Original Article

Phenotypic variability of hyperandrogenemia in females heterozygous for *CYP21A2* mutations

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ABSTRACT

Objectives: The objective was to seek evidence on the prevalence and consequences of heterozygous *CYP21A2* mutations in girls, adolescent, and adult females with clinical manifestation of androgen excess. **Patients and Methods:** The study included 64 girls diagnosed with premature adrenarche (PA) in childhood and 141 females with clinical hyperandrogenemia manifested in adolescence or adulthood. Direct DNA sequencing and multiplex ligation-dependent probe amplification analysis were used to identify mutations in the *CYP21A2* gene. **Results:** (1) Thirty-four patients were diagnosed with nonclassical-congenital adrenal hyperplasia (NC-CAH) based on the 17-hydroxyprogesterone (17-OHP) levels and the presence of two mutations in *CYP21A2* and therefore were excluded from the study, 66 were found to be heterozygotes and finally 105 had no identifiable mutations. The most frequent mutations among the carriers were the mild p.Val281 Leu and p.Qln318stop. Higher levels of mean stimulated 17-OHP were found in the carriers of the p.Val281 Leu. (2) A notable increased allelic frequency for the known p.Asn493 Ser polymorphism was observed in the pool of females with hyperandrogenemia in whom no mutation was identified. (3) In girls, who presented early with PA, 26.6% were diagnosed with NC-CAH and carried two mutations, 28.7% were identified as heterozygotes 43.7% had no identifiable genetic defect in the translated region of the *CYP21A2* gene. On the contrary, in the group of 141 females with late onset hyperandrogenemia, the presence of 2 mutations was detected in 12%, 1 mutation in 33.4% and no mutation in 54.6%. **Conclusions:** The carrier status for 21-OHD, may be an important factor in the variable phenotype of hyperandrogenism and may be a contributing factor for the early manifestation of the disease.

Key words: CYP21A2, hyperandrogenemia, polycystic ovary syndrome

Introduction

The most common form of congenital adrenal hyperplasia (CAH) (95% of all cases) is due to 21-hydroxylase deficiency (21-OHD) resulting from molecular defect in the steroid 21-OH (*CYP21A2*) gene, with an overall estimated incidence of 1:10,000–1:15,000 for the severe classic form and 1:500–1:100 live births for the nonclassical-CAH (NC-CAH).[1,2]

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The incidence of the genetic defects of 21-OHD has been extensively studied, and ethnic specific distribution of mutations has been reported. As expected from the prevalence of CAH and NC-CAH, the frequency of heterozygotes in the population for 21-OHD is quite common and varies considerably, ranging from 1 in 10 to 1 in 60 in certain population and ethnic groups and in some cases it may affect as 1 in 3 of Askenazi Jews.[3-12]

Approximately, 95% of the mutated *CYP21A2* alleles are the result of recombination events between the homologous CYP21A2P pseudogene and the active *CYP21A2* gene, while the remaining 5% represent new mutations.[13] Several studies in the Mediterranean region, including studies from our group have reported as the most prevalent genetic defects, the mutations c. 655A/C > G (IVS2–13A/C > G), c. 1994C > T (p.Qln318stop), c. 1683G > T (p.Val281 Leu), c. 329_336del (8 bpdelE3).[6,7,14,15]

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Compared to normal female individuals, female carriers for 21-OHD frequently demonstrate an exaggerated secretion of the 21-OH precursors 17-OH progesterone (17-OHP) and P4[16] and lower levels of 11-deoxycorticosterone[16] and aldosterone[17] after adrenocorticotropic hormone (ACTH) administration. In fact, between 50% and 80% of carriers exhibit a 17-OHP level after ACTH stimulation that is above the 95th percentile of the control value.[16,18,19]

The purpose of this study was to seek evidence on the prevalence and consequences of heterozygous *CYP21A2* mutations in girls, adolescent, and adult females with clinical signs of androgen excess.

Patients and Methods

Clinical characteristics

A total of 205 female patients with clinical signs of hyperandrogenemia was evaluated. The inclusion criteria were the clinical manifestation of premature adrenarche (PA) in prepubertal girls and the presence of clinical hyperandrogenemia in adolescent and adult females. Sixty-four presented with PA, which was defined as the development of pubic hair and/or axillary hair prior to 8 years of age. One hundred and forty-one females presented with hyperandrogenemia, which was defined as the presence of hirsutism (Ferriman Gallway score >8)[20] and/or severe acne according to Androgen Excess Society (AES).[21] Polycystic ovary syndrome (PCOS) criteria included irregular ovulation, hyperandorgenism, and/or polycystic ovaries as defined by AES.[21] Basal 17-OHP was measured in all patients. Symptoms of hyperandrogenemia and the elevated 17-OHP levels were used to diagnose the patients with the NC form of CAH. The patients with the NC form had basal 17-OH*P* values more than 15 nmol/l (often >60 nmol/l probably due to stress during sampling) and/or 17-OH*P* values, after intravenous administration of 250 μg of ACTH (1–24), >30 nmol/l (normograms for the diagnosis of 21-OHD). Possible heterozygote carriers were considered those patients with baseline 17-OHP > 6 nmol/l and those with baseline 17-OHP <6 nmol/l and ACTH stimulated <30 nmol/l.[2,5] Stimulated 17-OH*P* values >30 nmol/l revealed the possibility of mutations in more than one allele.[2,5] Serum 17-OHP concentrations were measured with the commercial Radioimmunoassay method (Beckman Coulter). Three hundred females of Cypriot origin were recruited from the cohort of healthy individuals seeking biochemical evaluation prior to their marriage from the National Unit for Thalassemia at the Makarios Hospital in Nicosia, Cyprus. The numbers were calculated based on the inheritance recessive model of the disease. Informed consent was obtained from all individuals, and the study was approved by the Cyprus National Bioethics Committee.

Amplification of the CYP21A2 gene

The *CYP21A2* genes of all patients were analyzed using genomic DNA isolated from peripheral blood samples. Molecular analysis was performed according to a cascade strategy as previously described.[6,7,14] The primers P1–P48[22] were used to amplify the fragment containing the-370 bp *CYP21A2* promoter, the 5' untranslated region of the *CYP21A2* gene that is mainly located in the first 167 nucleotides upstream the ATG codon and the 3' untranslated region that is 536 nucleotides downstream the TGA stop codon of the *CYP21A2* gene.[23] The polymorphism p.Asn493Ser is found in exon 10 of the *CYP21A2* gene, and therefore, was covered with the direct sequencing analysis.

Multiplex ligation-dependent probe amplification analysis

DNA from female patients in this study analyzed by direct sequencing was also examined with the multiplex ligation-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, Netherlands). MLPA was employed to investigate any possible large gene deletions and large gene conversions in the *CYP21A2* gene. The kit detects mutations for exons 1, 3, 4, 6, and 8; among these are c. 329_336del (8 bpdelE3), c. 999T > A (p.Ile172Asn), c. 1380T > A; c. 1383T > A; c. 1389T > A (Cluster E6) and c. 1994C > T (p.Qln318stop) mutations. Furthermore, this kit contains three *CYP21A1P*-specific probes, three *TNXB* probes, one *C4A* probe, one *C4B* probe, and one probe for the *CREBL1* gene which is located q-telomeric of *TNXB*. In addition, two other probes located on chromosome 6p21.3, 1 Y-chromosome specific gene (*UTY*) and 16 reference probes are included. Briefly, 50–200 ng DNA was denatured and hybridized overnight at 60°C with the SALSA probe mix. Samples were then treated with Ligase-65 enzyme for 15 min at 54°C, the reactions were stopped by incubation at 98°C for 5 min. Finally, polymerase chain reaction amplification was carried out using BigDye terminator v1.1, cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Amplification products were run on an automated Applied Biosystems 3130 *xl* Genetic Analyzer. The raw data were analyzed by using Coffalyzer 9.4 Software (MRC Holland, Amsterdam, Netherlands). The size of migration of exon-specific peaks was identified according to their migration relative to Gene Scan 600 LIZ size standard (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Comparisons of 17-OHP levels were performed using parametric methods (*t*-test procedures). A two-tailed alpha level of 0.05 was used to establish statistical significance

for the p.Asn493Ser polymorphism. All statistics analyses were performed using Statistical Analysis System (SAS) software Version 9.2, SAS Institute Inc., Cary, NC, USA.

Results

CYP21A2 genotypic analysis

Thirty-four patients were diagnosed with NC-CAH based on the 17-OHP levels and genotypic analysis (i.e. identified to carry 2 mutations) and were excluded from the study, 66 were identified as being heterozygotes for mutations in *CYP21A2* [Table 1] and 105 had no mutation in the *CYP21A2* [Tables 2-5].

The frequency of the molecular defects detected in the 66 heterozygote females (19 girls with PA and 47 adolescents and adults with hyperandrogenemia) are shown in Table 1. In the total pool of the 66 unrelated heterozygote alleles, the most frequent mutation was c. 1683G > T (p.Val281 Leu) (53.0%), followed by c. 1994C > T (p.Qln318stop) (18.2%), c. 2665C > T (p. Pro482Ser) (10.6%), c. 1752G > A (p.Val304Met) (6.1%), c. 2578C > T (p.Pro453Ser) (6.1%), c. 2296G > A (p.Ala391Thr) (1.5%), large deletion/conversion exons 1–4 (1.5%), large deletion/conversion exons 6–8 and c. 707_714delGAGACTAC (8 bpdelE3) (1.5%). The rare c. 1752G > A (p.Val304Met) was first found in a 7-year-old girl with PA and two adolescent females with PCOS. The large deletion/conversion of exons 1–4 and large deletion/conversion of exons 6–8 were, respectively, found in two young girls at the age of 7 and 9 years, both presented with PA.

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In 32, out of the 47 (68.1%), heterozygote adolescents and adults with hyperandrogenemia, who fulfilled the criteria of PCOS the most frequent mutation was p.Val281 Leu (73.9%).

Baseline and stimulated 17-OHP levels were compared according to mutation found in the 66 carriers. There was no statistically significant difference in the baseline mean 17-OHP levels. Stimulated 17-OHP, however, was higher in carriers of the c. 1683G > T (p.Val281 Leu) mutation compared to the mean 17-OHP found in carriers of the other mutations (21.1 nmol/L vs. 16.6 nmol/L, $P = 0.02$).

Table 1: Mutation frequency of affected alleles from 66 unrelated hyperandrogenic female patients

CYP21A2 mutations	Total	
	Number of alleles	Percentage of alleles
p.Val281Leu	35	53.0
p.Qln318stop	12	18.2
p.Pro482Ser	7	10.6
p.Val304Met	4	6.1
p.Pro453Ser	4	6.1
p.Ala391Thr	1	1.5
Deletion/conversion exons 1-4	1	1.5
Deletion/conversion exons 6-8	1	1.5
8bpdelE3	1	1.5

Table 2: Genotypic analysis, basal, and ACTH stimulated 17-OHP levels in 19 *CYP21A2* heterozygote girls with premature adrenarche. The polymorphism p.Asn493Ser was identified in heterozygosity in patients 1, 2 and 18

Number	Age of diagnosis	Genotype	17-OHP, nmol/l, basal	17-OHP, nmol/l ACTH stimulated
1	7	Large del 1_4	2.4	14.5
2	7	p.Val281Leu/X	6.4	28.3
3	7	p.Pro453Ser/X	3.9	17
4	10	p.Val281Leu/X	6.4	25.4
5	7	p.Val281Leu/X	3.5	23.5
6	7	p.Val281Leu/X	4.3	18.4
7	7	p.Pro453Ser/X	2.2	14.7
8	7	p.Val304Met/X	8.3	13.7
9	8	p.Val281Leu/X	3.7	18.5
10	8	p.Val281Leu/X	7.7	24.2
11	8	p.Val281Leu/X	6.6	20.0
12	8	p.Val281Leu/X	4.2	21.7
13	8	p.Val281Leu/X	3.7	19.8
14	9	p. Val281Leu/X	2.5	17.6
15	9	p.Qln318stop/X	5.3	13.4
16	9	p. Val281Leu/X	4.8	21.2
17	3	p.Qln318stop/X	11.4	17
18	9	Del EX6-8	6.5	11.7
19	9	p.Val281Leu/X	4.3	18.2

17-OHP: 17-hydroxyprogesterone, ACTH: Adrenocorticotropic hormone

A notable increased allelic frequency of 54.3% (57/105) for the known p.Asn493Ser polymorphism was observed in the pool of females with hyperandrogenemia and no mutation when compared with age-matched controls of Cypriot origin (37% [111/300], $P = 0.0001$) [Table 5]. The allelic frequency of p.Asn493Ser was 19.7% (13/66) in the group of the 66 *CYP21A2* heterozygotes ([$P = 0.01$] vs. controls and [$P = 0.0001$] vs. no mutation). In the subgroup of girls with PA, the known polymorphism p.Asn493Ser was detected in 18/28 (64.3%) patients, who concurrently had no identified mutations in the *CYP21A2*: ($P = 0.008$) versus controls and ($P = 0.02$) versus 26.3% in the group of 19 heterozygotes [Table 5].

In the total group of 64 girls, who presented early with PA, 17 (26.6%) were diagnosed with NC-CAH and carried 2

Table 3: Clinical features, genotypic analysis, basal, and ACTH stimulated 17-OHP levels in 17 *CYP21A2* heterozygote adolescent girls with hyperandrogenemia. The polymorphism p.Asn493Ser was identified in heterozygosity in patients 3-5, 13 and 16

Number	Age of diagnosis	Genotype	17-OHP, nmol/l, basal	17-OHP, nmol/l ACTH stimulated	Clinical presentation
1	14	p.Qln318stop/X	4.7	10.3	A, H
2	14	p.Val281Leu/X	2.6	11.0	A, H
3	15	p.Val281Leu/X	5.2	28	PCOS
4	17	p.Pro482Ser/X	6.7	14.7	IM
5	16	p.Val281Leu/X	4.0	17.2	PCOS
6	15	p.Qln318stop/X	6.3	14.8	PCOS
7	16	p.Pro482Ser/X	3.5	17.1	IM
8	15	p.Val281Leu/X	4.8	22.5	A, H
9	16	p.Val281Leu/X	7.1	19.4	IM
10	17	p.Val304Met/X	2.4	9.6	IM, A
11	17	p.Val281Leu/X	12.4	22.2	A, H
12	16	p.Val281Leu/X	5.7	26.3	IM, H
13	16	p.Qln318stop/X	9.2	10.5	PCOS
14	17	p.Val281Leu/X	5.1	15.7	PCOS
15	17	p.Val304Met/X	5.1	11.8	PCOS
16	16	p.Qln318stop/X	6.7	19.6	H, IM
17	15	p.Qln318stop/X	4.2	14.3	A, H

17-OHP: 17-hydroxyprogesterone, ACTH: Adrenocorticotropic hormone, PCOS: Polycystic ovary syndrome, IM: Irregular menses, A: Acne, H: Hirsutism (Ferriman–Gallwey score >8)

Table 4: Clinical features, genotypic analysis, basal, and ACTH stimulated 17-OHP levels in 30 *CYP21A2* heterozygote adult females with hyperandrogenemia. The polymorphism p.Asn493Ser was identified in heterozygosity in patients 4, 10, 23, and 27

Number	Age of diagnosis	Genotype	17-OHP, nmol/l, Basal	17-OHP, nmol/l ACTH stimulated	Clinical presentation
1	24	p.Val281Leu/X	4.5	28.2	PCOS
2	20	p.Val281Leu/X	5.4	15.8	IM
3	18	p.Val281Leu/X	7	18.2	A, IM
4	20	p.Val281Leu/X	9.2	29.4	PCOS
5	19	p.Ala391Thr/X	6.1	15.8	PCOS
6	24	p.Pro453Ser/X	6.2	19.8	IM
7	20	p.Val281Leu/X	4.1	14.6	IM, A
8	22	p.Val281Leu/X	3.8	21.4	A, H
9	21	p.Qln318stop/X	4.3	14.7	IM
10	26	8bpdelE3/X	8.8	12.3	IM, H
11	19	p.Val281Leu/X	7.2	19	IM, H
12	22	p.Qln318stop/X	7.6	16.8	PCOS
13	23	p.Pro482Ser/X	8.9	14.7	A
14	23	p.Pro482Ser/X	3.8	10.4	PCOS
15	24	p.Val281Leu/X	5.6	27.1	IM, A
16	26	p.Pro482Ser/X	1.7	6.7	A, H
17	26	p.Val304Met/X	2.2	15.6	PCOS
18	35	p.Val281Leu/X	4.3	22.8	A, H
19	35	p.Val281Leu/X	12	26.8	IM, H
20	35	p.Qln318stop/X	4.6	3.7	IM, H
21	22	p.Pro482Ser/X	4.3	8.9	IM
22	26	p.Qln318stop/X	7.9	11.4	A, H
23	20	p.Val281Leu/X	10.3	19.8	IM, H
24	19	p.Pro453Ser/X	4.4	11.1	IM, H
25	19	p.Qln318stop/X	10.7	18.1	IM
26	20	p.Val281Leu/X	3.4	17	IM, H
27	19	p.Val281Leu/X	5.0	16.5	PCOS
28	22	p.Val281Leu/X	6.1	11.3	PCOS
29	30	p.Pro482Ser/X	4.1	12.7	IM, A
30	22	p.Val281Leu/X	4.4	18.6	PCOS

17-OHP: 17-hydroxyprogesterone, ACTH: Adrenocorticotropic hormone, PCOS: Polycystic ovary syndrome, IM: Irregular menses, A: Acne, H: Hirsutism (Ferriman–Gallwey score>8)

mutations, 19 (28.7%) were identified as heterozygotes, and 28 (43.7%) had no identifiable genetic defect in the translated region of the *CYP21A2* gene. On the contrary, in the group of 141 females with late onset hyperandrogenemia, the presence of 2 mutations was detected in 17 (12%), 1 mutation in 47 (33.4%) and no mutation in 77 (54.6%) [Figure 1].

Table 5: Allelic frequency of p.N493S polymorphism in 300 controls, girls with PA with and without *CYP21A2* mutations, and adult females with and without *CYP21A2* mutations

Subjects	Number	Allelic frequency of the *CYP21A2* p.Asn493Ser variant
Controls females	*n*=300	37.0% (*n*=111)
Girls with premature adrenarche	Heterozygote for *CYP21A2* mutation (*n*=19)	26.3%† (*n*=5/19)
	Negative for *CYP21A2* mutation (s) (*n*=28)	64.3%** (*n*=18/28)
Adult females with hyperandrogenemia	Heterozygote for *CYP21A2* mutation (*n*=66)	19.7%*** (*n*=13/66)
	Negative for CYP21A2 mutation (s) (*n*=105)	54.3%* (*n*=57/105)

*P=0.0001 versus controls, **P=0.008 versus controls, ***P=0.01 versus controls, P=0.0001 versus 105 females negative for *CYP21A2*, †P=0.02 versus controls

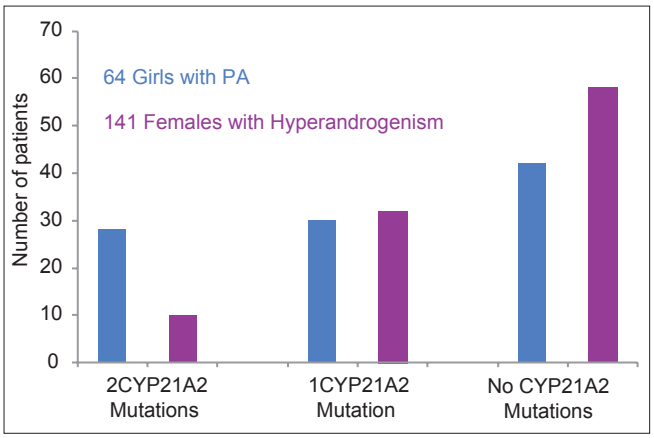

Figure 1: Girls with premature adrenarche (*n* = 64) and females (*n* = 141) who exhibited 2, 1, and no mutations in the *CYP21A2* gene

Discussion

The present study was designed to seek evidence on the prevalence and consequences of heterozygous *CYP21A2* mutations in females with clinical hyperandrogenemia. Most of the females with clinical signs of hyperandrogenism exhibited a significant elevation of stimulated 17-OHP and had identifiable heterozygous mutations in the *CYP21A2* gene. This finding was indicative of carrier status for 21-OHD and was further confirmed with direct DNA sequencing and MLPA analysis for mutations in the *CYP21A2* gene.

Several investigators have shown a high rate of heterozygosity for *CYP21A2* mutations in girls, female adolescents and women with clinical symptoms of androgen excess.[24-28] The presence of heterozygous *CYP21A2* mutations is associated with an increased risk of clinical and biochemical hyperandrogenism.[4,24,29,30] The clinical expression of *CYP21A2* heterozygosity prepubertally is consistent with the study by Dacou-Voutetakis and Dracopoulou,[31] who reported a high incidence of molecular defects of the *CYP21A2* gene in girls with PA, although in a significant number of cases the genotype could not be predicted by the age of onset of PA. A high prevalence of heterozygous mutations was also reported in a cohort of French Mediterranean girls with isolated premature pubarche.[25] In a similar manner as in the case of the 19 heterozygous girls of the present study, the French girls with PA were found to exhibit high 17-OHP levels.[25] In addition, a number of other studies have described both males and females with the milder form of NC-CAH carrying only 1 mutation in their *CYP21A2* gene.[32]

The most frequent mutation among these identified female carriers was the mild c. 1683G > T (p.Val281 Leu) with a frequency of 53.0%. Back in 2006 Admoni *et al*.[24] proposed a dominant-negative effect of the mutant c. 1683G > T (p.Val281 Leu) allele on the wild type by drastically reducing its activity. The mutant enzyme may interfere or compete with the wild type for the conversion of 17-OHP to 11-deoxycortisol. The c. 1683G > T (p.Val281 Leu) mutation is quite common in our population and a high rate seen in the symptomatic carriers group of the present study can be attributed to the high rate observed.[6,7,14] In a random screening of healthy individuals, often used as controls in our laboratory, c. 1683G > T (p.Val281 Leu) was determined in 26 out of 600 alleles or 1 in 23.[33]

The question of the contribution of the *CYP21A2* heterozygosity to the pathogenesis of PCOS has been recently studied by Settas *et al*.[34] who reported no significant input as the frequency of the *CYP21A2* heterozygous mutations in PCOS women and controls did not differ. This discrepancy in the literature could be partly explained by the different methodologies used and the diversity of the clinical phenotype among the patients.

The higher ACTH stimulated 17-OHP mean values exhibited in the group of carriers with the c. 1683G > T (p.Val281 Leu) mutation compared with carriers of other mutations suggest increased exposures to androgens and point to more severe impairment of 21-OHD in the symptomatic c. 1683G > T (p.Val281 Leu) carriers. This finding supports the already identified notion that carriers of the mild c. 1683G > T (p.Val281 Leu) missense mutation exhibit higher ACTH-stimulated 17-OH*P* values and higher rates of either PCOS[24] irregular menses or hirsutism.[35] Patients that exhibit stimulated 17-OHP levels >60.5 nmol/l are consistent with either the homozygous or the compound heterozygous state.

In the present study, the rare missense c. 1752G > A (p.Val304Met) mutation and the less frequent severe large deletions del1–4 and del6–8 were identified. We highlight that c. 1752G > A (p.Val304Met) is a mild and rare mutation and after expression in *COS-1* cells the mutated enzyme was found to have residual activity of 46% for conversion of 17-OHP and 26% for conversion of progesterone compared with the normal enzyme.[36] To our knowledge, c. 1752G > A (p.Val304Met) missense mutation was reported only by our group in Cypriot patients with the NC-CAH form[14] and in a 24-year-old female patient of Asian origin who presented with hirsutism, acne, and alopecia.[36] The c. 1752G > A (p.Val304Met) missense mutation is located at a region suggested to be involved in substrate interaction in a model of the protein.[37]

In the females with clinical hyperandrogenemia in whom the direct sequencing of the coding region and the proximal promoter region of *CYP21A2* gene did not identify any putative new mutation, the polymorphism p.Asn493Ser was identified to be the leading sequence alteration. Among these 57 patients, six were homozygotes with the rest 51 being heterozygotes for the p.Asn493Ser substitution. Most of the patients with p.Asn493Ser presented with elevated baseline or ACTH-stimulated 17-OHP levels and clinical signs of androgen excess. Taking into account the finding from a previous study by our group that the allelic frequency of p.Asn493Ser in the general population of Cyprus is quite high 37.0%[33] and also that in the *CYP21A2* heterozygotes (n = 66) also tested in the present study to be significantly lower to 19.7%, it may be suggested that a possible implication of this alteration could contribute in the pathogenesis of hyperandrogenemia.

Various studies demonstrated this substitution as a naturally occurring polymorphism[38] and some as a disease-causing mutation[39] but its influence on residual enzyme activity has never been tested *in vitro*. A recent study on a molecular model of human *CYP21A2* demonstrated unchanged the electrostatic charge of the non-conserved amino acid alteration p.Asn493Ser.[40] Such a study identified in Mexican CAH patients and healthy controls a higher proportion of homozygosity for the p.Asn493Ser substitution.[41] The presence of elevated basal and ACTH-stimulated 17-OHP, PA, hyperandrogenism, and PCOS in the female patients of the present study identified in heterozygosity or homozygosity only with p.Asn493Ser and with no other pathogenic mutation (s) is suggestive of a direct role of this substitution in the disease manifestation of NC-CAH.

Although no association could be found between the presenting symptom and the type of the molecular defect, it is clear that heterozygote females have a tendency to manifest androgen excess in early life. Heterozygotes for mutations in the *CYP21A2* gene may have mild biochemical abnormalities and in general they do not display a serious endocrine disorder.[42] Regardless of these general correlations, the 21-OHD phenotype does not always correlate precisely with the genotype,[43] suggesting that other genes influence the clinical manifestations.

The novelty provided by the present study is the tendency of heterozygote females to manifest androgen excess and PA in early life. In addition, the high carrier rate of the p.Asn493Ser in female patients with androgen excess with no other pathogenic mutation (s) is suggestive of a possible role of this substitution in the disease manifestation of NC-CAH.

Conclusion

The carrier status for 21-OHD, may be an important factor in the variable phenotype of hyperandrogenism and may be a contributing factor for the early manifestation of the disease. Although the clinical expression of NC-CAH is not solely depended on the genotype, discrimination between mild and severe alleles should be made. The clinical relevance of the early detected molecular defect is emphasized as these girls have a higher probability of having earlier onset of puberty, clinical hyperandrogenemia at puberty, menstrual disturbances, and fertility problems in adulthood and moreover compromised final height. The detection of patients with NC-CAH phenotypes tested negative for mutations in the *CYP21A2* gene underlines the importance to screen for other genes responsible for NC-CAH.

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