

## Carrier determination in a Hemophilia B family using single strand conformation polymorphism (SSCP) and sequencing

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### Abstract

Hemophilia B is an X-linked recessive bleeding disorder caused by heterogeneous mutations in factor IX gene. In about one-third of cases it arises by a new mutation in germ-line cells. In this study carrier testing was performed for females of a family with only one affected individual by single strand conformation polymorphism (SSCP). Results indicated that the SSCP band shift in the propositus was *de novo* and his mother and also sisters were not carrier. This finding was also confirmed by sequencing.

**Keywords:** Hemophilia B, Mutation, SSCP, Carrier Testing

Hemophilia B is an X-linked recessive coagulopathy resulting from defect in blood coagulation factor IX (FIX) (Lillicrap, 1998). It occurs in approximately 1 out of every 30,000 male births and accounts for about 15-20% of the hemophiliac disorders (Bolton-Maggs and Pasi 2003). The disease (OMIM # 306900) is produced by a wide range of mutations in FIX gene (Giannelli *et al.*, 1992). The FIX gene (F9) is approximately 34 kb in length, located at Xq27.1 and contains eight exons coding a 415 amino acid long polypeptide (Yoshitake *et al.*, 1985). More than 800 different types of molecular anomalies, most of which are point mutations, have been described in FIX gene so far (Giannelli *et al.*, 1998).

The female relatives of hemophilia B patients are at risk of being carrier. The carrier testing for familial cases could be performed by conventional indirect methods such as restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNP), short tandem repeat (STR), etc., which are currently the most widely used methods (Bicocchi *et al.*, 2003). These polymorphic markers, due to some limitations, can not be used in families with a single case (Bowen, 2002).

The application of PCR for the detection of hemophilia B carriers has advanced the molecular evaluation of families with hemophilia B. It was only after the development of PCR and PCR-based technologies, such as SSCP (Orita *et al.*, 1989), heteroduplex analysis (HA) (Driscoll *et al.*, 1996), denaturing gradient gel electrophoresis (DGGE) (Ghanem *et al.*, 1993), conformation sensitive gel electrophoresis (CSGE) (Hinks *et al.*, 1999) and recently denaturing high performance liquid chromatography (DHPLC) (Oldenburg *et al.*, 2001) that the mutation detection and carrier testing became easily possible. SSCP allows the detection of single base changes in short DNA fragments due to mobility differences of single-stranded DNA molecules on a non-denaturing polyacrylamide gel.

There are about 900 hemophilia B patients in Iran (Iran Hemophilia Society) and a high number of females which are potential carriers (each female having fifty percent chance of being carrier). Here we report the use of SSCP and sequencing in carrier detection of females from an Iranian family with one hemophilia B patient only.

A patient suffering from severe hemophilia B (factor

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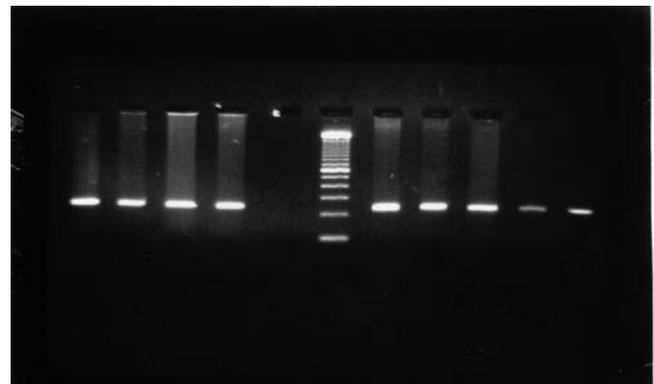
IX activity <1%) was referred to our center for DNA studies. This study was done during genotyping programme of Iranian hemophilia B patients. The family had only one affected male, with no other hemophilia B patient in the relatives. Carrier testing was requested for mother and four at-risk girls of this family.

Genomic DNA was extracted from 5 ml anticoagulated blood samples from the index case and his mother and sisters according to Sambrook and Russell (2001). For index patient, all exons and intron/exon boundaries, promoter and 5' and 3' untranslated regions were amplified. The exon 8 (E8) was subdivided into four fragments. Primers were from Montejo *et al.*, (1999), except for exons 1 and 3, which were designed for this study (unpublished data). After detection of a SSCP band shift for the index case, which was in the third fragment of exon 8 (bases 31015-31222, numbering based on Yoshitake *et al.*, 1985), PCR amplification on this fragment was performed for the patient and the females in the family. For exon eight PCR, each PCR mixture (25 µl) contained the following components: 10 pM of each primer (Genset, France), 0.5 U Taq polymerase (Eurobio, France), 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP (Roche, Germany) and 100 ng of genomic DNA. The temperature cycling (initial denaturation 5 min at 95°C, 30 cycles; denaturation 1 min at 94°C, annealing 1 min at 55°C and extension 1 min at 72°C and final extension 5 min at 72°C) was performed in a DNA thermal cycler (Mastercycler, Eppendorf, Germany). The PCR products were run on 2% agarose gel, stained with ethidium bromide, and visualized by UV.

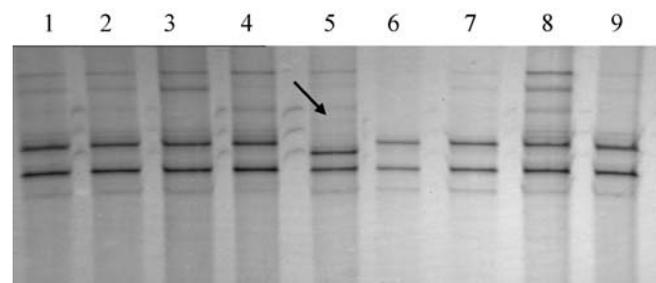
SSCP analysis was done on PCR products from the patient and his mother and sisters according to published procedure (Karimipoor *et al.*, 2003). Briefly, 5 µl of PCR products from the index case, his mother, four sisters and three normal samples were mixed with SSCP loading buffer, denatured five minutes at 95°C and immediately transferred onto ice. Samples were applied onto an 8% non-denaturing polyacrylamide gel and electrophoresis was carried out at 1000V for 6 hours at 4°C. After electrophoresis the gel was stained by silver-staining method (Sambrook and Russel 2001). For the patient and his mother PCR amplification was done in 200 µl reaction and PCR products purified by gel extraction purification kit (Qiagen, Germany). Sequencing of these fragments was done by chain termination method on ABI 377 sequencing machine (GATC Co., Germany). Sequencing results were analyzed using FASTA programme

(www.ebi.ac.uk/fasta33) and the mutation was compared against the hemophilia B mutation database (www.kcl.ac.uk/petergreen/haemB.html).

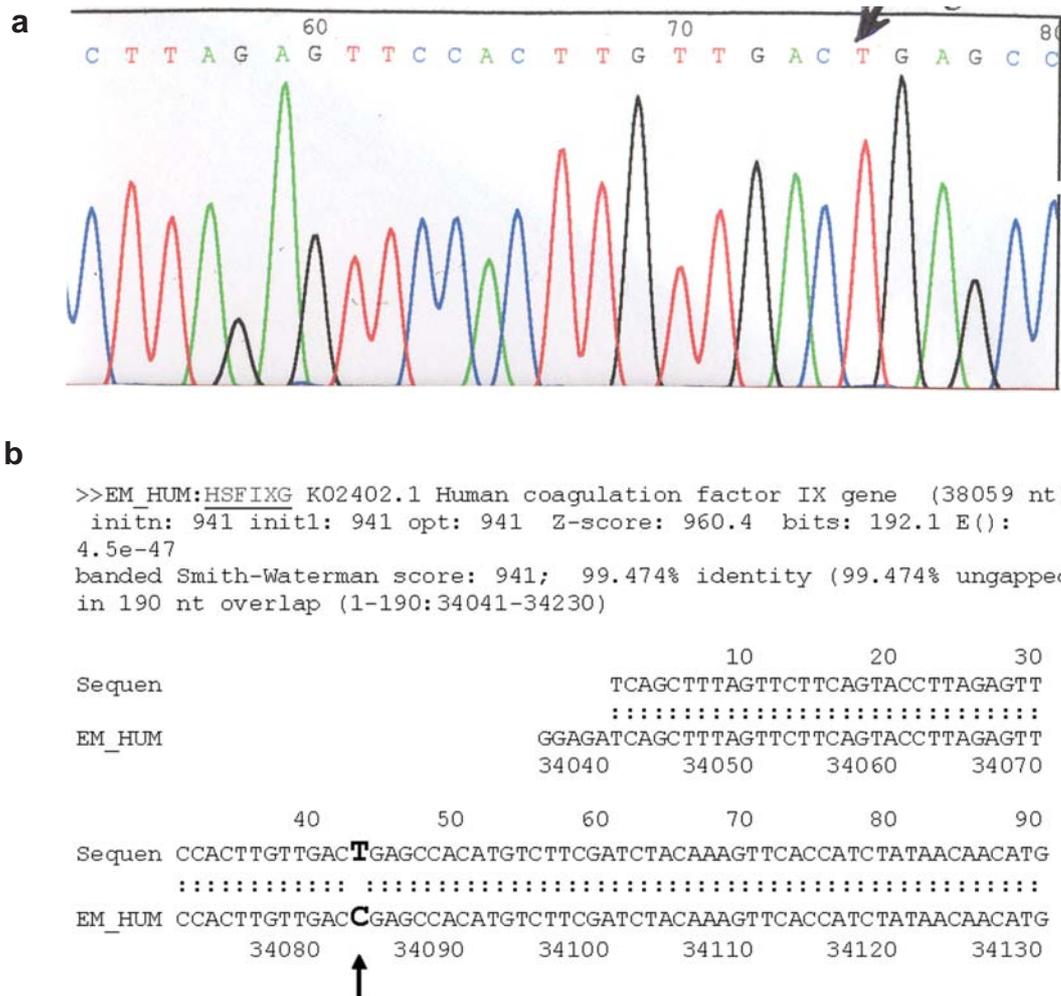
In this study, we first analyzed the functional regions of the FIX gene, including promoter, exons, exon/intron flanking sequences and 5' and 3' untranslated regions on the index case. This work was carried out during a genotyping programme of some Iranian hemophilia B patients (the first report on finding mutations in fifty patients is in preparation). SSCP showed a definite abnormal migration pattern at the fragment three of exon eight in the index case. Then PCR amplification (Fig. 1) and SSCP analysis (Fig. 2) were performed for other female members of the family, including the mother and four sisters. The figure shows a clear band shift for the hemophilia B patient, but not for other family members. Hence, the analysis of SSCP pattern indicated that none of the females could be carrier and the index patient interpreted as a sporadic case.



**Figure 1.** Amplification of a part of exon eight in the members of an Iranian family with only one hemophilia B patient. Lanes 1, 2, 3, 4, 5 and 6: the patient, his mother and sisters. Lanes 7-9: normal controls. B: blank and M: 100 bp molecular weight marker.



**Figure 2.** SSCP analysis of factor IX (E8, fragment 3) in an Iranian family with one hemophilia B patient. A band shift in index case is easily observed (lane 5), and his mother (lane 2) and sisters (lanes 3, 4, 6, 7) exhibit normal pattern similar to normal controls (lanes 1,8 and 9).



**Figure 3. (a)** A part of ABI electropherogram for the exon eight on FIX gene in the Iranian hemophilia B patient shows a C to T change. This causes Arg 333 to stop codon. **(b)** FASTA analysis of the graph indicates C to T change in 34082 (31118 according to Yoshitake *et al.*, 1985 numbering).

The mutation was then identified as C31118T (nucleotide numbering based on Yoshitake *et al.*, 1985) by direct sequencing (Figures 3a and 3b). This causes an Arginine to stop codon at residue 333 (Arg333X) in factor IX protein. This mutation has already been reported by several authors and causes a severe phenotype (hemophilia B mutation database). No such mutation was detected in the maternal DNA sample.

As mentioned, X-linked disorder such as hemophilia B are due to new mutations in approximately one-third of cases, making carrier testing and prenatal diagnosis especially problematic. Thus direct scanning methods based on PCR should be exploited instead of linkage analysis by intragenic polymorphisms. One of these methods is SSCP, which has enough sensitivity

and extensively used for detection of mutation and polymorphisms for many diseases (Glavac and Dean 1993), carrier detection (Martinez *et al.*, 1994) and prenatal diagnosis (Karimipoor *et al.*, 2003). While not regarded as “high-tech,” SSCP remains the most widely used mutation scanning technique (Cotton 2001).

We analyzed the DNA samples for this family by SSCP and were able to provide accurate carrier detection. Our results were subsequently confirmed by sequencing the exon 8 on the index case and his mother. Thus we can conclude that SSCP, where informative, can be used with relative ease and low cost for carrier detection.

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