Detection of Mutations in the CYP21 Gene Using the DCode System

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**Introduction**

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is caused by mutations in the gene CYP21 encoding the enzyme steroid 21-hydroxylase. In addition to deletions, approximately 20 different point mutations have been reported and novel mutations are still detected. This makes genetic diagnosis as well as carrier detection of 21-hydroxylase deficiency a complicated matter. We have used the denaturing gradient gel electrophoresis method (DGGE) using the DCode universal mutation detection system to detect mutations in the coding sequence and intron-exon junctions of CYP21.

**Materials and Methods**

**Samples**

DNA from healthy individuals and patients with 21-hydroxylase deficiency was isolated from peripheral blood lymphocytes using the salting-out method by Miller.

**Polymerase Chain Reaction (PCR)**

For amplification of CYP21 and selection against CYP21P, PCR was performed according to Wedell. The resulting two PCR products were purified and subsequently used as template for the amplification of DNA fragments for DGGE. The PCR reactions were performed with GC-clamped primers. The GC-density of almost all CYP21 exons are very high requiring long GC-clamps to obtain a single melting domain of the sequence of interest.

**Site-Directed Mutagenesis**

In order to obtain mutant control samples for all exons it was necessary to introduce mutations in some DNA fragments. This was done by PCR-based site-directed mutagenesis. The mutant PCR product was mixed with the corresponding wild type PCR product; the mixture was placed at 96 °C for 10 minutes to denature the PCR products and subsequently left at room temperature for gradual renaturation, thereby generating heteroduplex molecules.

**Denaturing Gradient Gel Electrophoresis**

Denaturing gradient gel electrophoresis was carried out using the DCode system (Bio-Rad). Fifteen µl of PCR product was loaded on a gel containing a polyacrylamide gradient ranging from 6–12% and a gradient of urea and formamide. The gels were run in 1x TAE buffer at 80 V overnight. After electrophoresis, the gels were stained in TAE buffer containing ethidium bromide and subsequently the resolved bands were visualized by ultraviolet (UV) transillumination.

**Fig. 1.** DGGE analysis of CYP21. Each exon is represented by a mutant and a normal control sample. Exon 2 (lane 1–2), Exon 5: (lane 3–4), Exon 6: (lane 5–6), exon 7: (lane 7–8). For exon 2 and 5 the mutant control samples were obtained by site-directed mutagenesis at the following nucleotide positions: exon 2: 2011 (A→C) and exon 5: 2851 (T→G). Nucleotide positions are given in accordance with CYP21, GenBank accession numbers: M12792; M23280. For exon 6 the mutant control sample represents the cluster-E6 mutation (lane 5) and for exon 7 the Val281Leu mutation (lane 7). Additional information about assay conditions will be given on request.
Results
The results of the DGGE analysis of exons 2, 5, 6, and 7 are presented in Figure 1. The presented DGGE analysis was carried out using a denaturing gradient of 30–70%. To validate the method, one wild type control sequence and one heterozygous mutant sequence was analyzed for each exon. For exons 2 and 5 the mutant controls were generated by site-directed mutagenesis at the following nucleotide positions: exon 2: 2011 (A→C) and exon 5: 2851 (T→G). Nucleotide positions are given in accordance with CYP21, GenBank: accession numbers M12792; M23280. The mutant control sample for exon 6 contains the cluster-E6 mutation and for exon 7 the Val281Leu mutation.

Discussion
To perform complete genotyping of 21-hydroxylase disease alleles and reliable carrier diagnosis, it is essential to use a technique that not only detects the most frequent previously identified point mutations but also both undefined and rare point mutations. The DGGE analysis fulfills these criteria, and therefore constitutes a fast and reliable procedure for genetic analysis of 21-hydroxylase deficiency.

References