ORIGINAL ARTICLE

A p.P30L MUTATION AT THE CYP21A2 GENE IN MACEDONIAN PATIENTS WITH NONCLASSICAL CONGENITAL ADRENAL HYPERPLASIA

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ABSTRACT

Nonclassical congenital adrenal hyperplasia (NCAH) is an autosomal recessive imbalance in cortisol synthesis with adrenal androgen excess. Although rarely recognized in infants, it may cause premature adrenarche and pubarche, virilization in young women and variable symptoms in young men. It is commonly caused by mutations in CYP21A2, the gene for steroid 21-hydroxylase. Patients with the p.P30L allele tend to have pronounced evidence of androgen excess but are categorized as nonclassical. We used direct molecular detection of the p.P30L mutation in CYP21A2 in 11 Macedonian NCAH patients and in 17 members of their families using polymerase chain reaction/amplification created restriction site (PCR/ACRS) analysis and digestion with restriction enzymes. The p.P30L mutation was found in a homozygous state in seven (63.6%) and in a heterozygous state in four (36.4%) patients. Of the latter, one was also heterozygous for the p.Q318X mutation. The p.P30L mutation was found in a heterozygous state in 10 (58.8%) and in a homozygous state in one (5.9%) of the family members. These findings support a role of the p.P30L mutation in NCAH.

Key words: CYP21A2 gene, 21-Hydroxylase deficiency, Nonclassical congenital adrenal hyperplasia (NCAH), p.P30L Mutation

INTRODUCTION

Nonclassical congenital adrenal hyperplasia (NCAH), an autosomal recessive disease, affects about 1 in 30 Ashkenazi Jews and 1 in 1,000 non Jewish Caucasians of mixed ethnicity [1]. It is commonly due to a mutation in the CYP21A2, a gene for steroid 21-hydroxylase [2]. Impaired enzyme activity (20-50% of wild type enzymatic action) leads to adrenal androgen excess. Unlike more severe forms of congenital adrenal hyperplasia (CAH), NCAH is rarely recognized in infants but may lead to premature adrenarche and pubarche, virilization in young women, and variable symptoms in young men [3]. Typically, infant girls do not have genital ambiguity but those who carry combinations of mild and severe mutant CYP21A2 alleles may have mild clitoromegaly or a partial urogenital sinus. In adolescence, females are more seriously affected than males, and may develop distressing

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features of androgen excess, such as hirsutism and acne. Oligomenorrhea, which is rare in adolescence, may be more severe in NCAH. Reproductive problems are also more common in NCAH women than in the general population [4]. Although there is no gender predilection for CAH, males are not readily detected with androgen excess after childhood. Oligospermia and infertility have been described, but less often than in classic CAH [5,6]. Differentiation of 21-hydroxylase deficiency from other enzyme defects is determined by the ACTH-stimulation test, which in patients with NCAH, raises serum levels of 17-hydroxyprogesterone (17OH-P) to 50-300 nmol/L [7].

The CYP21A2 gene is located on 6p21.3, within the class III region of the highly polymorphic HLA histocompatibility complex [8], with the pseudogene (CYP21A1P), with which it shares 98% homology in the exonic sequences. CYP21A2 consists of 3.2 kb pairs and contains 10 exons [9]. It is highly polymorphic, more than 100 alleles having been identified in patients and families with severe, moderate and mild forms of CAH [10,11]. Few alleles are specifically associated with NCAH. Most commonly, late-onset 21-hydroxylase deficiency results from a homozygous conservative point mutation p.V281L, p.P30L, and p.P453S [12]. We used direct molecular detection of the p.P30L mutation in CYP21A2 in 11 Macedonian patients with NCAH and in an equal number of their relatives.

MATERIALS AND METHODS

Nine unrelated girls and two boys with NCAH and 17 of their parents or relatives were studied. Diagnosis was made at age 4 to16 (8.5 ± 3.6) years at the Department of Endocrinology and Genetics, University Children's Hospital, Skopje, Republic of Macedonia. The presenting signs were: premature pubarche/adrenarche, hirsutism and/or oligoamenorrhea, and diagnosis of NCAH was based on these and on advanced bone age, abnormally elevated ACTH-stimulated 17OH-P serum levels and molecular gene analysis.

The samples were screened for the pP30L mutation. The samples heterozygous for pP30L or non carriers for this mutation were also tested for 10 other different mutations (IVS-II-655 C/A>G, 8 bp frameshift deletion at codons 111-113, p.I172N, p.I236N, p.V237E, p.M239K, p.V281L, p.F306+T, p.Q318X and p.R356W) according to the polymerase chain reaction/amplification created restriction site (PCR/ACRS), method. Thus, 90% of all the mutations identified in CAH patients were covered [13,14].

Molecular Analysis. Genomic DNA was extracted from peripheral blood lymphocytes following the standard phenol/chloroform protocol [15]. The primary differential PCR amplification of the active CYP21A2 gene, without contamination from the highly homologous pseudogene sequence, was performed with 20 pmol of each CYP21A2 (21BF/21BR) specific primer (21BF: 5'-TCG GTG GGA GGG TAC CTG AAG-3' and 21BR: 5'-AAT TAA GCC TCA ATC CTC TGC AGC G-3') in a final volume of 100 μL containing 1 μg of genomic DNA, 200 μM of each dNTP, 1.5 mM $Mg(OAc)_2$ and 4U r*Tth* DNA polymerase, XL (GeneAmp XL PCR kit; Applied Biosystems, Branchburg, NJ, USA). The *Eco*RI digestion of the 3.2 kb PCR active gene product, with two fragments (1.0 and 2.2 kb) as an end result, ensured that only the gene sequence had been amplified and analyzed (Figure 1).

The primary PCR product was then used as a template for secondary PCR amplification using ACRS with a pair of primers specific for direct mutational detection of p.P30L mutation (C1N: 5'-CTA CAC AGC AGG AGG GAT GGC-3' and C2: 5'-AGC AAG TGC AAG AAG CCC GGG GCA AGc tG-3'). The secondary ACRS PCR was carried out in a final volume of 50 μL, containing primary PCR product, 50 pmol of each primer, 200 μM of

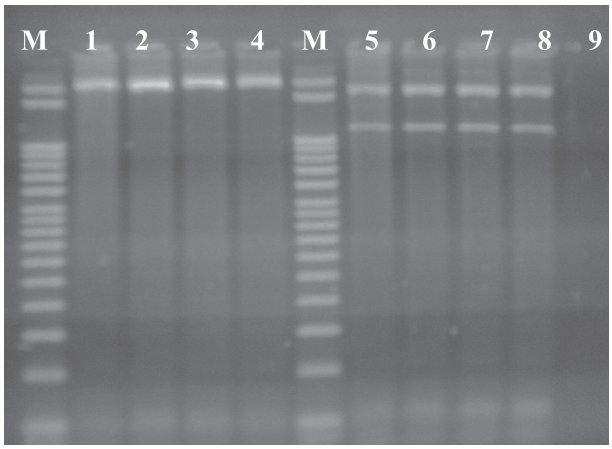

Figure 1. A 3.2 kb PCR product of CYP21A2 (lanes 1-4) and *Eco*RI digestion of CYP21A2 gene (lanes 5-8); lane 9: blank, M: marker (50 bp).

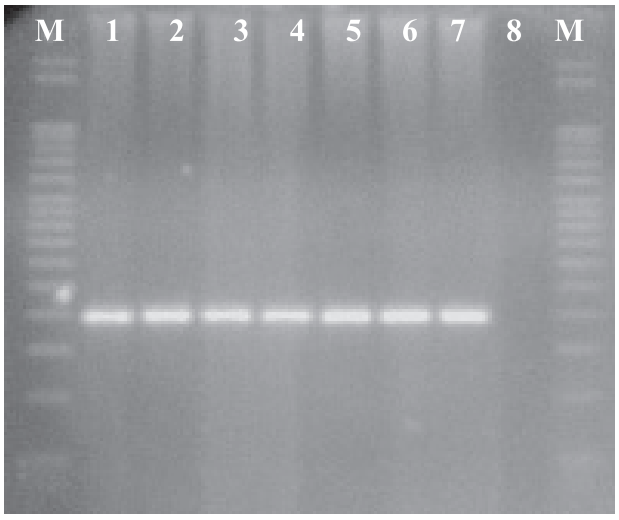

Figure 2. A 195 bp PCR product of ACRS/PCR mutational analysis on exon 1 of CYP21A2 gene (lanes 1-7), lane 8: blank, M: marker (50 bp).

each dNTP, 1.5 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Ampli Taq Gold; Applied Biosystems). A 195 bp PCR product was obtained (Figure 2). The amplified fragments were digested with 5-10 U of a *Pst*I restriction enzyme at 37°C overnight. The digestion products were run on 2% high resolution agarose gel and visualized under UV light after ethidium bromide staining.

The identification was based on presence of a recognition site for the restriction enzyme *Pst*I. Region specific primers create a *Pst*I restriction site in the mutant allele at codon 30 which yields two fragments (164 and 31 bp). In the normal allele there is no restriction site for *Pst*I (Figure 3). Subsequent restriction analysis also allowed determination of the zygosity of the mutation.

RESULTS AND DISCUSSION

Premature pubarche/adrenarche was present in six girls, hirsutism and oligoamenorrhea in two, and amenorrhea in one. The boys presented with adrenarche, typical development of genitalia, and increased rate of growth. Bone age was advanced by 3.6 ± 1.2 years (range: 1 to 4.5). The peak of ACTH-stimulated 17OH-P serum level was higher than 60 nmol/L in all patients.

The p.P30L mutation was detected in all NCAH patients: seven patients (63.6%) were homozygous (five girls and two boys) and four girls (36.4%) were heterozygous. One of the heterozygous patients was also heterozygous for a p.Q318X mutation, as previously reported [14].

Patients with the p.P30L allele, although still categorized as nonclassical, tend to have pronounced evidence of androgen excess [16]. A genotype that contains two mild mutant alleles produces clinical symptoms of NCAH, the allele that produces most enzyme activity determining the phenotype [17,18]. Thus, the patient who was heterozygous for p.P30L and p.Q318X presented with a nonclassical phenotype. However, women who have NCAH may give birth to children with a classical form of the disease if the father is heterozygous for a severe mutation. Prenatal diagnosis in the case of such a combination of parents is strongly recommended. However, the correlation of genotype to phenotype that we found in all of our NCAH patients strengthens the concept that the genotype is predictive of phenotype.

Besides the p.P30L mutation which was derived from pseudogene, mutations not found in the pseudogene, p.R339H, p.P453S [19], and p.H62L [20,21] are also associated with partially impaired *in vitro* activity of 21-hydroxylase but together probably account for no more than 5% of NCAH alleles [22,23]. The p.P30L mutation was found in 11/17 of the family members (64.7%), 10 were

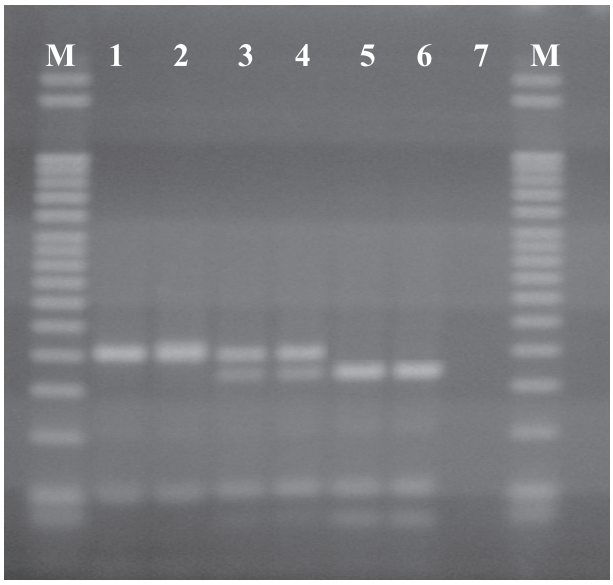

Figure 3. Digestion with *Pst*I restriction enzyme, lanes 1 and 2: normal (195 bp); lanes 3 and 4: heterozygous (195 bp/164+31 bp); lanes 5 and 6: homozygous (164+31 bp); lane 7: blank, M: marker (50 bp).

heterozygotes (58.8%) and one was a homozygote (5.9%). Only the mother (homozygous for p.P30L) and father (homozygous for p.Q318X) of the compound heterozygous patient, as previously reported, had infertility and oligospermia, respectively [18].

The previously reported genetic analysis confirmed a significant genotype to phenotype correlation and a prevalence of NCAH of 1:7000 in the Macedonian population [24,25]. Although p.P30L is classified as a mild mutation in CYP21A2, its role in increased virilization remains to be elucidated.

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