The Use of Intron 22 Mutation in Detection of Haemophilia A Carrier in Patient's Siblings

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Abstract:

Hemophilia A is an X-linked recessively inherited bleeding disorder characterized by deficiency of pro-coagulant factor VIII (FVIII). Genetic diagnosis is the most accurate method available for carrier detection in families at-risk.

Aim of the study:

To detect intron 22 XbaI mutation in haemophilic patients and their families in order to formulate accurate molecular diagnosis of the carrier.

Methods:

88 subjects screened for intron 22 mutation using XbaI restriction enzyme with long distance PCR.

Results:

Allele frequency of positive XbaI was 46%, 23%, 40% and 27% in patients, mothers, sisters and control respectively. Expected heterozygosity was 0.35 in mothers of the patients compared with 0.39 in the female control group while 0.48 in sisters of the patients. Observed heterozygosity was 46% in mothers compared with 54% in the control while 80% in sisters.

Conclusion:

Linkage analysis with Long distance PCR of intron 22 XbaI marker is a good method for carrier detection of hemophilia A.

Keywords:

Carrier detection, FVIII, Haemophilia A, Long PCR, XbaI.

Introduction:

Haemophilia A (HA) is the most common inherited bleeding disorder caused by defects in coagulation factor VIII (FVIII). It is an X-linked recessive disorder[1]. The worldwide incidence of haemophilia A is approximately 1 case per 5000 male individuals, with approximately one third of affected individuals not having a family history[2]. In Egypt, according to Egyptian Haemophilia Society (2004): there is no complete register for haemophilic patients, but at a national level a rough estimation

indicates that the number of registered cases is about 4000[3]. Clinical symptoms consist of recurrent bleeding in multiple sites, usually in joints and muscles[4]. The severity of haemophilia is directly linked to the magnitude of coagulation factor deficiency and is generally classified as mild, moderate, or severe[5]. FVIII gene spans 186 kilo base (kb) on the long arm of the X-chromosome at Xq28[6]. Genetic diagnosis is the most accurate method available for carrier detection and prenatal diagnosis in families at-risk. Because of the large size of the F8C gene and the heterogeneous nature of its mutations, direct mutation analysis in haemophilia A families is not cost-effective. Thus, carrier-screening methods often depend on linkage analysis, a common affordable approach for genetic diagnosis, especially in developing countries[4]. Linkage analysis largely depends on the availability of informative markers.

One such marker is the XbaI polymorphism which is located in intron 22[7]; int22h-1 region (intron 22 homologous region 1) that has two other copies (int22h-2 and int22h-3) distal to the factor VIII gene[8]. The polymorphic status of the XbaI locus was studied by either Southern blot or PCR that amplified all three copies. Liu et al (1998)[9] reported additional DNA sequences in the intron 22 (int22h-1) regions that allowed the amplification of factor VIII-intron 22-specific sequences in the int22h-1 region[7]. Management of hemophilia A involves FVIII replacement. However, in Egypt, FVIII therapy/prophylaxis is difficult because it is expensive and has limited availability. Moreover, it has drawbacks like development of FVIII inhibitors and blood-transmitted infections. In order to identify the females in whom prenatal diagnosis should be carried out, it becomes important to detect carriers of hemophilia A[10].

Aim of the study:

Aim of the study was to detect intron 22 XbaI mutation in haemophilic patients and their families in order to formulate accurate molecular diagnosis of the carrier.

Subjects And Methods:

Eighty eight subjects enrolled; Group 1, twenty six cases suffering from haemophilia A, their mothers, 10 of their sisters recruited from the Hematology Clinic at New Children Hospital, Cairo University. Group 2, twenty six normal unrelated females, at same age group of the cases and with no family history of haemophilia A enrolled as control group.

The clinical diagnosis was based on detailed family history, physical examination and pedigree chart was done accordingly. Bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT), FVIII assays were carried out in all cases on the basis of FVIII activity into mild (>5%), moderate (1-5%) and severe (<1%).

Molecular study started by DNA extracted from all fresh blood samples collected from all subjects; using (AXYGEN BIO SCIENCE Catalog No. AP-MN-BL-GDNA-50), DNA amplification by PCR using Long PCR Enzyme Mix (Fermentas Life Science (Catalog NO#k0181)) and primers (AccuOligo) sequenced as forward primer (F): 5'GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC 3' and reverse primer (R):5' TTC ACC ACG ACC ACC ATC TCT CAA GTG GCC 3', detection of PCR amplification products using agarose gel electrophoresis and ultra-violet light transillumination[7], restriction enzyme cleavage using XbaI enzyme (Fermentas Life Sciences (Catalog No.#ER0681, 1500u)) lastly, detection of XbaI polymorphism in the FVIII gene using gel electrophoresis and ultraviolet transillumination (figure 1).

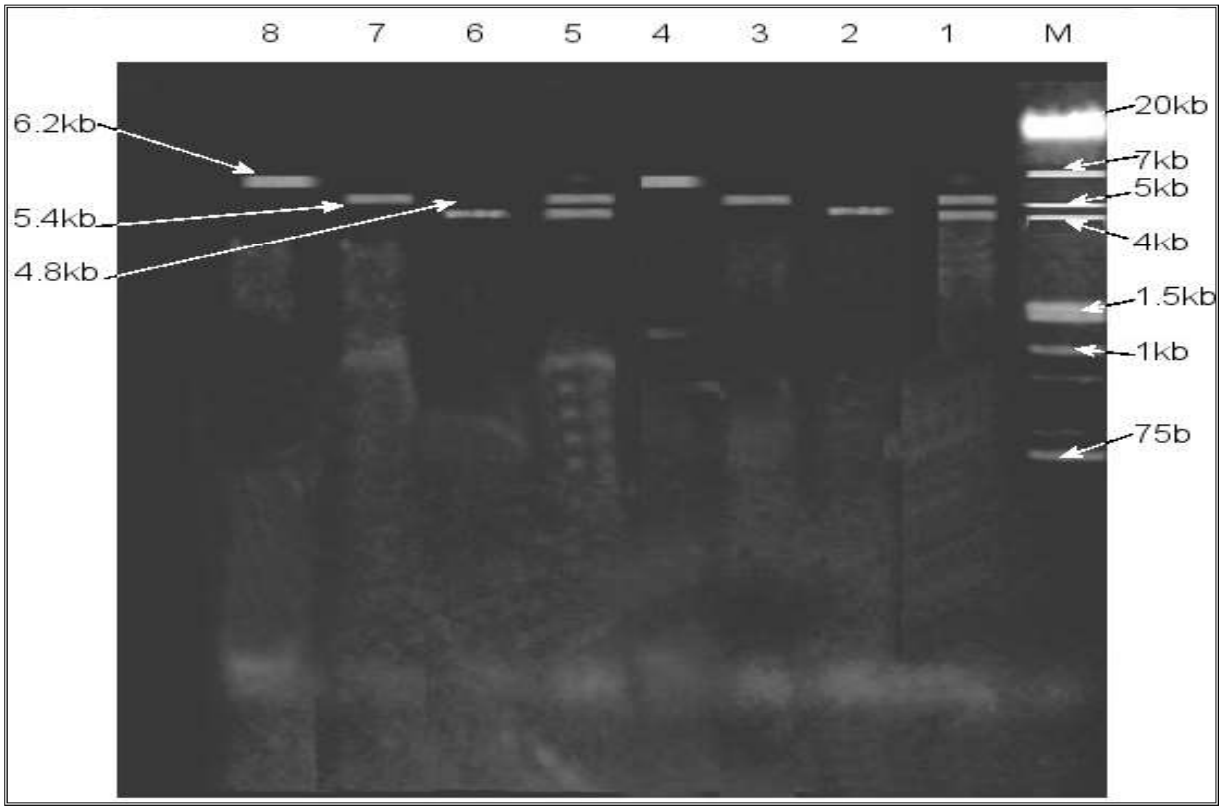

Figure (1): Agarose Gel Electrophoresis of the Long PCR and XbaI Digestion Products.

Results:

Descriptive data of the patients were shown in table (1). Patients were classified into 11 (42.4%) had severe haemophilia A, 9 (34.6%) had moderate and 6 (23%) had mild hemophilia A. Putting the growth parameters of the patients on the Egyptian growth charts revealed that all of them lying within the normal centile range also height on the WHO child growth standard Z-score show all the patients were between (-1, 2) standard deviation Z-scores.

Table (1): Patient's Descriptive Data

	Mean ± SD	No.	%
Age (Years)	6.1± 3.67		
Age at diagnosis (months)	7.46±11.38		
No. of cases with positive family history		11	42.3
No. of cases with parental consanguinity		7	27
No. of cases with female siblings		10	38.4
Clinical Data:			
No. of cases with bleeding:			
Haemarthrosis		20	77
Haematoma		4	15
Orificial		5	19
Ecchymosis		15	58
No. of cases with chronic arthropathy (boggy joint)		16	61.5
No. of cases with viral hepatitis infection		12	46.2

Molecular analysis showed that allele frequency of positive XbaI was 46%, 23%, 40% and 27% in patients, mothers, sisters and control respectively. Table (2) shows the heterozygosity rates of intron 22 XbaI polymorphism; the expected heterozygosity of the cases was (0.5), the expected heterozygosity of

the sibs was (0.48) and of the mothers was (0.35) while of the control group was (0.39). Also show the observed heterozygosity rate (46%), (80%) and (54%) for the mothers, sibs and control group respectively.

Table (2): Heterozygosity Rates of Intron 22 XbaI Polymorphism

Group (N)	No. Of Studied Chromosomes (Total)	Positive Allele		Negative Allele		Heterozygosity Rate* (Expected)	Heterozygosity Rate (Observed)
		No.	%	No	%		
Cases (26)	26	12	46	14	54	0.5	-
Mothers (26)	52	12	23	40	77	0.35	46%
Sibs (10)	20	8	40	12	60	0.48	80%
Control (26)	52	14	27	38	73	0.39	54%

Allele frequencies and the expected heterozygosity rate were calculated by using Hardy-Weinberg principle.

Table (3): Incidence of Intron 22 XbaI Polymorphism in patients with Different Clinical Severity

Factor VIII Level	Clinical Severity No.	No of cases with positive XbaI polymorphism		No of cases with negative XbaI polymorphism		X^2	P- Value
		No.	%	No.	%		
< 1	Severe (11)	5	45.5	6	54.5	0.71	>0.05
1-5	Moderate (9)	5	55.6	4	44.4		
5-40	Mild (6)	2	33.3	4	66.7		

Intron 22-specific long PCR for the XbaI polymorphism was shown in (figure 1). Agarose gel electrophoresis of the long PCR and XbaI digested products for all possible genotypes. Lanes M: marker; lane 1& 5: heterozygote female (-/+); lane 2&6: male (+) or homozygous female (+/+); lane 3&7: male (-) or homozygous female (-/-); lane 4&8: undigested product.

Discussion:

Haemophilia A is a hereditary bleeding disorder caused by the absence, severe deficiency, or defective functioning of plasma coagulation factor VIII, it inherited in an X-linked manner. Bleeding in the joints (haemarthrosis) is the hallmark in persons with haemophilia A[11]. Intramuscular haemorrhages are the second most common bleeding types after joint bleedings in haemophilia[12]. In the current study haemarthrosis represented (77%) of the studied cases as one type of bleeding that they suffer. And approximately (58%) of the cases exposed to skin bleeding as ecchymosis while orificial and intramuscular bleeding (haematoma) were (19%), (15%) respectively. Similar to the present study results Ahmed et al., (2006)[13] reported that joints bleeding was (60%) of their cases, (53%) of cases exposed to skin bleeding, intramuscular bleeding was (11%), and orificial bleeding was (36%). Haemarthrosis which represented (77%) of the studied cases as one type of bleeding were distributed on the joints as (57%) of the cases had knee joint haemarthrosis, (8%) of the cases had ankle joint, (4%) had both knee and ankle joints and (8%) had both knee and elbow joints. Srivastava et al., (2005)[14] reported that the incidence of haemarthrosis into different joints was (45%) of knee joint, (30%) of elbow joint, (15%) of ankle, (3%) of shoulder, (3%) of wrist, (2%) of hip and (2%) other joint.

Carrier-screening methods often depend on linkage analysis, a common affordable approach for genetic diagnosis, especially in developing countries[4]. Carrier detection by indirect gene analysis has been the method of choice in many developing countries. For the FVIII gene analysis, the use of intragenic markers has been recommended in place of extragenic markers as the chances of

recombination are more using intragenic than extragenic markers[15]. Such marker was XbaI. The presence of restriction site recognized by XbaI on the gene was indicated as positive allele. The incidence of positive allele in our patients was similar to that reported in Russian Slav (46%) and close to that reported in North India (43%)[16] but much higher than that reported in Uzbek (14%)[17] However, it was relatively lower than that reported in Polynesian (50%)[18]. And lower than that reported in Korean (72%)[19] and in Caucasian (57%)[20]. Expected heterozygosity among sibs of affected subjects was (0.48) and their mothers was (0.35) compared with (0.39) in the control group. The carrier state (observed heterozygosity) could be identified in 12/26 (46%) of the mothers by using this marker alone. Also carrier state (observed Heterozygosity) could be identified in (80%) of the patients siblings.

This marker was informative in the mothers by (46%); hence, by using this marker alone (46%) of the studied families could be tracked for haemophilia A. The XbaI long PCR marker was found to be less informative in the current study (46%) than that reported in Russian Slav (50%)[17] and Polynesian (50%)[18] while close to that reported in Caucasian (48%)[18] and Chinese (49%)[21]. It was slightly higher than that observed in Western European (41%)[22] and Italian (42%)[23]. In other studies using a combination of informative polymorphisms would increase the informativity; Graham et al., 1990 reported that intron 18/BCL1 and intron 22/XbaI loci show linkage disequilibrium and their combined use provides more informativity than either of them alone. In whites the informativity for combined use of XbaI and BCL1 is 65% vs. 49% if used on its own. However, XbaI occurs within intron 22 (int22h-1) and the corresponding positions in the extragenic homologues int22h-2/ 3 designated XbaI B and C makes the use of this polymorphic locus less useful[24] so long distance PCR was performed in the current study specifically for the intragenic homologues (int22h-1). Raza et al., (2009) reported the heterozygosity in terms of informativity of the markers was 53% for BclI, 44% for HindIII, and 34% for XbaI. Combined informativity of these markers was 77%. Review of world literature shows a marked variation in the informativity of polymorphic sites[16].

Results:

Results of the present study show incidence of intron 22 XbaI polymorphism in cases with different clinical severity; 12 out of 26 (46%) of cases were positive for XbaI polymorphic marker; 5 of positive cases were severe, 5 of positive cases were moderate and 2 of positive cases were mild haemophilia A. While in another study that was done by Omran et al., (2007)[25] 12 out of 40 (30%) of cases were positive for XbaI polymorphic marker; 3 of positive cases were severe, 7 of positive cases were moderate and 2 of positive cases were mild. Prabhavathi et al., (2002)[26] reported that 5 out of 15 (33%) were positive for XbaI polymorphic marker; 2 of positive cases were severe, 2 of positive cases were moderate and 1 of positive cases were mild. In the current study no significant relation was found between the XbaI polymorphic status and different clinical severity (P-value >0.05) in agree with Omran et al., (2007) and Prabhavathi et al., (2002).

We can conclude that linkage analysis with Long distance PCR of intron 22 XbaI marker is a good method for carrier detection of hemophilia A and it is cost-effective. However the use of other polymorphic marker such as Bcl1/ RFLP, HindIII/ RFLP and/ or dinucleotide (STR) CA repeats in combination with long distance PCR of XbaI should raise the informativity gained for genetic diagnosis.

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الملخص

استخدام الطفرة الوراثية للانترون٢٢ فى اكتشاف حاملى مرض الهيموفيليا "أ" من أخوات المرضى

هيموفيليا "أ" من أمراض الدم الوراثية المتنحية والمرتبطة بالجنس. تتميز بنقص العامل الثامن للتجلط (FVIII)، ويعتبر التشخيص الوراثى هو الأسلوب الأكثر دقة للكشف عن الناقل.

الهدف من الدراسة:

الكشف عن طفرة إنترون ٢٢ XbaI فى المرضى وعائلاتهم من أجل صياغة التشخيص الجزيئى الدقيق للناقل.

الطرق:

٨٨ من المواضيع تتعرض للكشف عن طفرة الإنترون ٢٢ باستخدام انزيم XbaI بطريقة Long distance PCR.

النتائج:

وكانت ترددات الأليل من XbaI الإيجابية ٤٦%، ٢٣%، ٤٠% و٢٧% فى المرضى والأمهات والأخوات والضوابط على التوالي. وكان Expected Heterozygosity ٠,٣٥ فى أمهات المرضى بالمقارنة مع ٠,٣٩ فى مجموعة الضوابط بينما ٠,٤٨ فى الأخوات للمرضى. وكانت Observed Heterozygosity ٤٦% فى الأمهات بالمقارنة مع ٥٤% للضوابط بينما كانت ٨٠% فى الأخوات.

الخلاصة:

تحليل الربط مع PCR مسافة طويلة من علامة الإنترون XbaI 22 هو طريقة جيدة لكشف الناقل من الهيموفيليا "أ"

الكلمات الرئيسية:

الكشف عن الناقل، العامل الثامن، هيموفيليا "أ"، Long Distance PCR، XbaI.