SHORT COMMUNICATION

Denaturing high-performance liquid chromatography (DHPLC)-based prenatal diagnosis for tuberous sclerosis

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Tuberous sclerosis (TSC) is a frequent autosomal-dominant condition (affecting 1 in 6000 individuals) caused by various mutations in either the hamartin (TSC1) or the tuberin gene (TSC2). This allelic and non-allelic heterogeneity makes genetic counseling and prenatal diagnosis difficult, especially as a significant proportion of TSC cases are due to de novo mutations. For this reason the identification of the disease causing mutation is mandatory for accurate counseling, yet current mutation detection methods such as single-strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) are labor intensive with limited detection efficiency. Denaturing high-performance liquid chromatography (DHPLC) is a high-throughput, semi-automated mutation detection system with a reported mutation detection rate close to 100% for PCR fragments of up to 800 bp. We used a recently described DHPLC assay allowing the efficient detection of mutations in TSC1 to analyze the DNA extracted from a chorionic villus sample in order to perform a prenatal diagnosis for TSC. The fetus was found not to have inherited the deleterious mutation and the DHPLC diagnosis was confirmed by haplotype analysis. This represents the first DHPLC-based prenatal diagnosis of a genetic disease. Copyright © 2001 John Wiley & Sons, Ltd.

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also demonstrated the efficiency of DHPLC in the context of mutations screening in TSC1 and TSC2 (Choy et al., 1999; Jones et al., 2000).

In the family reported herein, mutation scanning of the TSC1 gene had led to the detection of a truncating mutation in exon 18. Subsequently, during the pregnancy of an affected individual of the family, the DHPLC process was successfully applied to the search for the causative mutation in DNA extracted from a chorionic villus sample. This, to our knowledge, constitutes the first DHPLC-based prenatal diagnosis of a genetic disease.

MATERIALS AND METHODS

Patients

An affected individual (II:5), belonging to a large TSC family, requested prenatal diagnosis during her first pregnancy (Figure 1). She had achromic patches, epilepsy, an abdominal neurofibroma, cortical tubers and a Koenen tumor. The morbid allele (3,3,3) had been inherited from her affected mother who also had achromic patches and a renal angiomyolipoma. Two of her sibs were very severely affected (II:3 and II:6). II:3 presented severe epilepsy attacks and died at 14 years of age having neither acquired language nor speech. The other brother (II:6), who had achromic patches and severe epilepsy, committed suicide at age 25. Considering the phenotypic severity and the familial history, prenatal diagnosis was considered to be justified.

Linkage analyses

Three polymorphic CA repeats close to the TSC1 gene, namely D9S1830, D9S164 and D9S114, were used for linkage analysis (Henske and Kwiatkowski, 1995). Genomic DNA (150 ng) was amplified in a 20 µL volume containing 1 µM primers, 0.2 mM dNTP mix, 1 U Taq polymerase (Applied Biosystems, Courtaboeuf, France) in the buffer supplied by the manufacturer. Samples were processed through 30 thermal cycles at 96°C, 55°C and 72°C for 30 s at each temperature. PCR products were incubated at 94°C for 10 min before being loaded onto a 6% acrylamide, 7.5 M urea gel. Electrophoresis was carried out at 1800 V, 65 W for 4 h at 50°C. The gel was then blotted onto a nylon membrane and probed with [CA]n oligonucleotides labeled by chemiluminescence according to the manufacturer’s instructions (ECL direct nucleic acid labeling and detection system; Amersham Pharmacia Biotech, Courtaboeuf, France).

PCR for DHPLC

Genomic DNA was extracted from peripheral lymphocytes and the trophoblast sample. DNA (100 ng) was submitted to PCR amplification using the TSC1 exon 8 primers according to Jones et al. (1997). Amplification products were denatured for 10 min

Figure 1—Pedigree of a family affected with TSC and TSC1 haplotypes. The numbers placed next to the ladder represent the different alleles for polymorphic markers D9S1830, D9S164 and D9S114 as indicated in the box in which is given an estimation of the genetic distances between the different loci.

at 96°C and then allowed to reanneal gradually by slowly decreasing the temperature to 30°C before being placed in the DHPLC refrigerated chamber for analysis.

**DHPLC and sequencing conditions**

The crude PCR products were loaded on a DNA separation column (Transgenomic, Santa Clara, CA, USA) using a Dynamax automatic sample injector model AI-1A. Amplification products were then separated (flow rate: 0.9 ml/min.) over a 4.5 min. period through a linear acetonitrile gradient (54–63% buffer B) at a temperature of 60°C (Bénit et al., 2000). The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (TEAA) pH 7.0 with (buffer A) or without 25% acetonitrile (buffer B).

The mutation was characterized by direct sequencing using the Big Dye terminator cycle sequencing kit (Perkin Elmer, Courtaboeuf, France).

**RESULTS**

Prior to prenatal diagnosis of the pregnancy of patient II:5, linkage analysis using microsatellite markers close to the TSC1 and TSC2 loci was performed in the family. Linkage to TSC2 was ruled out (results not shown), but segregation of D9S1830, D9S164 and D9S114 were consistent with linkage of the disease with TSC1. All affected individuals tested shared a single haplotype (3,3,3) (Figure 1, I:1, II:5 and II:6) that was not found in healthy family members.

DNA from individual II:5 and normal controls were subsequently used to screen the full coding sequence of the TSC1 gene by DHPLC. Exon 18 amplified product gave an abnormal elution pattern (Figure 2). Figure 2A shows a normal profile from control DNA: a single narrow peak is eluted at 3.85 min corresponding to a single homoduplex double-stranded DNA population. Figure 2B shows the abnormal pattern of individual II:5 which displays a strong retarded band corresponding to the heteroduplex DNA formed of wild-type and mutant DNA molecules. The PCR product was sequenced and a heterozygous C2227T mutation was detected which corresponded to the Q743X nonsense mutation, previously described as a TSC-causing mutation (Young et al., 1998; Kwiatkowska et al., 1998).

At 12 weeks’ gestation a CVS was performed in individual II:5. TSC1 exons 18 from the fetal DNA, heterozygous affected and unaffected controls were amplified and analyzed by DHPLC as described previously (Figure 2). Analysis of the DNA extracted from the chorion sample (Figure 2C) clearly showed the same pattern as in Figure 2A, therefore indicating an unaffected fetus.

Diagnosis was confirmed by linkage analysis. The haplotype observed was indeed consistent with the wild-type diagnosis achieved through DHPLC analysis (Figure 1). Individual II:5 has now delivered a healthy baby boy.

**DISCUSSION**

Very few data regarding DNA-based prenatal diagnosis of TSC are currently available. Until recently, prenatal diagnosis of TSC was based upon the detection of either cardiac rhabdomyomata by ultrasonography (Journel et al., 1986; Muller et al., 1986) or cerebral manifestations such as microgyria or cortical tubers by magnetic resonance imaging (Mirlesse et al., 1992; Werner et al., 1994, Sonigo et al., 1996). However, these manifestations usually occur late in the pregnancy, commonly in the third trimester. Moreover, a normal imaging does not rule out the diagnosis of TSC and a significant fraction of lesions remain undetected (Werner et al., 1994; Sonigo et al., 1996).

The mapping and identification of TSC1 and TSC2 genes have recently provided useful tools for earlier assessment in couples with significant risks of having TSC-affected offspring. However, prenatal diagnosis using DNA markers is often hampered by (1) the lack
of informative meioses due to small family size, and (2) the high rate of de novo mutations precluding the identification of the disease-causing gene (TSC1 or TSC2). Moreover, even when indirect analysis based on the segregation of the mutant allele is theoretically possible, the risk of germline mosaicism strongly undermines the validity of this strategy for prenatal diagnosis. For these reasons, direct mutation detection is necessary, but technical difficulties in screening all the TSC1 and TSC2 coding sequences remain to be overcome to provide an efficient genetic service. Hence, novel technological approaches are required in order to improve sensitivity and rapidity of mutation scanning. Methods such as SSCP, DGGE or PTT are widely used for mutation detection, however they are extremely time consuming, often require long and difficult protocol optimization (DGGE, PTT) and are only 30–80% efficient in detecting mutations (SSCP, PTT). DHPLC, however, has marked advantages over classical approaches in term of time, cost and flexibility.

When using DHPLC compared to other methods several factors contribute to the gain of time: (1) automation of the DHPLC procedure precluding the need for any time-consuming gel preparation and loading, (2) the larger size of PCR fragments which can be analyzed and (3) the ease of optimization of the elution time and column equilibrium for each PCR fragment. All these parameters contribute to the high throughput capacity of this technique thus enabling the analysis of up to 200 PCR products a day with a single instrument. Moreover, the high sensitivity and specificity of the method (close to 100%) challenges the long, unfruitful and frustrating searches encountered when implementing more classical approaches (Liu et al., 1998; O'Donovan et al., 1998; Gross et al., 1999). It is also worth noting that the running cost of the DHPLC (about $1 per PCR product) is markedly low, regardless of the number of samples to be analyzed. Indeed, in contrast to gel-based mutation detection methods, small series of samples can be readily analyzed by DHPLC without wasting time and reagents.

Previous work in the laboratory demonstrated the increased efficiency of DHPLC over other screening methods such as SSCP or PTT for mutation detection in the TSC1 gene. Indeed nucleotide substitutions detected by either SSCP or PTT were all picked up by DHPLC which additionally detected three other sequence variations. Moreover, all abnormal DHPLC patterns were subsequently accounted for by TSC1 sequence variation (100% specificity) (Bénit et al., 2000).

In conclusion, the reproducibility and simplicity of this technique makes it directly applicable for prenatal diagnosis without the need for any extra optimization. DHPLC should therefore be considered by all centers involved in mutation detection and diagnostic procedures.

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