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ORIGINAL ARTICLE

MOLECULAR ANALYSIS OF A FAMILY WITH CONGENITAL ADRENAL HYPERPLASIA – GENOTYPE/PHENOTYPE DISCREPANCY

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ABSTRACT

Congenital adrenal hyperplasia (CAH) is a common autosomal recessive disease with a variable clinical presentation caused by a spectrum of different mutations. A significant association of genotype with phenotype has been reported.

The molecular analysis of a girl with a mild form of CAH presenting with precocious pubarche, confirmed that she was heterozygous for two mutations of the CYP21 gene (exon 1, codon 30/exon 8, codon 318). Her mother was homozygous for the codon 30 mutation and her father was homozygous for the codon 318 mutation. The only anomaly in the parents was a difficulty in conceiving. The molecular analysis of this family confirmed the variability of presentation in carriers of different mutations, which caused difficulties in decisions about the timing of therapy and in genetic counseling.

Key words: Congenital adrenal hyperplasia (CAH), CYP Mutations, Phenotype

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a heterogenous group of hereditary autosomal recessive disorders characterized by deficient synthesis of adrenal steroid hormones. It is the most frequent cause of adrenal insufficiency and ambiguous genitalia in infancy. The disease is caused, in most cases (more than 90%), by defects in the gene coding for steroid 21 hydroxylase, CYP21 [1,2].

Classical forms of CAH can manifest as lethal mineralocorticoid and cortisol insufficiency, or as a simple virilation. The late onset form presents in girls and young women with only hirsutism and oligoamenorrhea, whereas cryptic forms have no clinical symptoms and are detected only when family studies are performed [3-7]. Such heterogeneity has imposed difficult questions about the genetic background of the disease.

The incidence of different forms of the disease varies in different populations. Throughout Caucasian and Oriental populations the average incidence is 1:15,000 newborns. In general, three-quarters of the affected children have the salt-wasting form and only 20% have simple virilization.

The CYP21 gene is frequently modified through mutations and conversions from the very homologous pseudogene CYP21P [8-10]. A strong relation of genotype to phenotype exists. Aberrant splicing in intron 2 at nucleotide (nt) 656, an 8 bp frameshift deletion at codons 111-113, a thymine insertion at codon 306, a nonsense mutation at codon 318, and a single base substitution at codon 356, result in a complete inactivation of 21-hydroxylase and are found in the severe classical form of salt-wasting disease [3-5]. A

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single base change in exon 1 at codon 30, in exon 3 at codon 105, in exon 7 at codon 281, and in exon 10 at codon 453, are associated with the milder non classical form of CAH, in which there is only partial loss of 21-hydroxylase activity [4,6]. The simple virilization found in some CAH patients is associated with a mutation in exon 4 at codon 172, which abolishes 21-hydroxylase activity [4,7]. Combination of these mutations can cause different phenotypes [11,12].

We present a young girl who had a combination of severe and mild mutations and presented as simple virilization and whose parents have CAH which manifested only as a difficulty to conceive.

CASE REPORT

The patient was a 5 and a half-year-old girl, born as the first child of healthy young parents who had been. treated for sterility for 9 years. She was conceived by *in vitro* fertilization. No data were available about the cause of infertility. Mother did not have any health problems. She did not have hirsutism, nor precocious puberty. The father was a healthy individual, and was told that he had a poor sperm count during the sterility treatment. Both parents are Macedonians of Slavic descent. The birth weight of the girl was 3,000 g and birth length was 50 cm. She was developing normally. At the age of 5 years, pubic hair appeared and she was referred to the hospital for hormonal work-up. Clinical examination revealed a healthy and well-developed female child with pubic hair P2 according to Tanner [13]. The clitoris was enlarged (stage 2 according to the Prader classification of sexual ambiguity) [14]. No other pubic signs or abnormalities were detected. Her growth was at 90%o, and her weight at 75%o according to the Tanner-Whitehouse charts [15]. Bone age was advanced and was 9.5 years at diagnosis [+2.5 standard deviation (SD)]. 17-OH Progesterone was 4.0 ng/mL (within the normal range), but after stimulation with ACTH, reached 257 nmol/L at 2 hours (normal peak up to 121 nmol/L). The testosterone level was 2.51 nmol/L (normal range 0.19-2.67 nmol/L), and Dihydroepiandroster-one-S levels were normal.

She was diagnosed with a simple virilizing form of CAH. Treatment with hydrocortisone (7.5 mg twice daily divided) stopped the development of pubic hair, and improved the height/bone age ratio. Three years after diagnosis she grew at 75%o and her pubic hair is P3 according to Tanner.

MATERIALS AND METHODS

Blood samples for molecular diagnosis of CYP21 mutations were obtained from the girl and both parents. Genomic DNA was isolated from leukocytes using the standard proteinase K/SDS digestion-phenol/chloroform extraction-ethanol precipitation method.

Because of the high homology between the CYP21 and CYP21P genes (98% of nt sequences), a strategy to amplify the CYP21 gene differentially was used, following the polymerase chain reaction (PCR) conditions described in Gene Amp XL (Extra Long) PCR Kit (Applied BioSystems, Foster City, CA, USA). The primary PCR product was used as a template for secondary PCR amplification. Using the amplification-created restriction site (ACRS) approach for direct detection of mutations [16], a secondary PCR was then performed using a panel of primers specific for 11 known CAH mutations, according to the method previously described [17].

Following the secondary ACRS PCR, 3 μL of the PCR product was incubated, overnight at 37°C, with 5 to10 units of a specific restriction enzyme (Table 1), then analyzed by 2% agarose gel electrophoresis. Subsequent restriction analyses allowed the detection and the determination of the zygosity of the mutation analyzed.

RESULTS AND DISCUSSION

To distinguish between the inactive pseudogene (CYP21P) and the active gene (CYP21), two pairs of oligonucleotide primers (21AF/AR and 21BF/BR, respectively) were used for differential amplification of these genes as previously described [17]. A 3.2 kb product was detected (Figure 1).

The PCR products generated from CYP21P and CYP21 were distinguishable by digestion with the restriction enzyme *Eco*RI, which for the CYP21P produced three fragments (0.5, 0.6 and 2.2 kb), whereas for the active CYP21 gene produced only two fragments (1.0 and 2.2 kb). The primary PCR products of the CYP21 gene were subjected to locus analysis by using secondary ACRS PCR (Table 1).

Two of the 11 CAH mutations analyzed were detected in the proband and in her parents (Figure 2).

Table 1. Amplification-created restriction site detection of mutations in the CYP21 genes

Primer Pair	Mutational Allele	Restriction Site		Fragment Size (bp)	
		Natural[a]	Created[b]	Normal	Mutant
C1N/C2	Exon1, codon 30	–	*Pst*I	195	164+31
C3B/C4A	Intron 2, nt 656	–	*Sac*I	115	85+30
C3B/C4A	Exon 3, codons 111-113	–	*Rsa*I	115	89+26
C5/C6	Exon 4, codon 172	–	*Mse*I	148	118+30
C7D1/C8	Exon 6, codon 236	–	*Mbo*I	114+26	140
C7E/C8	Exon 6, codon 237	–	*Taq*I	140	116+24
C7C1/C8	Exon 6, codon 239	–	*Mse*I	140	110+30
C9/C10-1	Exon 7, codon 281	*Apa*LI	–	116+101	213
C9A/C9B	Exon 7, codon 306	–	*Mwo*I	123+34	157
C11/C12	Exon 8, codon 318	*Pst*I	–	146+51	197
C11/C12	Exon 8, codon 356	–	*Msc*I	197	167+30

[a] Natural: in two cases, the mutations had naturally created recognition sites for direct restriction detection.
[b] Created: region-specific primers for nine known mutation loci were designed that would lead to the creation of new restriction recognition sites at these known mutation sites on secondary amplification.

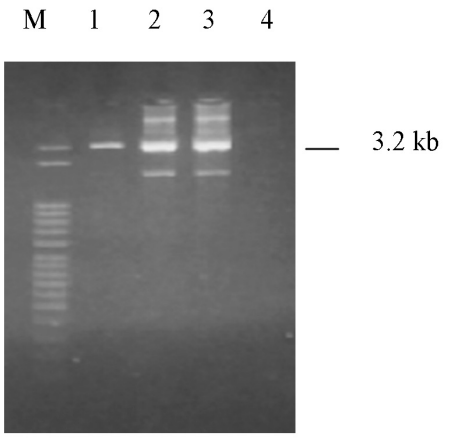

Figure 1. Differential amplification of the active CYP21 gene. Lanes 1, 2, 3: A 3.2 kb product of the active CYP21 gene; Lane 4: blank; M: marker (50 bp).

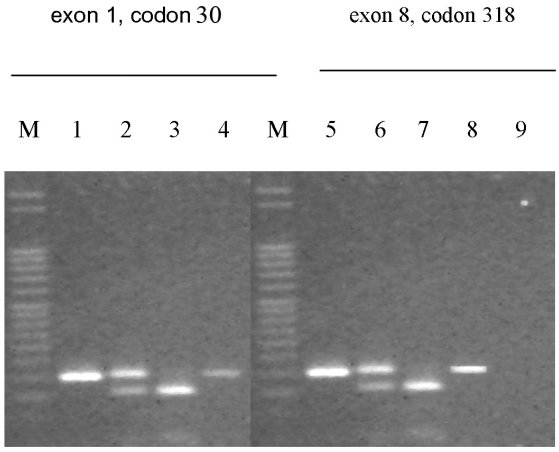

Figure 2. Mutations in exon1/codon 30 and exon 8/codon 318 of the CYP21 gene. M: marker (50 bp); lanes 1 and 5: PCR II product; lanes 2 and 6: patient; lanes 3 and 7: mother; lanes 4 and 8: father; lane 9: blank.

The proband was heterozygous for two mutations, one in exon 1 (at codon 30, which is considered to be a mild mutation), the second in exon 8 (at codon 318, considered to be a severe mutation). This genotype usually produces a moderate phenotype of the disease. The mother was homozygous for the mutation in exon 1 (at codon 30) and only had difficulty in conceiving. The father was homozygous for the mutation in exon 8 (at codon 318), a nonsense mutation usually associated with severe salt-wasting, and only suffered from difficulty in fathering a child, which is an unusual presentation of the disease. These results

indicated that the proband inherited the mutation in exon 1 at codon 30 from her mother and that in exon 8 at codon 318 from her father.

A clear genotype/phenotype correlation in CAH patients has been established by many large studies [18,19]. Classification of mutations into severe, moderate and mild, and their combination, can predict the severity of the clinical presentation in approximately 79% of patients [18]. However, there is a high degree of overlap between moderate and mild forms. The Pro30Leu anomaly, usually associated with a simple virilizing form of the disease, is one of the less frequent mutations, and has also been described in non classical mild forms of the disease [19]. Our patient carried one severe mutation (codon 318) and one moderate mutation (codon 30), and presented with a mild form of late onset and mild evolution. From her genotype, we would expect a more severe form of the disease with earlier presentation and more severe virilization. Treatment was effective in the progression of the puberty and bone maturation.

Both parents are also CAH patients on the basis of genotype. The mother, who was homozygous for the mild mutation (codon 30), did not have symptoms of the disease until the age of conception, when she was unable to conceive and was treated for sterility. No history of early virilization was present. Her clinical presentation is consistent with the genotype, although earlier presentation would have been expected.

The father, homozygous for the severe nonsense mutation (codon 318), which usually presents with a salt-wasting form accompanied by precocious puberty and frequently by sterility. Although improvement of salt-wasting has been shown with age in some studies [19], we were not able to find another published case of homozygosity for the nonsense mutation who survived childhood without therapy. All inquiries about the father's early puberty were also negative. Thus, his only presentation was a poor sperm count. *De novo* recombination and unequal crossover involving CYP21 have been documented in sperm [19], however, there is no evidence of this process in our patient. It is impossible to postulate which parent contributed most to the failure to conceive a child spontaneously.

One might speculate that there may be additional 21-hydroxylase activity not encoded by the CYP21 gene that contributes to the moderate clinical presentation. Modifying genes and transcription abnormalities of the CYP21 gene could also be involved.

Genotype/phenotype discrepancies pose difficulties, especially for genetic counseling of families. An extended pedigree with genotyping of more members of this family might be helpful in the future.

In conclusion, based on the genotype, a more severe phenotype would have been expected in the mother, the father and the child. It is possible that some protective mechanisms, yielding higher 21-hydroxylase enzyme activity, have been transmitted to the child from both parents. Further analyses of the enzyme activity are necessary to elucidate the unusual clinical presentation and genotype/phenotype discrepancies.

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