNA has a maximum UV absorption spectrum at 260 nm.

PCR technique

Most of the exons in the HB gene are relatively short. Direct sequencing of a complete exon are possible once it has been amplified from genomic DNA with a PCR technique therefore, all exons of the FIX gene are amplified by PCR (Wulff et al., 1995). Primers are listed in Table 1.

Polyacrylamide gel electrophoresis

To determine if amplification has responded to criteria expected (specificity, efficiency and absence of contamination), electrophoresis on acrylamide gel was performed. The electrophoresis technique is based on the separation of negatively charged nucleic acids under the effect of an electric field.

A polyacrylamide gel is a separation matrix used in electrophoresis. It consists of acrylamide which is the base unit and bisacrylamide which is the bridging agent. Depending on the different levels of these two substances, different meshes and thus different gel densities are obtained. The polymerization reaction was accomplished by the addition of two reactive substances: tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) which, upon reaction with light, become hyperactive reactive anions triggering the polymerization. The amplicons are separated according to their size; the smaller fragments will migrate faster.

The separation gel was 8%, after the electrophoretic migration, the gel was illuminated under ultraviolet light in order to observe the fluorescent DNA bands due to the Ethidium bromide and photographed with a digital camera. Although the color of the fluorescent DNA is orange-red, the photographs are published in black and white.

Sequencing

The HB mutation was identified by automated sequence analysis performed on an 3500 XLDX Genetic Analyzer (Applied Biosystems) using the Capillary electrophoresis method. The migration support is a liquid polymer (POP7) containing the separation polymer, salts required for migration and urea as a denaturing agent.

RESULTS AND DISCUSSION

All patients had a prolonged aPTT, low FIX activity and normal FVIII activity. None of the patients had inhibitor against FIX during the substitution treatment. Mutations were identified in 9 patients with an exception of 2 patients with FIX activity levels of 2% (Table 2). In all, 2 FIX gene mutations were detected, a point mutations (2 missense mutations). Sequencing of exon 4 of FIX gene helped in identifying 7 patients with a substitution G> A, at position 323 of exon 4 (c.323G> A). Consequence of this, is a change in the second base of codon TGT to TAT responsible for the substitution of a cysteine with a tyrosine at position 108 (p.Cys108Tyr) (Figure 1A). Sequencing of exon 8 of a FIX gene, helped to identify 2 cases in a substitution of G> A at position 881 of exon 8 (c.881G> A). Consequence of this, is a change in the second base of codon CGA to AAC responsible for the substitution of an arginine with a glutamine at position 294 (p.Arg294Gln) (Figure 1B).

This study is the first molecular analysis of Algerian HB patients. Identification and characterization of the molecular basis of HB was conducted from the region of Batna. In Algeria, the number of hemophiliacs founded in 2006 was 1128 a prevalence of 3.76 / 100 000; 82.81% are type A and 16.82% are type B (Belhani, 2009). HB occurs in approximately 1 in 30,000 male live births (Biocchi et al., 2002). For the woman, the abnormality of

Table 1. Primers for mutation analysis of FIX gene.

Exon	Primer sequence	Noun	Product size (base pair "bp")
Exon 1	5'-AGTCCAAAGACCCATTGAGG- 3' 5'-GACTCTTCAATATTGCTGTCAAATC- 3'	HEMBex1-M13	321
Exons 2-3	5'-TGCCCTAAAGAGAAATTGGC- 3' 5'-TGGGTTAGAGGGTTGGACTG- 3'	HEMBex2-3M13	637
Exon 4	5'-GAGGACCGGGCATTCTAAG- 3' 5'-CCAGTTTCAACTTGTTCAGAGG- 3'	HEMBex4-M13	236
Exon 5	5'-CAAAATTTCTCTCCCAACG- 3' 5'-GGTCTAATTCAAGCTACTGATATTTTC- 3'	HEMBex5-M13	411
Exon 6	5'-TGTAATACATGTTCCATTTGCC- 3' 5'-TAGCCTCAGTCTCCACCTG- 3'	HEMBex6-M13	342
Exon 7	5'-TTTCTGCCAGCACCTAGAAG- 3' 5'-ACCCTTCTGCCTTTAGCCC- 3'	HEMBex7-M13	353
Exon 8a	5'-GCCAATTAGGTCAGTGGTCC- 3' 5'-CTTCATGGAAGCCAGCAC- 3'	HEMBex8a-M13	458
Exon 8b	5'-TGTAAGTGGCTGGGGAAGAG- 3' 5'-TGAGAGGCCCTGTTAATTTTC- 3'	HEMBex8b-M13	388

Table 2. Mutations found in three Algerian families diagnosed with haemophilia B.

Patients	FIX activity Percentage (%)	Region	Nucleotide change	Amino acid substitution	Domain
Familie 1	<1	Exon 4	c.323G>A	p.Cys 108 Tyr	EGF1
Familie 2	<1	Exon 4	c.323G>A	p.Cys 108 Tyr	EGF1
Familie 3	<1	Exon 8a	c.881G>A	p.Arg 294 Gln	SP
Familie 4	2	-	-	-	-

EGF1, Epidermal growth factor 1; SP, Serine protease.

the gene located on one of the X chromosomes is generally compensated completely or partially by the other healthy X chromosome. As a result, women are rarely affected by this disease and only few examples of these women with HB have been reported (Ingerslev et al., 1989 ; Wollina et al., 1993 ; Lillicrap, 1998).

Some women will have hemophilia in exceptional situations; when the father is haemophiliac and the surrogate mother and when the woman undergoes a phenomenon of lyonization or inactivation of the X chromosome (she has an abnormal X chromosome and a normal X but inactive from the point of view of the manufacture of the coagulation protein). Databases that register HB mutations indicate a total of 1095 (Rallapalli et al., 2013).

Point mutations are the most common gene defect and are present in approximately 90% of HB patients (Nazia et al., 2008). This findings concurs with a number of other studies (Bowen, 2002; Morteza et al., 2009; Rallapalli et al., 2013; Pei-Chin et al., 2014; Tengguo et al., 2014; Surin et al., 2016). The majority of point mutations that have been reported in HB patients are missense and

nonsense (Nazia et al., 2008). According to Mannucci and Franchini (2013) missense mutations are prevalent in severe HB patients, this results are in concordance with all previous studies.

In our study, 2 point mutations (missense mutations) have been identified. The first was c.323G>A (Cys 108 Tyr) located in the epidermal growth factor1 (EGF1) domain; this is coded by exon 4, which has been implicated in binding to factor VIIIa (Rees et al., 1988; Hughes et al., 1993; Lenting et al., 1996). Cys108 is a buried residue in a beta sheet region of a FIXa structure. The substitution involves a conserved cysteine residue that forms a disulphide bridge. Breakage of this disulphide bridge may have serious effects on the stability of the domain. The second revealed missense mutation 881G>A (p.Arg 294 Gln) within the protease domain, the greater part (codons 280–451) is coded by the largest exon in the FIX gene (exon 8). According to Nazia et al. (2008), serine protease domain is responsible for cleaving FX to FXa, thus predicting a severe phenotypic expression of HB. Similar findings were observed in our results; both mutations located in

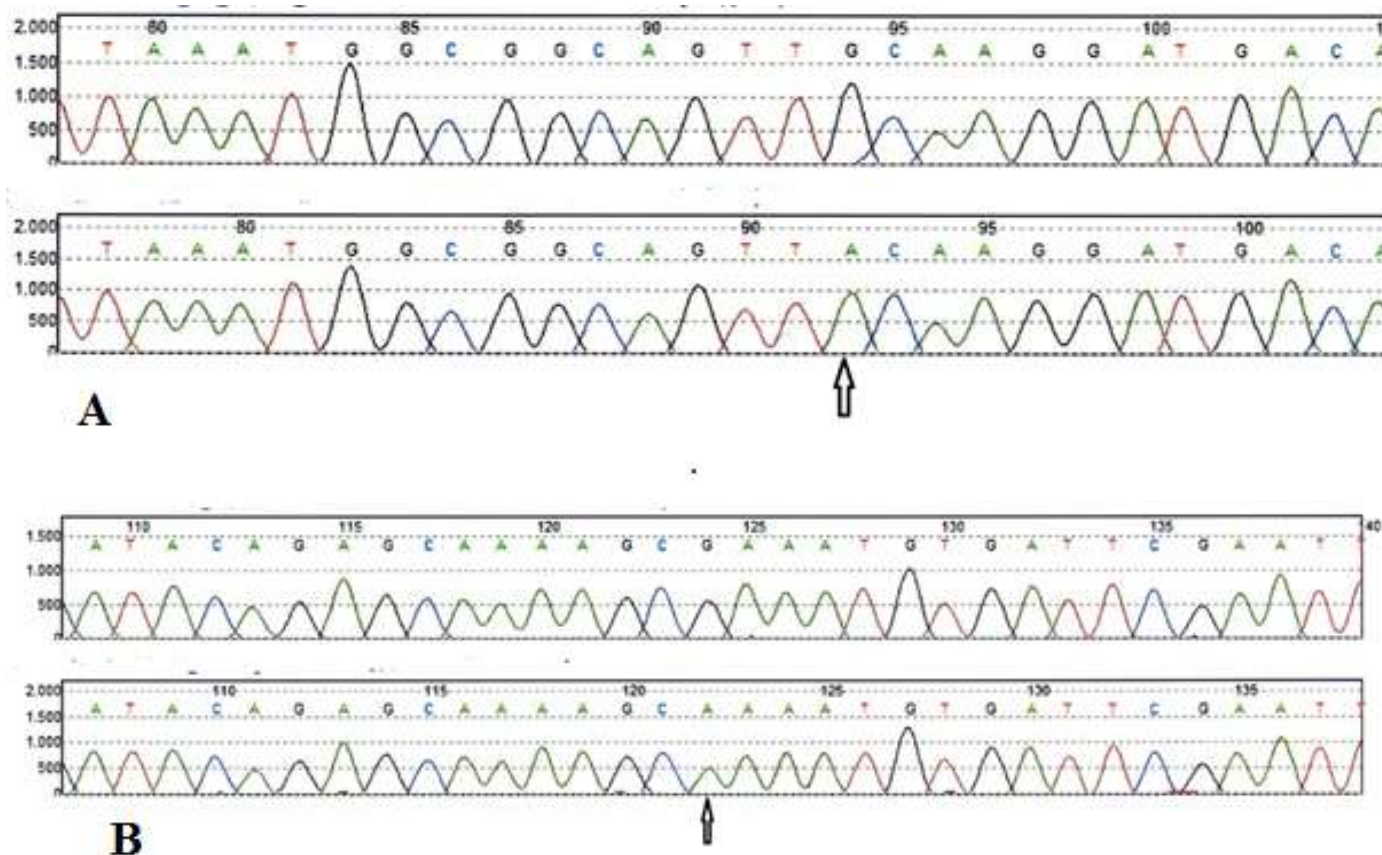


Figure 1. A. A point mutation (missense mutation) leading to the substitution of the 108th amino acid residue cysteine to tyrosine detected by sequencing analyses in a Algerian male patient with HB. **B.** A point mutation (missense mutation) leading to the substitution of the 294th amino acid residue arginine to glutamine detected by sequencing analyses in a Algerian male patient with HB.

EGF1 and serine protease domains were associated with a severe form. In general, there is good correlation between the type of mutation (location in the amino acid position and domain in the protein) and their functional outcome, yielding a predictable clinical severity (Ana et al., 2012).

The frequency of mutation c. 323 G> A is the highest in our patients; it was identified in 7 patients out of 11. This is explained and confirmed by the presence of maternal kinship between these patients. According to Guérois (2009), we find most of the time in the same family, the same type of hemophilia (either B or A) and the same degree of severity. This is generally related to the transmission of the same mutation among several individuals of the same family. Severe, moderate and mild forms of HB resulting from the replacement of Arg-294 by glutamine have also been reported earlier (Rallapalli et al., 2013). The second mutation (p.Cys 108 Tyr) is rare; which was previously found in three patients from United Kingdom, Germany, and USA and the last is in moderate form (Rallapalli et al., 2013).

No mutation was detected in two cases. The absence of mutations may be due to large rearrangements of the

FIX gene or due to a promoter mutation. The preferred treatment for hemophilia is factor replacement therapy. One of the complications of replacement therapy is the development of inhibitors. No FIX inhibitor was observed in either patient. Inhibitors are much less common in patients with HB than in those with hemophilia A. In two studies reporting hemophilia from Iran, neither of patients with HB developed inhibitor (Morteza et al., 2007; Mehdizadeh et al., 2009) similar to our study. Philip et al., 2012 affirm that, a small percentage of people with HB develop inhibitors of FIX. Potentially important host-related risk factors include positive family history for inhibitors (Gill, 1999; Astermark et al., 2001), African or Latino ethnicity (Gill, 1984; Aledort and Dimichele, 1988; Addiego et al., 1994), hemophilia genotype (Oldenburg et al., 2004; Oldenburg and Pavlova, 2006), and type of FIX product used (Gouw et al., 2007).

Conclusion

In this study, we used the direct DNA sequencing to detect the mutations causing HB in Algerian population.

Two mutations have been identified in the Batna (northeast Algeria) population and c.323G>A is the most frequent mutation type in our patients, followed by c.881G>A. Both of these mutations were associated with a severe form, and neither the patients developed inhibitor.

The study confirms the correlation between the type of mutation and severity form. Absence of inhibitors was similar to findings in other populations. The importance of molecular analysis, for the characterization of HB in patients was observed, where HB is the result of a genetic error. Identifying this error is very important for patients because, it helps to confirm the diagnosis and predict complications of treatment.

Conflicts of Interests

The authors have not declared any conflict of interests.

REFERENCES

- Addiego JR, Kasper C, Abildgaard C, Lusher M, Hilgertner M, Glader B, Aledort L, Hurst D, Bray G (1994). Increased frequency of inhibitors in African American hemophilia A patients. *Blood* 84(1):239.
- Aledort LM, Dimichele DM (1998). Inhibitors occur more frequently in African-American and Latino haemophiliacs. *Haemophilia* 4:68.
- Ana RJ, Claudia PB, Isaura AG, José de Jesús LJ, Hilda LZ, Johanna MMC, Jessica NMA, Mayra JVG (2012). Genotype-Phenotype Interaction Analyses in Hemophilia. *Hemophilia* pp. 15-32.
- Astermark J, Berntorp E, White GC, Kroner BI, MIBS Study Group (2001). The Malmo International Brother Study (MIBS): further support for genetic predisposition to inhibitor development in hemophilia patients. *Haemophilia* 7:267-272.
- Belhani M (2009). Epidémiologie de l'hémophilie en Algérie. *Alg. Hematol.* 1:32-35.
- Biocchi MP, Pasino M, Bottini F, Lanza T, Mori PG, Acquila M (2002). Mutation analysis is an essential strategy in the genetic counselling of sporadic hemophilia B families. *Haemophilia* 8:730-732.
- Bowen DJ (2002). Haemophilia A and haemophilia B: molecular insights. *Mol. Pathol.* 55:127-144.
- Gill FM (1984). The natural history of factor VIII inhibitors in patients with hemophilia A. *Prog. Clin. Biol. Res.* 150:19-29.
- Gill JC (1999). The role of genetics in inhibitor formation. *J. Thromb. Haemost.* 82:500-504.
- Gouw SC, van der Bom JG, Auerswald G, Carmen EE, Ulf T, Marijke B (2007). Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood* 109:4693-4697.
- Guérois C (2009). L'hémophilie aujourd'hui. *Kinesither Rev.* 88:32-36.
- Hughes, PE, Morgan G, Rooney EK, Brownlee GG, Handford P (1993). Tyrosine 69 of the first epidermal growth factor-like domain of human factor IX is essential for clotting activity. *J. Biol. Chem.* 268(24):17727-17733.
- Ingerslev J, Schwartz M, Lamm LU, Kruse TA, Bukh A, Stenbjerg S (1989). Female hemophilia A in a family with seeming extreme bidirectional lyonization tendency: abnormal premature X-chromosome inactivation?. *Clin. Genet.* 35(1):41-48.
- Lenting PJ, Christophe OD, Maat H, Rees DJG, Mertens K (1996). Ca²⁺ binding to the first epidermal growth factor-like domain of human blood coagulation factor IX promotes enzyme activity and factor VIII light chain binding. *J. Biol. Chem.* 271(41):25332-25337.
- Lillicrap D (1998). The molecular basis of haemophilia B. *Haemophilia* 4:350-357.
- Mannucci PM, Franchini M (2013). Is haemophilia B less severe than haemophilia A?. *Haemophilia* 19:499-502.
- Mehdizadeh M, Kardoost M, Zamani G, Baghaeepour MR, Sadeghian K, Pourhoseingholi MA (2009). Occurrence of haemophilia in Iran. *Haemophilia* 15(1):348-51.
- Miller SA, Dykes DD, Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16(3):1215.
- Morteza K, Sirous Z, Edward GT, Nafiseh N, Manijeh L, Peter G (2009). Molecular Characterization of the Factor IX Gene in 28 Iranian Hemophilia B Patients. *Iran. J. Blood. Cancer.* 2:43-47.
- Morteza K, Sirous Z, Nafiseh N, Edward GD, Manijeh L, Reza S (2007). Identification of factor IX mutations in Iranian haemophilia B patients by SSCP and sequencing. *Thromb. Res.* 120:135-139.
- Nazia N, Rashid H, Khalid M, Gulzar Niazi (2008). Molecular Basis of Hemophilia B in Pakistan: Identification of Two Novel Mutations. *World J. Med. Sci.* 3(2):50-53.
- Oldenburg J, Pavlova A (2006). Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* 12(6):15-23.
- Oldenburg J, Schröder J, Brackmann HH, Müller-Reible C, Schwaab R, Tuddenham E (2004). Environmental and genetic factors influencing inhibitor development. *Semin. Hematol.* 41(1):82-88.
- Pei-Chin L, Yi-Ning S, Yu-Mei L, Tai-Tsung C, Shih-Pien T, Hsiu-Lan S, Shyh-Shin C (2014). Efficient detection of factor IX mutations by denaturing high-performance liquid chromatography in Taiwanese hemophilia B patients, and the identification of two novel mutations. *Kaohsiung J. Med. Sci.* 30:187-193.
- Philip CJ, Sarkar BRS, Kumar LCS, Prathip BR, Amardeep P (2012). Factor IX deficiency (Christmas disease). *Med. J. Armed Forces India* 68:379-380.
- Rallapalli PM, Kemball-Cook G, Tuddenham EG, Gomez K, Perkins SJ (2013). An interactive mutation database for human coagulation factor IX provides novel insights into the phenotypes and genetics of hemophilia B. *J. Thromb. haemost.* 11:1329-1340.
- Rees DJ, Jones IM, Handford PA, Walter SJ, Esnouf MP, Smith KJ, Brownlee GG (1988). The role of beta-hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IX. *J. Eur. Mol. Biol. Organ.* 7(7):2053-2061.
- Surin VL, Demidova EY, Selivanova DS, Luchinina YA, Salomashkina VV, Pshenichnikova OS, Likhacheva EA (2016). Mutational Analysis of Hemophilia B in Russia: Molecular_Genetic Study. *Russ. J. Genet.* 52(4):466-473.
- Tengguo L, Connie HM, Jennifer D, Amanda BP, Dorothy E, Hooper WC (2014). Mutation analysis of a cohort of US patients with hemophilia B. *Am. J. Hematol.* 89(4):375-379.
- Wollina K, Bowen DJ, Syrbe G, Zintl F (1993). Female twins with severe Christmas disease (hemophilia B). *J. Thromb. Haemost.* 70(5):774-776.
- Wulff K, Schröder W, Wehnert M, Herrmann FH (1995). Twenty-five novel mutations of the factor IX gene in haemophilia B. *Hum. Mutat.* 6: 346-348.
- Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K (1985). Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 24:3736-3750.