Prenatal Diagnosis of Steroid 21-Hydroxylase Deficiency by the Modified Polymerase Chain Reaction to Detect Splice Site Mutation in the CYP21 Gene

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Abstract. A splicing junction mutation at nucleotide 656 (A-> G substitution, I2G) in the steroid 21-hydroxylase gene (CYP21) is the most frequently detected mutation in patients with the salt-wasting and simple-virilizing forms of steroid 21-hydroxylase deficiency (approximately 60%). In this disease, prenatal diagnosis and treatment to minimize the effects of excess androgen in affected females has been advocated. Therefore, to detect the I2G mutation rapidly, accurately, and without the use of radioisotope, we developed a modified polymerase chain reaction (PCR) with a mismatched 3′ nucleotide primer to introduce a new restriction site upon PCR amplification of the mutant allele. This allowed the mutant allele to be identified readily by restriction enzyme digestion of the PCR product, and subsequently this PCR product was subjected to restriction enzyme digestion for diagnosis. Chorionic villus biopsy samples (CVS) were obtained at 10 to 11 weeks gestation from two females carrying fetuses at risk for steroid 21-hydroxylase deficiency. Prenatal diagnosis was successful in both cases. One affected female was treated with dexamethasone to term. In the other case, treatment was withdrawn at an early stage when testing revealed a normal fetus. The results demonstrate the rapid and accurate detection of the I2G mutation by this method, thereby indicating the feasibility of for prenatal diagnosis of the I2G mutation.

Key words: Steroid 21-hydroxylase deficiency, Prenatal diagnosis, Polymerase-chain reaction, Chorionic villus sampling


CONGENITAL adrenal hyperplasia due to steroid 21-hydroxylase deficiency is one of the most common inherited metabolic diseases [1]. This disease is caused by deletions or several point mutations of the steroid 21-hydroxylase (CYP21) gene. Among several point mutations, the most frequently identified point mutation in the CYP21 gene in salt-wasting (SW) and simple virilizing (SV) patients with steroid 21-hydroxylase deficiency is an A-> G transition at nucleotide 656, causing aberrant splicing [2–9]. This mutation is detected in approximately 60% of Caucasian and Japanese patients [2–9]. In this disease, prenatal diagnosis and treatment are advocated to prevent ambiguous genitalia in female newborns [1]. The measurement of amniotic fluid hormone levels and polymorphic
HLA haplotypes markers has been used for prenatal diagnosis of steroid 21-hydroxylase deficiency [1]. For maternal dexamethasone treatment to be effective however, it must be started early in pregnancy. Therefore, early prenatal diagnosis is important to prevent unnecessarily prolonged exposure to dexamethasone of males and unaffected females. Recent diagnostic advances include early detection by chorionic villus sampling (CVS) combined with molecular analysis for direct detection of the mutations of the CYP21 gene by polymerase chain reaction (PCR) [10-13]. The previous methods using PCR and subsequent dot blot analysis for prenatal diagnosis have required a radioactive probe [10-12]. Recently, a nonradioactive PCR method for detection of eight frequent mutations of CYP21 gene has been reported and used for prenatal diagnosis [9,13], and this large study revealed that proper prenatal diagnosis and treatment with dexamethasone has been proved to be effective and safe [13].

In this study, to detect the most frequent I2G mutation in Japanese patients with 21-hydroxylase deficiency more rapidly and safely without the use of radioactive probes, an assay was developed that use a 3' PCR primer with one mismatched base pair in order to introduce a new restriction site during amplification of the allele bearing the I2G mutation. We report here the successful use of this modified PCR methodology in the prenatal diagnosis of steroid 21-hydroxylase deficiency.

**Materials and Methods**

In two families, pregnancies for prenatal diagnosis were considered, because these families had prior female children affected with the classic form of CAH due to 21-hydroxylase deficiency, exhibiting ambiguous genitalia at birth and requiring surgical correction. In these families, genetic diagnosis of the prior proband, father and mother was already determined by direct sequencing as described previously [7]. These results revealed that genotypes of the probands were homozygous for I2G mutation, and their parents were carrier for the I2G mutation [7].

Informed consent for prenatal diagnosis and treatment was obtained from two families. Maternal dexamethasone (20 μg/kg) treatment was commenced at 8 weeks gestation in both families [1]. Whole blood was collected from their mothers and fathers, and DNA was prepared by the standard procedure. At 10-11 weeks gestation, DNA from CVS cells was also prepared for the prenatal diagnosis.

Specific PCR amplification of the CYP21 gene was performed with primers A and B: A: 5'-TGGGCATCCCCAATCCAGGTCC-3' (134–156) B: 5'-ACCACGTGAGGCAGAGAT-3' (677–656). Primer A was specific for the CYP21 gene sequence [14]. To make a restriction enzyme site artificially at the mutation site of the I2G, we employed the mismatched primer of B described in Fig. 1. The 3' T base of primer B does not match the sequence of the CYP21 gene. In previous studies, single primer-template mismatch of one base from the 3' nucleotide of a primer has not been shown to significantly alter the PCR yield, and such mismatched primers have been used to introduce the restriction site for mutation detection in several genetic diseases [15, 16]. If the I2G mutation is present, the PCR product using primers A-B can be digested by Sau 3A-I and split into 129 and 23 bp bands (Fig. 1). In contrast, if the normal allele is amplified, a Sau3A-I recognition site does not occur, and PCR product shows only a 152 bp band (Fig. 1). The PCR amplification was carried out for 30 cycles with each cycle of incubation consisting of 30 sec at 94 °C for denaturation, 30 sec at 65 °C for annealing and then 45 sec at 72 °C for polymerization. Restriction digests of PCR products were carried out according to the manufacturer’s recommended protocols. After digestion, the reaction mixture was electrophoresed on a 2.5% agarose gel. The presence of the mutations was confirmed by direct sequencing as described previously [7]. The sex of the fetus was determined by amplifying the sex determining region of the Y chromosome (SRY) as described previously [17]. Postnatal genetic test of two cases was also performed by the above described method of restriction enzyme digestion and direct sequencing with DNA from whole blood.

**Results**

The results of prenatal diagnosis are given in the Table 1.
Family 1

In family 1, the each parent’s PCR product showed two bands, 152 and 129 bp, and they were heterozygous for the I2G mutation (Fig. 2, lanes 2 and 3) as expected from the previous study. By contrast the PCR product from the fetus (after Sau3A-I digestion) showed a single band of 129 bp, indicating that the fetus was either homozygous or hemizygous for the I2G mutation (Fig. 2, lane 1, Table 1). SRY could not be amplified from the CVS DNA, and therefore the fetus was diagnosed to be an affected female. This female was treated with dexamethasone to term. The female infant was born with normal female genitalia. Postnatal testing showed that she was also homozygous for the I2G mutation.

Family 2

In family 2, the father and mother were heterozygous for the I2G mutation, but the DNA from the CVS had only the 152 bp band, indicating that the fetus had inherited both normal alleles. An SRY band was also amplified, leading to the diagnosis of a normal male. Dexamethasone treatment was discontinued in view of these results. After the male infant was born, postnatal testing confirmed that he carried only normal alleles.

Discussion

The direct detection of point mutations of the CYP21 gene using PCR for the prenatal diagnosis of steroid 21-hydroxylase deficiency was reported [10-13]. These direct methods using PCR were sensitive and accurate, but employed
Recently, the method based on PCR without radionucleotide for the prenatal diagnosis was reported [13]. Our study describes a sensitive method without radioactive probe that allows the I2G mutation to be visualized rapidly rather than by allele-specific dot blot. Because the I2G mutation is the most frequent cause of 21-hydroxylase deficiency [2–8] and the frequency of the I2G mutation in Japanese population is higher than that in Caucasian, this method is useful for the carrier detection and prenatal diagnosis of the approximately 25% of Japanese families [2, 7], in which the disorder results from homozygosity for the I2G mutation. And for those families in which the affected individuals are compound heterozygotes for the I2G mutation and a different gene conversion event [2–8], this method could be combined in the future with a similar approach to detect the other known mutations of the CYP21 gene.

In conclusion, we demonstrate that the I2G mutation of the CYP21 gene can be detected readily by introducing a restriction enzyme site during PCR amplification with a mismatched primer. This simple, nonradioactive and inexpensive method should facilitate the prenatal diagnosis of steroid 21-hydroxylase deficiency.

References


