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Characteristics and prevalence of non-classical congenital adrenal hyperplasia with a V281l mutation in patients with premature pubarche

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Abstract

We aimed to determine the prevalence and clinical characteristics of non-classical congenital adrenal hyperplasia (NCCAH) with V281L mutation in patients with premature pubarche. An adrenocorticotrophic hormone (ACTH) stimulation test was performed in 14 of the 159 patients with premature pubarche (PP). Patients whose stimulated 17α-hydroxyprogesterone (17-OHP) level on the ACTH test was ≥10 ng/mL underwent a mutational analysis of the *CYP21* gene. NCCAH was defined in nine (5.7%) patients, all of whom had the V281L mutation. Four of the NCCAH patients were homozygote and four of them were heterozygote. One other patient was compound heterozygote for V281L mutation and the I2 splice mutation. One of the patients with V281L heterozygous mutation developed true precocious puberty and the other one had rapid progressive early puberty and developed polycystic ovary syndrome. ACTH stimulated 17-OHP ≥10 ng/mL in PP patients is load star to mutation analysis and heterozygote patients should be followed for clinical and biological hyperandrogenism up to completion of the whole 'genome sequence'.

Keywords: children; 21-hydroxylase deficiency; non-classical congenital adrenal hyperplasia; premature pubarche; V281L mutation.

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is a common autosomal recessive disorder caused by mutations in the *CYP21* gene. The disorder has traditionally been divided into classical and non-classical 21-OHD types according to expression severity. Non-classical congenital adrenal hyperplasia (NCCAH) is associated with different degrees of postnatal virilization that develop during childhood or at puberty. The clinical manifestations include premature pubarche (PP), hirsutism, acne, menstrual irregularities, and infertility (1). PP is biochemically characterized by mild to moderate oversecretion of adrenal androgens. In 80%–95% of cases, PP is related to idiopathic PP (IPP), the non-pathological exaggerated secretion of adrenal androgens (2). However, in 5%–20% of cases, the cause of PP is late-onset CAH, which is mainly due to NC 21-OHD (3–6).

The *CYP21* gene and a highly homologous inactive pseudo gene, *CYP21P*, are located on the short arm of chromosome 6 within the HLA class III region. The *CYP21* gene deletion and at least 10 sequence aberrations were probably transferred from *CYP21P* by gene conversion and have been identified as causing the different forms of 21-OHD (7). Previous studies relating mutations to enzymatic activity levels have shown that the mild mutations (V281L and P30L) result in an enzyme with 20%–50% of normal activity, and the severe mutations (gene conversion/gene deletion, exon 6 triple-codon mutation, I2 splice, Q318X, and I172N) in an enzyme with 0%–2% normal activity (1, 8). NCCAH is characterized by combinations of mutations that have a less severe effect on enzyme activity, such as P30L, V281L, P453S, or combinations of "severe" and "mild" alleles (9–12).

The aim of this study was to find the prevalence of NCCAH biochemically and perform molecular analysis, and to compare their clinical and laboratory characteristics to patients with IPP and to define the genotypic and phenotypic relationships in these patients.

Materials and methods

The study group was composed of 159 unrelated Turkish patients admitted to our Pediatric Endocrinology Clinic for PP. PP was defined as pubic hair onset before age 8 in girls and age 9 in boys (2). All patients underwent a physical examination, and medical histories were reviewed. Bone age (BA) was evaluated using the standards of Greulich and Pyle (13). Puberty was assessed according to Marshall and Tanner (14, 15).

An adrenocorticotrophic hormone (ACTH) stimulation test was performed in 14 of the 159 children referred for PP who had basal 17α-hydroxyprogesterone (17-OHP) levels ≥2 ng/mL (5). The test was conducted with an intramuscular administration of 0.25 mg Synacthen® (Novartis Pharma, Basel, Switzerland) in all patients at 09:00 h. Cortisol, 17-OHP, and dehydroepiandrosterone

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sulfate (DHEAS) levels were determined at 0, 30, and 60 min post-Synacthen administration. 17-OHP was determined by radioimmunoassay, whereas DHEAS, testosterone, and cortisol were analyzed by chemiluminescence (Roche E-70, Basel, Switzerland). Patients whose stimulated 17-OHP plasma level on the ACTH test was ≥10 ng/mL underwent a mutational analysis of the *CYP21* gene, and those with the mutation were considered to have NCCAH. Otherwise the other patients were considered to have IPP (5, 16, 17).

Peripheral blood samples were obtained from patients after they gave informed consent. Genomic DNA was prepared from peripheral blood leukocytes using standard procedures (18). *CYP21A2* was amplified using previously reported gene-specific PCR primers (19). The following mutations were screened: large gene deletion and 8-bp deletion in exon 3; seven point mutations: P30L in exon 1, A/C655G in intron 2 (I2 splice), I172N in exon 4, V281L in exon 7, Q318X stop codon in exon 8 and a cluster of three point mutations in exon 6 (I123N, V237E, and M239K), and R356W. First, we detected large gene deletions and the 8-bp deletion located in exon 3 by PCR.

The PCR reaction was carried out in a final volume of 100 µL containing approximately 0.7 µg of genomic DNA, 150 ng of each deoxynucleotide triphosphate (Larova, dNTP-set 1), 1.5 mM of $MgCl_2$ (Applichem A5324,0005), 1×PCR buffer, and 2.5 U of *Taq* DNA polymerase (Applichem, 101,005). Thirty-one cycles of amplification were used, each consisting of a denaturation step for 60 s at 95°C, annealing step for 60 s at 58°C, and extension step for 60 s at 72°C (20). The amplification products were analyzed by 1% agarose gel electrophoresis in the presence of ethidium bromide stain.

Four different specific amplifications using *CYP21A2* gene-specific primers were conducted on genomic DNA to analyze the seven point mutations, followed by digestion with restriction enzymes. The restriction endonucleases are listed in Table 1. Electrophoresis was performed on 3% agarose gels containing 0.5 µg/mL ethidium bromide. The lengths of the fragments were evaluated as shown in Table 1 to determine whether mutations were present. All patients were screened for all nine mutations even if a mutation had been detected in a patient.

Results

Among the 159 patients with PP, NCCAH was defined in nine and the remaining 150 patients had IPP. All of the NCCAH patients had the V281L mutation. Other than the V281L homozygote (four patients) and heterozygote mutations (four patients), case I demonstrated a compound heterozygote mutation for the V281L mutation and the I2 splice mutation together.

The gender distribution showed a similarity between NCCAH with the V281L mutation and patients with IPP (p=0.18). Approximately 91% (136/150) of IPP and 77.7% of the patients with NCCAH (7/9) were female (Table 2). Although 12.5% (2/16) of the male patients who were admitted for PP had a diagnosis of NCCAH with the V281L mutation, only 4.9% of the female patients (7/143) were diagnosed with NCCAH and the V281L mutation. The prevalence of NCCAH was higher in male patients with PP than in the females (p=0.001).

When compared with the IPP group, the NCCAH group with the V281L mutation had higher BA and BA-chronological age ratio (Table 3). However, chronological age, age at pubic hair onset, height, height standard deviation score, parental adjusted deficit in height, weight, and body mass index (BMI) were similar in both groups (Table 2). The NCCAH group with the V281L mutation had significantly higher basal 17-OHP, DHEAS, and testosterone levels than did those in the IPP group (Table 3). All nine patients whose peak 17-OHP levels in the ACTH stimulation test were ≥10 ng/mL had the *CYP21* gene mutation. Peak 17-OHP levels of the remaining five patients in whom the ACTH stimulation test was performed were significantly lower than those in patients with a positive mutation analysis (p=0.00).

Clinical and laboratory characteristics of the patients with NCCAH and the V281L mutation are shown in Table 4. A comparison of the clinical and laboratory characteristics of the patients homozygous or compound heterozygous for theV281L mutation with patients heterozygous for the V281L mutation is shown in Table 5.

Oral hydrocortisone therapy was started for all patients with NCCAH (10–15 mg/m^2, divided into three doses) because of progressive virilization, acceleration of growth, and/or advanced BA. Case VIII, with a V281L heterozygote mutation, developed premature thelarche in addition to PP, and thus a gonadotropin-releasing hormone (GnRH) stimulation test was performed. A pubertal luteinizing hormone response was defined as ≥5 IU/L (21); therefore, the patient was treated with an intramuscular GnRH agonist

Table 1 DNA fragments yielded in normal and mutant alleles after digestion.

Mutation	PCR fragment	Restriction endonuclease	Recognition site	DNA fragment	
				Normal	Homozygous
8-bp deletion	64 bp	–	–	64	56
Large deletion	789 bp	–	–	789	–
P30L	854 bp	Aci	CCGC	464, 167, 153, 43, 27	464, 196, 167, 27
IVS2-13A/C	126 bp	Alu I	AGCT	60, 51, 15	60, 34, 17, 15
I172N	330 bp	Bse1 I	ACTGGN	231, 95	217, 95, 14
E6 cluster	1460 bp	Mbo I	GATC	349, 336, 332, 257, 183	685, 332, 257, 183
V281L	1460 bp	Alw211	GTGCT	991, 205, 170, 91	1161, 205, 91
Q318X	1460 bp	Pst I	CTGCA	584, 300, 298, 154, 121	584, 452, 300, 121
R356W	1460 bp	Aci I	CCGC	420, 272, 189, 166, 131, 65, 56, 55, 34, 30, 12, 10, 9, 8	420, 272, 219, 166, 131, 65, 56, 55, 34, 12, 10, 9, 8

Table 2 Clinical characteristics for IPP and NCCAH with V281L mutation.

	IPP (n=150)	NCCAH with V281L (n=9)	p-Value
Age, years	7.7 (4.3–9.9)	8.4 (6.7–12.6)	NS
Sex, female/male	136/14	7/2	NS
Age at pubic hair onset, years	7 (2.5–8.75)	6.6 (5–8.19)	NS
Gestational age, weeks	38 (26–40)	38 (38–38)	NS
Birth weight, g	3200 (1000–4800)	2900 (2400–3400)	NS
Height, cm	127.2 (104.5–146.5)	133 (126–142.4)	NS
Height SDS	0.85 (–1.58/4.04)	0.61 (–2.04/2.43)	NS
Parental adjusted deficit for height	0.8 (–2.13/4.09)	1.38 (–0.04/2.03)	NS
Weight, kg	29.5 (16.5–57.7)	35.4 (23–55)	NS
BMI, kg/m^2	18 (12–31)	19.4 (15–27)	NS

NCCAH, non-classical congenital adrenal hyperplasia; IPP, idiopathic premature pubarche; SDS, standard deviation score; BMI, body mass index; NS, not significant.

(3.75 mg/4 weeks; leuprolide acetate, Ferring GmbH, Kiel, Germany) in addition to hydrocortisone for true precocious puberty. Two of the patients with NCCAH and the V281L mutation were followed long enough to have reached pubertal age and were thus re-evaluated at an age >12 years. Case III (V281L homozygous mutation) had normal somatic maturation and a BA appropriate to his chronological age in the third year of follow-up under treatment. In contrast, case VI, who had the heterozygous V281L mutation (medical history included PP and rapid progressive early puberty and early menarche in her first evaluation when she was 12.6 years old) did not use the prescribed treatment and developed polycystic ovary syndrome with hirsutism and oligomenorrhea when she reached 15 years of age.

Discussion

The frequency of NCCAH among children presenting with PP is variable between 5% and 43% based on diagnostic criteria (3–6). The percentage of NCCAH cases we observed in our cohort was similar to other studies with low rates. The high incidence of the V281L mutation previously reported for this disorder (22) was confirmed in our study. Detecting the V281L mutation in all of our patients with NCCAH suggests the possible high prevalence of this mutation rather than the other two known mutations in our population. Some reports have indicated that patients with NCCAH are taller, heavier, or have accelerated bone maturation than those with IPP (5, 23, 24). Although significant differences were observed among our groups for BA, and BA-chronological age ratio, no differences were found in height, height standard deviation score, parental adjusted height standard deviation score, weight, and BMI. These differences in those parameters might not be of statistical significance due to the small NCCAH group size.

The diagnosis of NCCAH is based on peak 17-OHP concentrations (5, 12, 25). Azziz et al. reported that a basal 17-OHP level >2 ng/mL was highly suggestive of NCCAH (26). Armengaud et al. proposed that a basal plasma 17-OHP level >2 ng/mL presented 100% sensitivity and 99% specificity for identifying NCCAH (5). A cut-off value for an ACTH-stimulated 17-OHP level of >10 ng/mL is currently used for diagnosing NCCAH (5, 16, 17). Based on the detection of genetic mutations in all of our patients with NCCAH, we suggest mutation screening for patients who have ACTH-stimulated 17-OHP levels ≥10 ng/mL. Although the peak 17-OHP response on the ACTH stimulation test was >15 ng/mL in patients with compound heterozygote and homozygote mutations, it was between 10 and 15 ng/mL in two of the four heterozygote cases (case VI, 12.7 ng/mL and case VIII, 13.5 ng/mL) (Table 4). Two patients from the heterozygote

Table 3 Laboratory data for IPP and NCCAH with V281L mutation.

	IPP		NCCAH with V281L		p-Value[a]
	Female (n=136)	Male (n=14)	Female (n=7)	Male (n=2)	
Basal 17-OHP, ng/mL	0.74 (0.1–3.7)	0.6 (0.29–2.2)	7.7 (2.4–8.6)	23.19 (8.19–38.2)	0.000
Peak 17-OHP, ng/mL	3.1 (0.9–9.6)	2.5 (1.5–8)	26.75 (12.7–100)	61.7 (27.5–95.9)	0.000
DHEAS, µg/dL	78.1 (17.7–240.8)	106.7 (26.2–325)	145 (101–192)	151.1 (98.2–204)	0.015
Testosterone, ng/dL	8.39 (0.5–55.3)	10.9 (2–21.7)	43 (10.8–59.12)	41.4 (39.9–43)	0.035
Bone age, years	7.75 (4.2–12)	9.5 (5.5–11)	10 (7–15)	11.3 (10–12.6)	0.004
Bone age-chronological age ratio	1.05 (0.76–2.04)	1.07 (0.87–1.23)	1.25 (1–1.46)	1.28 (1.17–1.39)	0.013

[a]p-Value for differentiation between IPP and NCCAH with V281L regardless of sex distribution. 17-OHP, 17-hydroxyprogesterone; DHEAS, dehydroepiandrosterone sulfate. Testosterone nmol/L=0.0347×ng/dL, DHEAS µmol/dL=0.0271×µg/dL, 17-OHP nmol/L=3.03×ng/mL.

Table 4 Clinical and laboratory characteristics of patients with NCCAH with V281L mutation.

Case no.	V281L/I2A/C656G compound heterozygous mutation	V281L homozygous mutation				V281L heterozygous mutation			
	Case I	Case II	Case III	Case IV	Case V	Case VI	Case VII	Case VIII	Case IX
Sex	Female	Male	Male	Female	Female	Female	Female	Female	Female
Age, years	7.58	8.49	9.3	7.1	8.44	12.6	8.79	7.39	6.9
Height, cm	136	137	137	123	131	142	126	133	125
Height SDS	+2.43	+1.44	+0.5	+0.61	+0.59	–2.04	–0.71	+2.08	+0.89
PAD for height	+1.7	+2.03	+1	–0.29	NA	+0.7	0	+1.76	+1.1
Bone age, years and months	10 years	10 years	12 years 6 months	7 years 10 months	11 years	15 years	8 years 10 months	10 years 8 months	7 years
Bone age SDS	+3.76	+1.95	+1.06	+1.44	+2.22	+1.83	+0.54	+5.08	+0.24
Basal 17-OHP, ng/mL	7.78	8.18	38.2	8.6	7.7	6.5	9.79	2.4	2.4
Peak 17-OHP, ng/mL	100	95.9	27.5	26.5	NA	12.7	27	13.5	55.5

PAD, parental adjusted deficit; NA, not available.

Table 5 Comparison of clinical and laboratory characteristics of patients with homozygous or compound heterozygous and V281L mutation to patients with heterozygous V281L mutation.

	Homozygous or compound heterozygous mutation (n=5)	Heterozygous mutation (n=4)
Accelerated growth	2/5	1/4
Advanced bone age	4/5	2/4
Peak 17-OHP >10 ng/mL	4/4[a]	4/4
Peak 17-OHP >15 ng/mL	4/4[a]	2/4

[a]Peak 17-OHP in ACTH stimulation test was not available in one patient with homozygous mutation.

carriers group had a peak 17-OHP concentration above 15 ng/mL (case VII, 27 ng/mL and case IX, 55.5 ng/mL), consistent with either the homozygous or compound heterozygous state. We performed a screening for the most common mutations, but the "genome sequence" had not yet been completed. We believe that these patients could carry another unidentified mutation on an alternate allele and are thus compound heterozygotes.

The frequency of the heterozygous *CYP21* gene mutation in girls with PP is approximately 45% in some studies, but this was based only on an ACTH stimulation test (27). Other studies have reported an incidence of approximately 30% based on a search for known *CYP21* mutations (22, 28, 29). Potau et al. studied 53 unrelated girls with a history of PP and 35 controls (28). Thirteen PP girls and eight control girls were heterozygous for one of the mutations studied. The frequency of the carrier status was similar between 25% in PP and 23% in control groups (28). In contrast to this study, Witchel et al. reported a higher proportion of carrier rate in PP patients (35%) compared with controls (3%) (29). The carrier rate of our study population was not compared with the control group. Paris et al., who had a lower number of patients with PP than our study, reported that heterozygosity for the *CYP21* gene mutation was detected in 22% of girls with PP, and most carried the mild p.V281L, similar to our patients (30). Much discussion has focused on whether the *CYP21* mutation heterozygous carrier state contributes to hyperandrogenism. Some studies have reported that heterozygous *CYP21* mutations are found more frequently in patients with hyperandrogenism than in the general population (31, 32), whereas others have found no difference (33, 34). Admoni et al. examined the differences between two groups of carriers and showed that the group of symptomatic carriers presented with PP had accelerated growth, advanced BA, and hirsutism, which are symptoms suggesting androgen excess, whereas family member carriers were almost all asymptomatic. The symptomatic carriers had higher ACTH-stimulated 17-OHP levels and a higher rate of the V281L mutation (58%) than did family member carriers (22%). The authors concluded that the higher ACTH-stimulated

17-OHP levels suggested increased exposure to androgens, and thus may point to more severe impairment of 21-OH enzyme activity in the symptomatic-carriers group. In a 13-year follow-up, they showed that 70% of the symptomatic carriers developed additional manifestations of androgen exposure over time, including low final height, true precocious puberty requiring GnRH agonist therapy, and accelerated growth with advanced BA and polycystic ovary syndrome (35). In our cohort, case VI, who had the heterozygous V281L mutation, did not use the prescribed treatment and developed polycystic ovary syndrome with hirsutism and oligomenorrhea. True precocious puberty has been described previously in NCCAH either at presentation or following therapy, when hyperandrogenism has resolved (36). In our cohort, case VIII, who had a V281L heterozygous mutation, developed precocious puberty and was treated with an intramuscular GnRH agonist in addition to hydrocortisone for true precocious puberty. Furthermore, case III, who had a homozygous mutation, had normal somatic maturation and appropriate BA in the third year of follow-up under treatment. Explanations for the phenotypic heterogeneity among individuals carrying the same mutation are still hypothetical and include the presence of other still unidentified mutations, the activity of other genes encoding proteins with extra-adrenal 21-OH activity (37), individual variation in the amount of protein produced (38), differences in intra-adrenal concentrations of progesterone (7), and differences in sensitivity to androgens (39). Additionally, because of incompleted complete gene analysis, there is a possibility that patients with the heterozygote mutation are compound heterozygotes.

Based on our findings, we suggest that if ACTH-stimulated 17-OHP is ≥10 ng/mL, then a mutation analysis should be conducted in patients with PP. Heterozygous patients should be followed for clinical and biological hyperandrogenism up to completion of the complete gene analysis, and a treatment option should be considered.

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