Original Paper

HORMONE RESEARCH IN PÆDIATRICS

Horm Res Paediatr 2011;75:180–186
DOI: 10.1159/000320040

Received: December 30, 2008
Accepted: August 4, 2010
Published online: September 14, 2010

Molecular Defects of the *CYP21A2* Gene in Greek-Cypriot Patients with Congenital Adrenal Hyperplasia

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Key Words
Congenital adrenal hyperplasia · *CYP21A2* gene · Mutations

Abstract
Background/Aim: To determine the mutations in the *CYP21A2* gene in Greek-Cypriots with congenital adrenal hyperplasia (CAH) and attempt a genotype-phenotype correlation. ***Subjects and Methods:*** Molecular analysis was performed by multiplex ligation-dependent probe amplification and direct sequencing of PCR products of the *CYP21A2* gene in 32 CAH patients. ***Results:*** The most frequent genetic defect in the classic salt-wasting and simple virilizing forms was the IVS2-13A/C>G (55%) mutation, followed by Large lesion (20%) and in the non-classical form, the p.V281L (79.5%). Genotypes were categorized in 4 mutation groups (null, A, B and C). All 3 patients in the null group manifested the salt-wasting form and all 6 patients in mutation group A presented with the classical form. One patient in group B had the simple virilizing form and 22 patients in group C exhibited the non-classical form. ***Conclusion:*** The spectrum of mutations of the *CYP21A2* gene in our population is comparable to the most common reported in similar ethnic groups. The knowledge of the ethnic specificity of the *CYP21A2* mutations represents a valuable diagnostic tool for all forms of CAH.

Introduction

Congenital adrenal hyperplasia (CAH) comprises a group of autosomal recessive disorders of cortisol biosynthesis, which is caused by the loss or severe decrease in the activity of one of the enzymatic steps required for cortisol biosynthesis in the adrenal cortex. The most common form of CAH (95% of all cases) is due to 21-hydroxylase deficiency (21-OHD) resulting from molecular defect in the steroid 21-hydroxylase *(CYP21A2)* gene, with an overall estimated incidence of 1:10,000 to 1:15,000 live births [1–8].

The *CYP21A2* gene is located on the short arm of chromosome 6, within the region of the major histocompatibility complex, at a distance of 30 kb from a highly homologous (>95%) pseudogene, designated *CYP21A1P*. The location of the *CYP21A2* gene makes it vulnerable

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to relatively large genomic recombinations with its homologous gene, *CYP21A1P*. The proximity of these genes and their location within the *HLA* region, which has a high rate of recombination, facilitate such events [6, 9, 10]. The molecular defects of *CYP21A2* may result from two types of recombinations between the *CYP21A2* and the *CYP21A1P* pseudogene: unequal crossingover during meiosis leading to deletion of *CYP21A2* and conversions that result in transfer of altered sequences from *CYP21A1P* to *CYP21A2*, where they become detrimental [11].

Genetic defects in the *CYP21A2* gene are classified into three categories depending on the residual enzymatic activity and typically correspond to the three types of 21-OHD: salt-wasting (SW), simple virilizing (SV), and non-classical (NC) CAH. In vitro studies have shown that mutations resulting in complete inactivation of 21-hydroxylase activity are associated with the SW phenotype, those that reduce 21-hydroxylase activity to approximately 2% are associated with the SV phenotype, whereas those that reduce 21-hydroxylase activity to 10–75% are associated with the NC phenotype [12]. In the great majority of cases there is a correlation between genotype and phenotype, although it is not always possible to predict the phenotype on the basis of genotype with accuracy [13]. Most patients are compound heterozygotes, and the severity of the disease is determined by the activity of the less severely affected allele [14–16].

CYP21A2 is one of the most polymorphic human genes, and more than 100 alleles have been identified in patients with CAH [Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/ac/index.php]. The incidence of the genetic defects of 21-OHD has been extensively studied and ethnic-specific distribution of mutations has been reported [17]. There is no data about the mutations of *CYP21A2* gene in our population, which is mainly of Greek origin influenced by a genetic impact of surrounding countries. Studies in neighboring countries in the Mediterranean region have reported as the most prevalent genetic defects: IVS2-13A/C>G, p.Q318X, p.V281L and large lesions of the gene [18–23]. Therefore, we aimed to analyze the types and frequencies of mutations in the Greek-Cypriot patients with 21-OHD and study the correlation between genotype and phenotype.

Subjects and Methods

Patients

Thirty-two unrelated Greek-Cypriot CAH patients were studied. In 85% of the cases, DNA samples from parents and siblings were also analyzed. All patients were previously characterized as follows: 22 patients were classified as having the NC form, 8 suffered from the SW form, and 2 from the SV form of CAH. The patients were characterized on the basis of clinical, biochemical findings and an elevated plasma 17-hydroxyprogesterone (17-OHP) [2, 18]. The patients were classified in the SW form when clinical and biochemical findings of renal salt wasting were evident (virilization of external genitalia, failure to thrive, hyponatremia, hyperkalemia, high renin (PRA) values, and 17-OHP value >75 nmol/l in the first month of their lives). Severe clinical symptoms of CAH without electrolyte imbalance (virilization of any degree without clinical evidence of salt loss, acceleration of height and bone age development in early childhood, 17-OHP values >75 nmol/l, and normal/elevated PRA values) were characteristic in patients diagnosed with the SV form. Symptoms of hyperandrogenemia in peripubertal years (premature appearance of pubic hair, severe acne or hirsutism at puberty, with or without menstrual disorders and complete lack of virilization except an isolated clitoromegaly) and 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-OHP values >15 nmol/l (often >60 nmol/l probably due to stress during sampling) and/or 17-OHP values, after intravenous administration of 250 μg of ACTH (1–24), >30 nmol/l (normograms for the diagnosis of 21-OHD).

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. The *CYP21A2* gene was amplified by PCR of two fragments overlapping the third exon, so as to avoid amplifying the *CYP21A1P* pseudogene that has the 8-bp deletion [24]. The first fragment extended from the 5′ end of the *CYP21A2* gene to the third exon, and the second fragment extended from the third to the tenth exon.

In a first round of PCR, primer 1 (P1) was used together with primer 2 (P48) and primer 3 (P55) with primer 4 (P4) to amplify fragments of 1,148 and 2,211 bp, respectively. The conditions of the PCR amplification of the *CYP21A2* gene and the sequence of the primers were the same as described in Wedell and Luthman [24]. For the detection of mutations in the fragment 1 covering exons 1–3 and fragment 2 covering exons 3–10, primers were designed that covered the complete exonic region and partial intronic areas using the Primer 3 program of the Whitehead Institute for Biomedical Research [16]. The PCR primers used in direct sequencing for the detection of mutations in the fragment 1 covering exons 1–3 and fragment 2 covering exons 3–10 of the *CYP21A2* gene will be available on request. The sequence information of *CYP21A2* gene was obtained from www.ensembl.org (ENSG0000016482).

A total of 2,946 bases out of the 3,359 bases amplified from the first-round PCR were sequenced. Direct sequencing was employed on an automated Beckman Coulter CEQ 2000 sequencer using the CEQ 2000 Dye Terminator Cycle Sequencing Kit according to the manufacturer's procedure.

The 8-bp deletion in exon 3 is used to distinguish between the functional *CYP21* and the *CYP21A1P* pseudogene. For the detection of the 8-bp deletion in exon 3, DNA was amplified with primers 1, 2 and 5 (P49) and subsequent digestion with *Taq*I since a restriction site is present only in *CYP21A1P* [24, 25].

Multiplex Ligation-Dependent Probe Amplification Analysis

DNA from the 32 CAH patients in this study analyzed by direct sequencing was also examined with the multiplex ligation-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, The 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 the Del 8bp, p.I172N, Cluster E6 (nomenclature) and p.Q318X mutations. Furthermore, this kit contains 3 *CYP21A1P*-specific probes, 3 *TNXB* probes, 1 *C4A* probe, 1 *C4B* probe and 1 probe for the *CREBL1* gene located q-telomeric of *TNXB*. In addition, 2 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, PCR amplification was carried out with the specific SALSA PCR primers. Amplification products were run on an automated Beckman Coulter CEQ 2000 Genetic Analyzer. The raw data were analyzed by using Coffalyzer 7.0 Software (MRC Holland). The size of migration of exon-specific peaks was identified according to their migration relative to the FRAG-600 size standard (Beckman Coulter, Fullerton, Calif., USA).

Categorization in Mutation Groups

The disease-causing mutations were divided into four mutation groups according to a previous description [26].

The null group contained alleles with mutations resulting in an enzyme with no activity (classical known mutations: Large lesion, p.F306insT, and p.Q318stop [27–29]). Group A contained genotypes composed by the mutation IVS2-13A/C>G in a homozygote status or with a null allele in trans. The IVS2-13A/C>G splice site mutation is also known to result in an enzyme with minimal residual activity [30, 31].

Group B contained genotypes composed by the mutation p.I172N in a homozygote status or with a mutation from null or A group in trans. Regarding the p.I172N, the residual enzyme activity in in vitro expression experiments is about 2% [32].

Group C contained genotypes composed by a mild mutation on at least one allele: the p.V281L, p.P30L or p.P482S. The other allele bears a mild or a severe mutation belonging to groups null, A or B [12, 29, 33, 34]. The mild mutations p.V281L, p.30L, p.P453S and p.P482S result in 30–60% enzyme activity [12].

Genotypes categorized in groups null and A were predicted to result in SW CAH. Those in group B were expected to manifest as a SV phenotype, and those in group C as NC-CAH.

Results

The overall frequency of the molecular defects detected in our patients is depicted in table 1. There are 32 unrelated patients, corresponding to 64 unrelated CAH alleles. In the 64 unrelated alleles, the most frequent point mutations were p.V281L (54.7%) and IVS2-13A/C>G (21.9%). In patients with the classic form the most frequent mutation was that of IVS2-13A/C>G (55%), followed by large lesions (7.8%), whereas in subjects with the NC form the most prominent defect was the p.V281L mutation (79.5%), followed by IVS2-13A/C>G (6.8%) and p.P30L (4.5%).

Table 1. Mutation frequency of 64 affected alleles from 32 unrelated patients with 21-OHD

Mutation	Number of alleles			% of alleles		
	classic	NC	total	classic	NC	total
p.V281L	0	35	35	0	79.5	54.7
IVS2-13A/C>G	11	3	14	55	6.8	21.9
Large lesion	4	1	5	20	2.3	7.8
p.P30L	0	2	2	0	4.5	3.1
p.I172N	2	0	2	10	0	3.1
p.F306insT+p.V281L[1]	2	0	2	10	0	3.1
p.Q318stop	1	1	2	5	2.3	3.1
p.P482S	0	1	1	0	2.3	1.6
p.M283V	0	1	1	0	2.3	1.6
Total	20	44	64	100	100	100

[1] Mutation p.F306insT+p.V281L falls under the category of multiple mutations because it is found in *cis* on the same allele.

The type of the molecular defects in the 10 patients with the classic form of CAH is shown in table 2, where relevant clinical and biochemical data are also presented. Five patients were found to be homozygote for the IVS2-13A/C>G mutation and 1 patient was homozygote for the Large lesion. The remaining 4 patients had a combination of compound heterozygote genotypes made of severe mutations such as: Large lesion, p.Q318stop, IVS2-13A/C>G, and p.F306insT. One patient affected with the SW form was associated with the rare genotype p.F306insT+p.V281L/p.F306insT+p.V281. The above genotype was detected both on the paternal and the maternal alleles. Although the parents are not related, they originate from the same village. The mutation p.I172N was identified in homozygote state in only 1 male patient, who presented with clinical signs of GnRH-independent precocious puberty, also called precocious pseudo-puberty.

The type of the molecular defects with clinical and biochemical data in the patients with the NC form of CAH is shown in table 3, where relevant clinical and biochemical data are also presented. The common mutation p.V281L was present in 21 patients with the NC form and in 14 of them it was found in the homozygote state. The combination of classic/non-classical mutation genotype was detected in 4 patients. The first patient (No. 15) with

Table 2. Types of molecular defects with clinical and biochemical data in the patients with classic CAH

	Genotype	Form	Sex	Age of diagnosis	Clinical phenotype	Hyponatremia hyperkalemia	17-OH P nmol/l	ACTH <60 pg/ml	Renin PRA ng/ml/h (0.2–2.8)
1	IVS2-13A/C>G/IVS2-13A/C>G	SW	F	neonate	Prader 3	yes	>75.7	1,450	10.3
2	IVS2-13A/C>G/IVS2-13A/C>G	SW	F	neonate	Prader 3	yes	>75.7	1,355	9.4
3	IVS2-13A/C>G/IVS2-13A/C>G	SW	M	neonate	adrenal crisis	yes	>75.7	>2,100	11.4
4	IVS2-13A/C>G/IVS2-13A/C>G	SW	M	neonate	adrenal crisis	yes	>75.7	>2,100	10.7
5	IVS2-13A/C>G/IVS2-13A/C>G	SV	M	5.5 years	GnRH independent precocious, puberty	no	43.7	282	1.23
6	IVS2-13A/C>G/Large lesion	SW	M	neonate	adrenal crisis	yes	>75.7	2,352	9.8
7	p.F306insT+p.V281L/ p.F306insT+p.V281L	SW	F	neonate	Prader 4	yes	>75.7	>2,100	12
8	Large lesion/Large lesion	SW	M	neonate	adrenal crisis	yes	>75.7	>2,100	8.5
9	Large lesion/p.Q318stop	SW	M	neonate	adrenal crisis	yes	>75.7	1,680	11.3
10	p.I172N/p.I172N	SV	F	4.5 years	peripheral, precocious puberty	no	>75.7	392	8.2

Table 3. Types of molecular defects with clinical and biochemical data in the patients with the NC form of CAH

	Genotype	Sex	Age in years of diagnosis	Clinical presentation	17-OHP, nmol/l basal	17-OHP, nmol/l stimulated
1	p.V281L/p.V281L	M	8	PP	27.2	>75.7
2	p.V281L/p.V281L	M	8	PP	39.6	>60.5
3	p.V281L/p.V281L	F	21	O	>60.5	>60.5
4	p.V281L/p.V281L	F	6.5	PP	>75.7	>75.7
5	p.V281L/p.V281L	F	16.5	O, H	>60.5	>60.5
6	p.V281L/p.V281L	F	7	PP	36.8	>60.5
7	p.V281L/p.V281L	F	6.5	PP	>60.5	>60.5
8	p.V281L/p.V281L	F	8	PP	35.9	>75.7
9	p.V281L/p.V281L	F	15	O, H	12.7	45.6
10	p.V281L/p.V281L	F	13.5	PP, O	>60.5	>60.5
11	p.V281L/p.V281L	M	8	PP	10.5	>60.5
12	p.V281L/p.V281L	F	8.5	PP	>63.6	>63.6
13	p.V281L/p.V281L	F	8	PP	40.8	>63.6
14	p.V281L/p.V281L	F	15	H	14.5	>60.5
15	p.V281L/IIVS2-13A/C>G	F	6	PP	>75.7	>75.7
16	p.V281L/IVS2-13A/C>G	F	15	O, H	11.8	>60.5
17	p.V281L /Large lesion	F	22	O	>60.5	>60.5
18	p.V281L/p.Q318stop	F	14	O	>75.7	>75.7
19	p.V281L/p.P482S	F	15	O, A	12.53	57.9
20	p.V281L/ p.P30L	F	11	H	16.3	>60.5
21	p.V281L/p.M283V	M	8	PP	22.6	>63.6
22	p.P30L/IVS2-13A/C>G	F	6.5	PP, clitoromegaly	>75.7	>75.7

PP = Premature pubarche; O = oligomenorrhea; H = hirsutism; A = acne.

the genotype p.V281L/IVS2-13A/C>G presented at the age of 6 years with premature pubarche. She was 134 cm tall (+3.8 SD) and she had a bone age of 11 years. She had no signs of virilization. Her basal 17-OHP was >75.7 nmol/l and androstendione was 25.5 nmol/l. The second patient (No. 16) with the same genotype presented at the age of 15 years with oligomenorrhea and hirsutism, and had a baseline of 17-OHP 11.8 nmol/l, with stimulation >60.5 nmol/l. The third patient (No. 17) with the genotype p.V281L/Large lesion presented at the age of 22 years

Table 4. Genotypes grouped according to predicted severity of involved mutations and phenotypes in 32 patients

Group	Genotype	Patients	Phenotype		
			SW	SV	NC
Null	p.F306insT+p.V281L/ p.F306insT+p.V281L	1	1		
	Large lesion/Large lesion	1	1		
	Large lesion p.Q318stop	1	1		
A	IVS2-13A/C>G/IVS2-13A/C>G	5	4	1	
	IVS2-13A/C>G/Large lesion	1	1		
B	p.I172N/p.I172N	1		1	
C	p.V281L/p.V281L	14			14
	p.V281L/IVS2-13A/C>G	2			2
	p.V281L/Large lesion	1			1
	p.V281L/p.M283V	1			1
	p.V281L/p.Q318stop	1			1
	p.P30L/p.V281L	1			1
	p.V281L/p.P482S	1			1
	p.P30L/ IVS2-13A/C>G	1			1
Total		32	8	2	22

with oligomenorrhea and had a baseline 17-OHP >60.5 nmol/l. Overall, a Large lesion was detected in 4 patients, 3 affected with the SW form (patients 6, 8, 9; table 2) and 1 affected with the NC form (patient 17; table 3). The fourth patient (No. 18) had the genotype of p.V281L/p.Q318stop and presented at the age of 14 years with oligomenorrhea and had a baseline 17-OHP >75.7 nmol/l. The less frequent missense mutations p.P482S and p.M283V were found in 2 patients with NC-CAH in a compound heterozygote state with p.V281L (No. 19 and No. 21, respectively).

Furthermore, a genotype/phenotype correlation was carried out according to their predicted functional consequences (table 4). All patients, who belonged in the null group, manifested the SW form as expected. All patients in mutation group A as well presented with the classical form of CAH. There was only 1 patient in group B who had the SV form in accordance with the genotype I172N. All patients who were categorized as group C exhibited the NC form. The patient who carried the p.P30L/IVS2-13A/C genotype manifested a severe phenotype of the NC form. She was initially seen at the age of 6.5 years and by history she presented at the age of 4 years with premature pubarche. On physical examination she was noted to have acne and signs of virilization (clitoromegaly). Her height was 131.5 cm (+2.6 SD) and she had advanced bone age (12 years). She had basal 17-OHP >75 nmol/l, and elevated levels of androstenedione (38 nmol/l), testosterone (9.6 nmol/l) and ACTH (71 pg/ml) with normal for age and pubertal status DHEAS: 17.2 nmol/l. The overall genotype-phenotype correlation in this study was 100%.

Discussion

In this study, we described the spectrum and frequency of *CYP21A2* alleles, as well as the genotype-phenotype relationship in unrelated Greek-Cypriot CAH patients of the 21-OHD form. The classical form of CAH seems to be rare in Cyprus, since we had only 10 new cases diagnosed over the past 30 years (1979–2008). Given the total number of births during this period, which is 309,470 [www.mof.gov.cy], the incidence of classical CAH in live births is 1:30,000, much less than expected [1, 3, 7, 8].

The most frequent molecular defect in the SW form was the splice site mutation IVS2-13A/C>G. The high frequency of the IVS2-13A/C splice mutation is in agreement with most studies reported so far [13, 18, 26, 35–46]. The mutation p.Q318stop was detected in 1 patient as Large lesion/p.Q318stop. This frequency is lower than that of the Hellenic population (14.3%) and other ethnic groups [18, 40, 42, 43], and could be attributed either to differences in our genetic background or to our small number of patients. Similarly, the I172N, which is found in 13.5% in Greek patients, was identified in only 1 SV patient. This genetic defect was detected in 4% in patients in Turkey, in 30% in Morocco and in no patients in Sicily [20–22].

In the NC form, the prevalent mutation was the p.V281L, as in most populations studied [18, 26, 36, 39, 40, 44, 47]. The overall frequency of p.V281L is however one of the highest found for this mutation both in Greek [18] and other populations in Europe and the Mediterranean area [19–22, 25, 35, 36, 48–51]. This mutation is quite common in our population. In a random screening of healthy individuals, often used as controls in our laboratory, p.V281L was detected in 2 out of 134 or 1/67 [unpubl. data].

The phenotypic expression of 21-OHD is primarily related to the type of the molecular defect and correlates with the less severely mutated allele, although it does not always reflect the underlying genetic defect [18]. In genotypes predicting NC expression of the disease, the concordance rate was not absolute. The girl who fulfilled the criteria of having a severe NC form and belonged to the C group of the genotypes was found to carry in compound heterozygosity the relatively milder p.P30L mutation with

the IVS2-13A/C and in homozygosity the polymorphic variant p.N493S. The influence of p.N493S variant on the residual enzyme activity has never been analyzed in vitro, and in a recent study a molecular model of human *CYP21A2* showed that the non-conserved amino acid alteration does not change the electrostatic charge [52].

This milder missense p.P30L mutation, although known to reduce enzyme activity and generally associated with NC form, is often present in patients with more severe signs of androgen excess [13, 53, 54]. A study on the Hellenic population identified the mutation p.P30L present in at least 1 of the 2 chromosomes in 5 out of 6 NC genotypes, associated with the SV phenotype [18]. Similar findings were also reported by Krone et al. [35], where the prediction was stronger in patients in group C when excluding the p.P30L mutation. The presence of p.P30L was found in 23 out of 93 patients with the SV form either in homozygosity or in a compound heterozygote state with a second mutation or gene deletion/conversion [36]. The diversity of the clinical phenotype in patients carrying p.P30L is further supported by its presence in patients with all forms of CAH [50].

In conclusion, the previously described major mutations are found to dominate the mutation spectrum of Greek-Cypriot patients with CAH. There is an excellent genotype-phenotype correlation in all patients, although differences in phenotypic appearance may appear and caused by still undefined factors modifying 21-OH gene expression. Knowing the genetic defects in correlation with the phenotypes produced will be of immense help in detecting heterozygote carriers in antenatal diagnosis and genetic counseling.

Acknowledgment

We would like to thank A.G. Leventis Foundation for supporting this research.

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