Rapid Identification of Family-Specific Mutations in the Factor VIII Gene by One-Step DGGE

Ayşe ANIL TİMUR*, Davut ALBAYRAK, S. Hande ÇAĞLAYAN***

* Department of Molecular Biology and Genetics, Boğaziçi University, İstanbul,
** Department of Pediatric Hematology, Faculty of Medicine, Ondokuz Mayıs University, Samsun, TURKEY

ABSTRACT

A one-step denaturing gradient gel electrophoresis (DGGE) strategy for the rapid detection of mutations in the factor VIII gene of haemophilia A patients is described. All coding (except the middle part of exon 14) and flanking intronic regions of the gene corresponding to approximately 6.6 kb were amplified in 27 fragments using four PCR programs. Heteroduplex formation was performed for each fragment. A common denaturant gradient gel (35-65%) was chosen that allowed the simultaneous analysis of all PCR amplified regions on a single gel and run for 3.5 h at 160 V. This method was implemented for a patient whose family was seeking carrier determinations. An abnormal pattern was detected in exon 23 and the family-specific mutation was found by subsequent DNA sequencing. One-step DGGE is a promising rapid method for the carrier detection and prenatal diagnosis in haemophilia A families when immediate results are required and when polymorphic markers fail to give information.

Key Words: Hemophilia A, Denaturing gradient gel electrophoresis (DGGE), Family-specific mutations, Carrier identification, Prenatal diagnosis.

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INTRODUCTION

Hemophilia A is an X-linked bleeding disorder caused by the deficiency or absence of the coagulation protein Factor VIII (FVIII). Molecular analysis of the FVIII gene has revealed a variety of mutations almost unique to each family including single base substitutions, deletions, duplications and insertions that are listed in the haemophilia A database (1; http://europium.csc.mrc.ac.uk). One type of inversion, however, disrupting the FVIII gene in intron 22 accounts for 40-45% of the severe cases[2].

The detection of family-specific mutations provides the most accurate and reliable hemophilia A diagnosis and carrier detection. However, due to the large size and complexity of the FVIII gene, it is very difficult to locate mutations by direct sequencing. A number of screening methods have been implemented for the detection of haemophilia A mutations including chemical cleavage of mismatch (CCM)[3,4], single-strand conformation polymorphism (SSCP)[5,6], and denaturing gradient gel electrophoresis (DGGE)[7-9].

DGGE has been shown to detect DNA molecules differing by as little as a single base change[7,10]. The efficiency of the method is estimated to be about 98-100%. Although it has been reported that DGGE is one of the most sensitive methods used to identify FVIII mutations, the detection power of DGGE is shown to be between 82-86% for the FVIII gene[8,9,11]. Consequently, the point that mutations that cause hemophilia A may reside in regions not routinely screened in the FVIII gene or in some other genomic loci.

A fine DGGE screening of the FVIII gene involves the analysis of each fragment separately since each fragment requires a different denaturant gradient. This is the most systematic and economic way for screening large samples of patients. In this manner 32 hemophilia A mutations have been identified in Turkish patients[12]. However, the method is not feasible for the detection of a mutation in only one family. Similar difficulties are encountered in other large genes where heterogenous point mutations cause the disease. A modified DGGE method called "broad-range" was first applied to human phenylalanine hydroxylase (PAH) gene by adapting a 0-80% gradient gel to 13 exonic fragments so that they are all analyzed in one gel[13]. Herein, we report a one-step DGGE system that allowed the simultaneous analysis of all PCR-amplified fragments of the FVIII gene, enabling scanning of all coding regions of a patient on a single DGGE gel.

MATERIALS and METHODS

Polymerase chain reactions (PCRs) for all 25 exons, all corresponding flanking regions, and 5' and 3' portions of exon 14 (14a and 14b, respectively) were carried out in 25 µL reaction mixture containing 1X PCR buffer containing of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5-10 pmoles of each primer, 0.5 U of MBI Fermantas Taq DNA Polymerase (Vilnius, Lithuania) and 0.5-1 µg of genomic DNA on a MJ Research PTC-200 Peltier Thermal Cycler (Massachusetts, USA). The T7 Sequenase™ PCR Product Sequencing Kit and α-[^{35}S]-dATP were used for the sequence analysis. DNA sequencing reactions were performed according to manufacturer's protocols (Amersham LIFE SCIENCE, Inc., Arlington Heigths, Illinois, USA).

Primer and GC-clamp sequences are as determined by Schwaab et al, 1995 with the exception of primers for exon 11-13,15,19, 21 and 26 that are very sligthly different than these primers (Exon 11: F-$(GC)_x$TGGTTTTGCTTGTGGGTAG, R-ATAAGGGGACATCAACTGAGAATGAA; Exon 12: F-$(GC)_x$CCTTTCAATATATGTAATTAACAG, R-ATTCACCACCCACTGGACTTAAGTGCTG; Exon 13: F-$(GC)_x$GATGTGTCTAAATCTCT TTTCCCCATTG, R-ATATAATAACTAACCTGG GTTTTCCATC; Exon 15: F-$(GC)_x$CACCTAGGAAAATGAGGATGTGAGGC, R-TTCTTGTAA TTCCACTGTCCTTAACT; Exon 19: F-$(GC)_x$TT AGTGAAAAGAAATAATTTCTGTT, R-GTAGGC TGAGTAGGTAGGGAACCTCTG; Exon 21: F-

GAATTTAATCTCTGATTTCTCTAC, R- F-$(GC)_x$ GAGTGAATGTGATACATTTCCCATCA; Exon 26: F- F-$(GC)_x$AGCGTCTGTGCTTTGCAGTG, R-TGACGGCAGTGGCAGGTGCT; $(GC)_x$ =39). For PCR amplification, after an initial denaturation at 94°C for 5 min, 35 cycles were performed involving a 35s denaturation at 94°C, 1 min 30s annealing at 51-61°C, and 1 min synthesis at 72°C. PCR was completed with a 5 min extension at 72°C. The matching PCR annealing temperatures of 27 were grouped and amplified in four different thermal cycler programs. PCR products were between 207-388 bp including a 39 bp GC-clamp. For heteroduplex formation, equal amounts of PCR products from patient and normal DNA were combined for each exon, separately. The mixture containing about 20 ng of PCR product was denatured for 10 min at 98°C and allowed to reanneal for 10 min at 60°C.

One-step DGGE was carried out at 160 V for 3.5 h on a Hoefer Pharmacia SE600 apparatus (Hoefer Pharmacia Biotech Inc., San Francisco, California, USA) at a 59°C constant temperature of the 1X tris-acetic acid-EDTA (TAE) buffer. The gel slabs were 0.75 mm-thick, 15 cm-wide and 16 cm-long. Gels were visualised by silver staining and appropriate PCR products were loaded for proper and stronger visualisation[14].

RESULTS

A 35-65% denaturing concentration range with a 7% acrylamide content (37.5:1) was tested to be optimal to analyse all fragments in one-step DGGE using known mutations in 19 different exons as controls. One-step DGGE was applied for the detection of point mutations in a haemophilia A patient screening all 27 PCR fragments comprising 25 exons and two portions of exon 14. Patient DNA was heteroduplexed with a normal DNA for all fragments and applied on DGGE gels. Figure 1A and 1B show the DGGE pattern. Heteroduplexes were detected in exon 23 (Fig 1B).

Some artefacts are present in exons 11, 12, 13; under the real bands and in exon 2 and exon 24; above the real band. However, they can be distinguished from real bands as they are generally stained lighter with silver nitrate. They possibly result from unspecific PCR products, macromolecular impurities, detergent micelles or other anionic material in the sample or in the buffer.

The presence of a point mutation in exon 23 of the patient was confirmed by direct sequencing. The patient had a novel G to C transversion at codon 2163 (Arg2163Pro) in exon 23 corresponing to a CpG site of the FVIII gene (patient 147HA581, Timur et al. submitted).

DISCUSSION

DGGE is proven to be a very sensitive mutation screening method with applications to both linkage and mutation analysis of haemophilia A. It can be costly, laborious and time-consuming to start with but once established, it can be simpler and routine. Using one-step DGGE strategy it became possible to scan all coding regions of the FVIII gene in a common denaturing gradient gel that takes only two days.

One-step DGGE is highly promising for the rapid detection of family-specific mutations and therefore, for the accurate carrier detection and prenatal diagnosis in haemophilia A when immediate results are required and especially when linkage markers are not informative. The banding pattern of the exon suspected to carry the mutation could also be used in linkage analysis to follow the inheritance of the disorder in the family without further DNA sequence analysis to identify the mutation.

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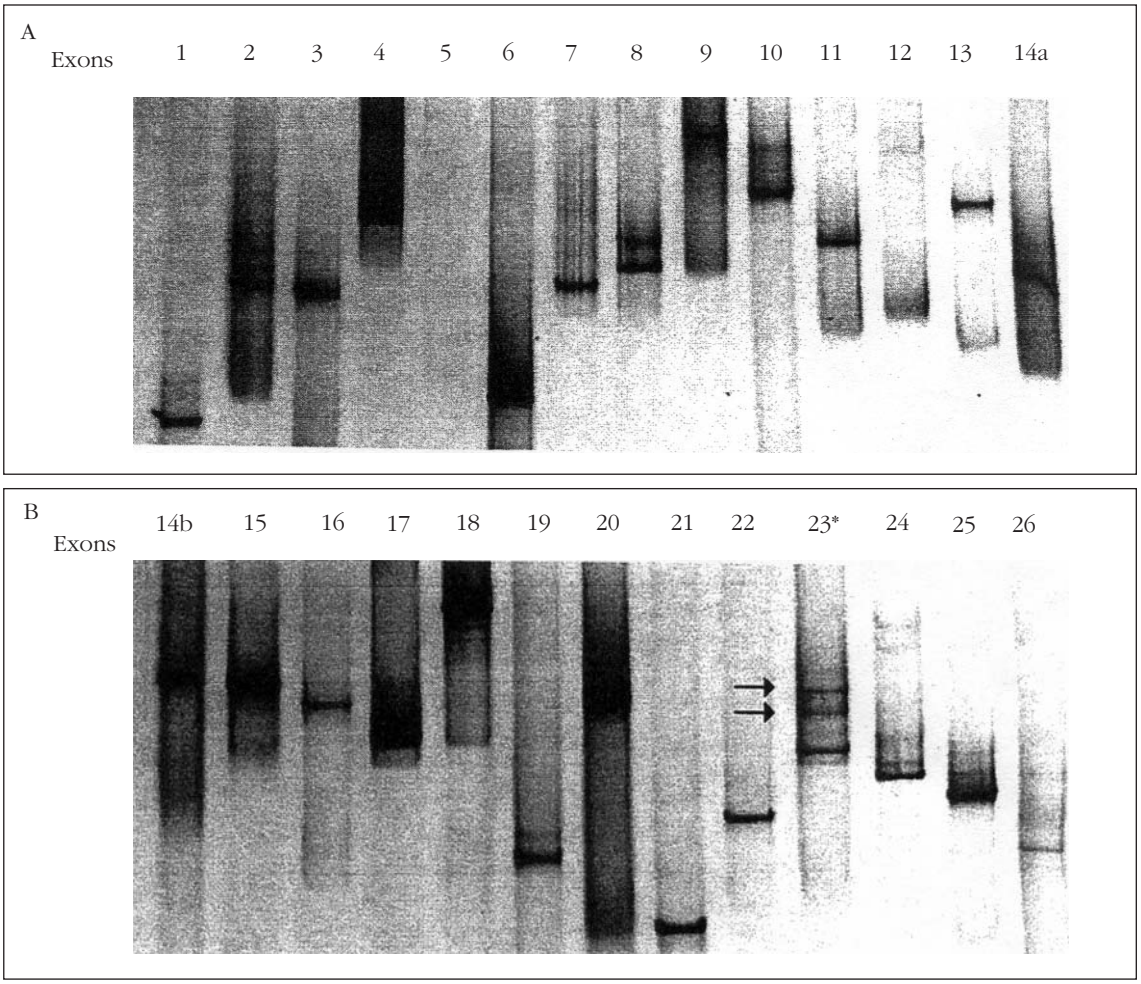

Figure 1 AB. One-step DGGE analysis of the FVIII gene. Numbers on each lane refer to exon numbers. A. Exonic fragments 1-14a. B. Exonic fragments 14b-26. *points to the lane where an abnormal pattern was observed in exon 23. Heteroduplex patterns are indicated by arrows.

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Address for Correspondence:

S. Hande ÇAĞLAYAN, MD

Department of Molecular Biology and Genetics
Boğaziçi University, 80815
Bebek, İstanbul, TURKEY