UDC 575.113.2; 616.151.5
Original scientific paper

GENETIC PREDICTION OF HEMOPHILIA A BY INT18/BCII RFLP ANALYSIS

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Rakićević Lj., D. Georgijević, G. Janković, D. Miković, and D. Radojković (2000): *Genetic prediction of hemophilia a by INT18/BCLI RFLP analysis.* – Genetika, Vol. 32, No. 2, 129-135.

Hemophilia A, an X linked genetic disease, is the most common coagulation disorder with an incidence of about 1-2 in 10 000 males. It is caused by mutations in the factor VIII coagulation gene. After cloning of the factor VIII gene, almost all types of mutations causing hemophilia A were characterized. The wide spectrum of different mutations in the factor VIII gene made direct DNA diagnosis of the disease not the method of choice. Indirect DNA diagnosis, using restriction fragment length polymorphisms (RFLPs) offers an alternative. The aim of our study was to provide carrier and prenatal diagnostics for affected families using the indirect approach. The genomic DNA of 67 members of 24 hemophilia A families were analyzed by polymerase chain reaction (PCR) amplification of the Bc*l*I polymorphic region at intron 18 of the factor VIII gene. Twelve families were informative for int18/Bc*l*I RFLP (50%). One prenatal diagnosis of hemophilia A was performed in the first trimester of gravidity, and the fetus was found to be a male affected by hemophilia A.

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Indirect DNA testing is straightforward, rapid and inexpensive to perform. Thus, in many families requiring genetic diagnosis of hemophilia A, as well as genetic counselling, the use of intragenic polymorphism analysis represents the diagnostic strategy of choice.

Key words: hemophilia A, prenatal diagnosis, carrier detection, RFLP

INTRODUCTION

The factor VIII gene comprises 26 exons spanning 186 kb of DNA located at the distal end of the long arm of the X-chromosome (GITSCHIER *et al.*, 1984; TOOLE *et al.*, 1984). It is transcribed and processed into a 9 kb mRNA which translates into a protein of 2351 amino acids. A marked mutational heterogeneity has been documented over the past decade (TUDENHAM *et al.*,1991; KEMBAL *et al.*, 1997) and a variety of nonsense and missense mutations, frameshifts, deletions, duplications and insertions have been described (HGMD, 2000). The same mutation has been observed occasionally in unrelated individuals. These data suggested that generally each family bears its own mutation. Defects in this gene resulting in a quantitative protein abnormality cause hemophilia A, the most common severe inherited bleeding disorder affecting about 1-2 in 10 000 males. Females heterozygous for hemophilia A (carriers) are asymptomatic and information provided by coagulation assays is equivocal, due to random X-chromosome inactivation or lyonization (LYON, 1962) causing a wide range of factor VIII levels.

In view of problems in carrying out direct mutation detection for haemophilia A, due to marked genetic heterogeneity, the majority of laboratories involved in genetic testing for carrier detection and prenatal diagnosis have relied upon linkage studies employing polymorphic markers of the factor VIII locus (JANCO *et al.*, 1987; TUDDENHAM *et al.*, 1987; PEAKE, *1992;* POON *et al.* 1992). These polymorphisms involve recognition sites for particular restriction enzymes; the presence or absence of the site providing a tag for the hemophilia mutation in pedigree analysis. Initially DNA polymorphism analyses relied on the use of radioactive Southern blotting, which is costly and labor-intensive. Nowadays, it is generally replaced with PCR based methods. In addition, two polymorphic microsatellite repeat sequences have been characterized within factor VIII gene (within intron 13 and 22) (LALLOZ *et al.*, 1991; LALLOZ *et al.*, 1994).

The purpose of this study was to introduce molecular diagnostics of hemophilia A using int18/Bc/I RFLP. The final aim was to investigate registered families with hemophilia A, and to determine informativeness for indirect molecular testing using int18/Bc/I RFLP. In that way, it would be possible to identify female carriers and provide adequate genetic counselling in families at risk.

MATERIAL AND METHODS

Genomic DNA samples were analysed from subjects from 24 hemophilia A families. These samples were obtained from the National Blood Transfusion Institute for carrier detection or prenatal diagnostic studies. Blood from hemophilic

patients and their families, and a CVS sample (in a case of prenatal testing) were obtained and genomic DNA was isolated (DEL SAL *et al.*, 1989).

Intragenic RFLP int18/Bc*l*I analysis was performed using PCR (KOGAN *et al.*, 1987). A 142 bp fragment flanking the polymorphic site in intron 18 was amplified in a 50µl final volume containing 50 mM KCl, 10mM Tris-HCl pH 9.0, 0,1% Triton X-100, 5mM $MgCl_2$, 200mM dNTPs, 20pmol each primer, 1,5U DNA polymerase (Perkin Elmer), and 200-500ng DNA. The reaction mixture was overlaid with a drop of mineral oil and the following cycling conditions were applied: initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of; 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, and final polymerization at 72°C for 10 minutes.

Typically, 10 µl amplified samples were digested with Bc*l*I enzyme (Bio-Labs) for two hours, and analysed by electrophoresis on 10% polyacrylamide gels. The gels were stained by silver staining (BASSAM *et al.*, 1991).

Determination of fetal sex was performed by amplification of a Y-chromosome-specific repeat sequence (KOGAN *et al.*, 1987).

RESULTS AND DISCUSSION

Hemophilia A, unlike diseases such as sickle cell anemia, thalassemias in certain populations, or phenylketonuria, stems from a wide variety of mutations. Thus, genetic prediction of hemophilia A depends upon the analysis of DNA polymorphisms in and near the factor VIII gene. In the factor VIII gene eight two allele restriction polymorphisms (Bc*l*I, XbaI, MspI and HindIII) and two multiallelic (int13CA and int22 CA) have been discovered. The various alleles are characterized by manifest ethnic variations. For example, the *BclI* marker is more useful in Mediterranean, Indian and Japanese populations, than in Caucasians, African American and Chinese (PEAKE *et al.*, 1993). For the Yugoslav female population, there are still no data regarding heterozygosity of the Bc*l*I marker.

Our study was carried out in 24 hemophilia A families involving 67 subjects using PCR amplification of the Bc*l*I polymorphic region in intron 18 of the factor VIII gene. Twelve families (50%) were informative for int18/Bc*l*I RFLP. In these families hemophilia A was inherited with the presence or absence of a polymorphic Bc*l*I restriction site within int18 of the factor VIII gene (figure1). The usefulness of a polymorphism depends on the frequency of women heterozygous for the allele. Our first results, although on a limited number of subjects, suggest that this marker enables precise carrier detection in half (12/24) of the affected families.

Prenatal diagnosis of hemophilia A is an important aspect of genetic counselling in a family at risk. Chorionic villus sampling (CVS) is the method most widely used today to obtain fetal material for diagnostic purposes. Transabdominal CVS is optimally performed in gestational weeks 10-12. The fetal chorion villus sample is first used for fetal sexing. In the case of a male fetus, further analysis using the PCR and RFLP technique is performed. The great advantage with CVS is that, in the event of an affected fetus, the pregnancy can be terminated

during the first trimester, which is not only simpler to do but also less stressful than termination in the second trimester.

So far, we have performed one prenatal diagnosis of hemophilia A. The data illustrating the DNA patterns obtained with an informative family are given in Fig. 1. The mutation in the factor VIII gene, in the patient with hemophilia A, was detected by the presence of a restriction site for the BclI site, giving a fragment of 96 bp. In this case, prenatal diagnosis was performed on CVS, taken from the mother in the first trimester of pregnancy. Determination of fetal sex by amplifcation of a Y-chromosome specific repeat sequence showed that the fetus was male. RFLP analysis showed the same DNA patern as the first son with hemophilia A.

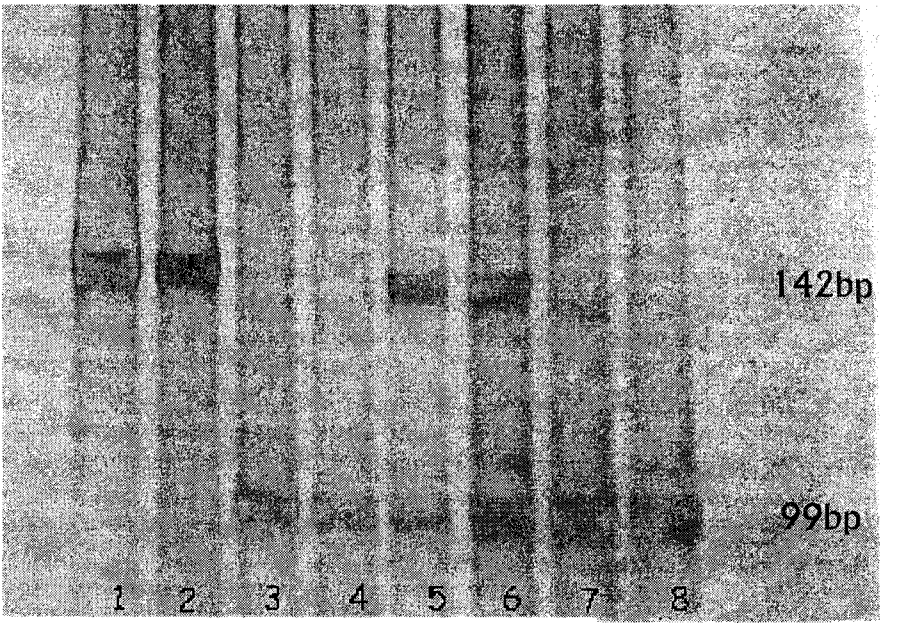

Fig 1. Analysis of a family informative for BclI polymorphism on 10% polyacrylamide gel A 144 bp PCR amplified polymorphic region (1) control PCR products without (2) and with BclI restriction site (3,4), control heterozygotic female (5), heterozygotic mother (6), the son with hemophilia A mutations (7) and male fetus with same patern as the son with hemophilia A (8)

In diseases of very high mutational heterogeneity, such as hemophilia A, where patients generally carry different mutations, carrier and prenatal diagnosis based on indirect criteria such as the interfamilial segregation of gene-specific polymorphic markers are of substantial help.

CONCLUSIONS

Increasing worldwide use of molecular genetic analysis is enabling accurate carrier detection for hemophilia to be made more widely available. Use of

DNA polymorphisms in linkage analysis is an accurate method for carrier detection applicable to the majority of families. Indirect DNA testing is straightforward, rapid and relatively inexpensive to perform. Our results showed that int18/*Bcl*I RFLP was informative in 50% tested families. Thus in many families, requiring genetic diagnosis of hemophilia A, as well as genetic counselling, the use of intragenic polymorphism analysis represents the diagnostic strategy of choice. For families remaining uninformative by these procedures, a range of point mutation screening techniques is available.

Received July 3rd, 2000
Accepted October 2nd, 2000

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GENETIČKO PREDVIĐANJE HEMOFILIJE ANALIZOM INT18/BCII RFLP

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Izvod

Hemofilija A je X vezana bolest, koja se javlja sa učestalošću od 1 do 2 na 10000 muškaraca. Ona predstavlja najčešći koagulacioni poremećaj i izazvana je mutacijama u genu koji kodira osmi faktor koagulacije (FVIII). Nakon kloniranja gena za FVIII okarakterisan je veliki broj mutacija koje dovode do hemofilije A, što otežava direktnu DNK dijagnostiku ove bolesti. Indirektna DNK dijagnostika primenom polimorfizama u dužini restrikcionih fragmenata DNK (RFLPs) predstavlja alternativni pristup. Cilj ovog rada je bio da se utvrdi informativnost porodica u kojima se javlja hemofilija A za prenatalnu dijagnostiku u narednim trudnoćama. Korišćenjem reakcije lančanog umnožavanja DNK (PCR), analizirano je 67 uzoraka DNK članova, 24 porodice, u kojima se javlja hemofilija A. Umnožavan je polimorfan region u intronu 18, gena za VIII faktor koagulacije i obrađivan restrikcionim enzimom BclI. Utvrđeno je da je 12 porodica (50%) informativno za 18/BclI RFLP. Urađena je i jedna prenatalna dijagnoza, kod žene u prvom trimestru trudnoće, i pri tom je kod fetusa muškoga pola detektovan isti obrazac restrikcionih fragmenata kao kod obolelog od hemofilije. S obzirom da je indirektna DNK dijagnostika brz i ekonomski isplativ metod, ona predstavlja dijagnostičku strategiju izbora u većini porodica u kojima je indikovana genetička dijagnostika hemofilije A.

Primljeno 3. VII 2000.
Odobreno 2. X 2000.