

Mutations Causing Haemophilia B: Identification of a Rare Mutation in the EGF1 Domain of Coagulation Factor IX in Patients with Haemophilia B in the BATNA Region (Northeast Algeria)

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Abstract

Haemophilia B (HB) (also known as Christmas disease) is a form of haemophilia characterized by a factor IX (FIX) deficiency leading to spontaneous or prolonged haemorrhage. It is inherited as x-linked recessive disorder which affects almost exclusively men. Depending on the nature of the genetic mutation that is the cause of the disease, the affected coagulation factor may be totally absent from the patient's organism or present but in a dysfunctional form. Different degrees of severity of haemophilia have been established: minor, moderate, severe, based on plasma anti-haemophilic factor activity. Generally, this circulating factor level correlates with observed clinical manifestations. Molecular studies of these mutations are essential to increase our understanding of their pathogenic effect causing the disorder.

Nine male patients with severe HB, were enrolled in this study. DNA was extracted by the salting out method. Direct sequencing of a complete exon is possible once it has been amplified from genomic DNA with the polymerase chain reaction (PCR) technique. The HB mutation was identified by automated sequence analysis using the capillary electrophoresis method.

We identified 2 point mutations (missense mutations). The first identified mutation is located in exon 4 and the second is situated in exon 8.

Keywords: Aemophilia B; Factor IX gene; Mutation

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Introduction

Haemophilia is a recessive hereditary disease related to the X chromosome. It involves a decrease or even a total deficiency of FVIII (haemophilia A) or FIX (haemophilia B) of coagulation, which leads to a coagulation disorder.

HB affects all populations on the planet with a prevalence of about 1 per 50,000 people. It is about five times less common than haemophilia A. Its exact incidence in Algeria is unknown due to the lack of national epidemiological data on the number of patients, their place of residence, types of haemophilia, the treatments received.

The FIX participates in an essential stage of haemostasis, its

absence or its malfunctioning is at the origin of the formation of a clot of poor quality and the development of a haemorrhagic phenotype in the absence of adequate treatment. All localizations of the haemorrhagic syndrome are possible but the most frequently observed are located at the articular and muscular levels.

The gene responsible for the synthesis of FIX, called the FIX gene. It is located on the long arm of chromosome X in the Xq27 region and consists of approximately 34,000 base pairs [1], with eight exons interspersed with seven introns. Almost 95% of the length of the gene is non-coding. The gene encodes a pre-factor IX which will be cleaved [2,3]. It encodes a 2.8 kb mRNA [4].

The FIX is divided into several domains. At the N-terminus there

is the GLA domain with many glutamic acid residues, it is derived from the translation of exons 2 and 3. FIX has two EGF-like domains (epidermal growth factor): EGF1 and EGF2, these are encoded by exons 4 and 5. Towards the C-terminus is located the activation domain, which is encoded by exon 6, and then the catalytic domain, which is encoded by exons 7 and 8 [5].

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 [6].

The objective of this study was, 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.

Material and Methods

Three families including 9 patients with severe HB of Algerian origin were enrolled in this study. At the time of the study their age ranged between 9 and 50 years. All the patients gave informed consent for molecular studies. The participants were from Batna region, cared from the association of haemophilia of the University Hospital, Batna.

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 [7] 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 is 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 [8]. 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 colour of the fluorescent DNA is orange-red, the photographs are published in black and white.

Table 1 Primers for mutation analysis of FIX gene.

Exon	Primer sequence	Noun	Product size (base pair "bp")
Exon 1	5'-AGTCCAAAGACCCATTGAGG- 3'	HEMBex1-M13	321
	5'-GACTCTTCAATATTGCTGTCAAATC-3'		
Exons 2-3	5'-TGCCCTAAAGAGAAATTGGC- 3'	HEMBex2-3M13	637
	5'-TGGGTTAGAGGGTTGGACTG- 3'		
Exon 4	5'-GAGGACCGGCATTCTAAG- 3'	HEMBex4-M13	236
	5'-CCAGTTTCAACTTGTTCAGAGG- 3'		
Exon 5	5'-CAAAATTTCTCTCCCAACG- 3'	HEMBex5-M13	411
	5'-GGTCTAATTCAAGCTACTGATATTTTC3'		
Exon6	5'-TGTAATACATGTTCATTTGCC- 3'	HEMBex6-M13	342
	5'-TAGCCTCAGTCTCCACCTG- 3'		
Exon 7	5'-TTTCTGCCAGCACCTAGAAG- 3'	HEMBex7-M13	353
	5'-ACCTTCTGCCTTAGCCC- 3'		
Exon 8a	5'-GCCAATTAGGTCAAGTGGTCC- 3'	HEMBex8a-M13	458
	5'-CTTCATGGAAGCCAGCAC- 3'		
Exon 8b	5'-TGTAAGTGGCTGGGGAAGAG- 3'	HEMBex8b-M13	388
	5'-TGAGAGGCCCTGTAATTTTC- 3'		

Sequencing

The HB mutation was identified by automated sequence analysis performed on a 3500 XLDX Genetic Analyser (Applied Bio systems) 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

In this study, 9 haemophilia B patients from 3 families were investigated. In total we identified 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). 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) (**Table 2**).

Our studies, the first of its kind in Algeria, represent an approach for the molecular diagnosis of HB in our country. According to Belhani, in 2006, the number of haemophiliacs founded in Algeria was 1128, a prevalence of 3.76 / 100,000; 82.81% are type A and 16.82% are type B [9].

In our study, 2 point mutations (missense mutations) have been identified. The frequency of mutation (p.Cys108Tyr) is highest among our patients; it was identified in 7 patients from 9. This mutation is rare; which was previously found in three patients from United Kingdom, Germany, and USA and the last is in moderate form. Severe, moderate and mild forms of HB resulting from (p.Arg294Gln) have also been reported earlier. The genotyping associated with the inventory of previously detected abnormalities and their pathological consequences makes it possible to evaluate very often the risks incurred for a new case of severe haemophilia.

The first mutation (p.Cys108Tyr) located in the epidermal growth factor1 (EGF1) domain; this is coded by exon 4, which has been implicated in binding to factor VIIIa [10-12]. 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.

[13], serine protease domain is responsible for cleaving FX to FXa, thus predicting a severe phenotypic expression of HB.

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 [14].

According to Tailhefer [5] by studying the Gibbs free energy of the mRNA on the basis of its secondary structure, the analysis suggests that the mutations causing severe HB perturb the mRNA more interestingly. A change in structure, stability and mRNA translation rate affects the severity of haemophilia as well as the change in physicochemical properties of the substituted amino acids.

Among the mutations listed, 1094 are linked to a single molecular event which 814 (74%) are point mutations [6]. The mutations on exons 4 and 8, respectively encoding the EGF-like domain and the catalytic domain are more frequent among the mutations described in haemophiliacs B. In a more recent analysis of FIX point mutations in 1 127 haemophiliacs B, the most commonly reported substitution was guanine by adenine (28%) and more generally that of a guanine base in the first or second Codon position (46%) [15].

Haemophilic patients are of course exposed to haemorrhagic complications, the occurrence of which is largely conditioned by the degree of severity of the pathology (**Table 3**).

In our serie, arthropathies are encountered in 6 patients with the same mutation (p. Cys 108 Tyr), whose FIX <1%, reflecting the severe form. The two patients with the mutation p. Arg 294 Gln, showed no complications despite the fact that the FIX level was also <1%. The mechanisms included in the appearance of haemophilic arthropathy are still insufficiently known, but they may be multiple factors. They include inflammatory and degenerative elements [16]. Our results are in agreement with the existence of a correlation between phenotype and type of mutation, but the discussion remains difficult because of the presence of several factors that may influence this correlation. However, the results have yet to be confirmed on a larger sample.

Repetition and therapeutic insufficiency of haemarthroses lead to the appearance of subacute and then chronic arthropathy, clinically manifested by hypertrophy of the joint, amyotrophy of the underlying muscles and static disorders, and, radiologically, rearrangements of the bone ends [9]. 30 patients from the study of Belhani [9] have arthropathies of which 23/35 (66%) severe haemophilia and 7/20 (35%) moderate haemophilia, one or more joints are affected in the same haemophiliac.

Table 2 Identified causative mutations in Algerian haemophiliacs B.

Family	Number of patient	FIX activity Percentage (%)	Severity	Mutation	Type	Location	Domain
Family1	4	<1	Severe	c.323G>A; p.Cys108Tyr	Missense	Exon 4	EGF1
Family2	3	<1	Severe	c.323G>A; p.Cys108Tyr	Missense	Exon 4	EGF1
Family3	2	<1	Severe	c.881G>A; p.Arg294Gln	Missense	Exon 8	SP

EGF1: Epidermal growth factor 1; **SP** : Serine protease.

Table 3 Joints complications and complications related to the treatment of Algerian haemophiliacs B.

Family (F) / Patient (P)	Age	Mutation	Joints complications	Viral infection	Inhibitors
F1 / P1	16	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F1 / P2	19	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F1 / P3	9	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F1 / P4	27	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F2 / P1	29	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F2 / P2	15	p.Cys108Tyr	Nothing to report	No	No
F2 / P3	26	p.Cys108Tyr	Arthropathy (Knees, elbows)	No	No
F3 / P1	45	p.Arg294Gln	Nothing to report	HCV	No
F3 / P2	50	p.Arg294Gln	Nothing to report	HCV	No

HCV: Hepatitis C virus

The occurrence of an inhibitor antibody by all immunization remains the major complication of alternative therapy for haemophiliacs. This complication presents 35% of cases of severe haemophilia A and 5% of cases of severe haemophilia B according to the latest studies [17-19].

FIX inhibitors have not been observed in our patients. Inhibitors are less frequent in patients with HB than in those with haemophilia A. In two Iranian studies, none of the patients with HB developed an inhibitor [20,21], similar to our study. Philip et al. state that a small percentage of people with HB develop FIXES inhibitors [22].

Based on the data in **Table 3**, two patients born before the 1980s were infected with HCV. Most patients treated with anti-haemophilic factors of plasma origin before the late 1980s were infected with HCV [23].

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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 p.Cys108Tyr is the most frequent mutation type in our patients, followed by p.Arg294Gln. Both of these mutations were associated with a severe form. The study confirms the correlation between the type of mutation and severity form.

Our study represents only an approach on the study of the mutations of the FIX gene in Algeria. Many other mutations remain to be sought and which would probably be specific to Algeria.

Molecular analysis is important; it can confirm the diagnosis, predict certain complications of the treatment and identify with certainty the women who will transmit the disease.

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