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CYP21A2 mutations in pediatric patients with congenital adrenal hyperplasia in Costa Rica

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ABSTRACT

Steroid 21-hydroxylase deficiency accounts for 95% of congenital adrenal hyperplasia (CAH) cases. Newborn screening has allowed for early detection of the disease, and currently, molecular analysis can identify the genotypes of these patients. Phenotype-genotype correlation has been well described in previous studies. In Costa Rica, there is no data about the genetic background of these patients, nor their phenotypic correlation.

Design: Observational, retrospective, descriptive study based on the review of patient records who had a diagnosis of CAH and were performed molecular analysis using gene sequencing or MLPA during the period from 2006 to 2018 ($N = 58$).

Objective: To describe the clinical and genetic characteristics of CAH patients due to 21-hydroxylase deficiency at the National Children's Hospital "Dr. Carlos Sáenz Herrera", Caja Costarricense de Seguro Social (CCSS) in Costa Rica.

Results: 53% (31/58) of the patients were male and 80% (37/46) were born full term; 72% (42/58) had salt wasting phenotype, 9% (5/58) simple virilizing phenotype and 19% (11/58) non-classic phenotype. The most frequent variants were c.292+5G>A in 26% (15/58) of patients and Del/Del in 21% (12/58) of them.

Conclusions: The most frequent mutation in our study population was the c.292+5G>A, which was found in 15/58 patients. This rare variant has only been reported in three other studies so far but as an infrequent mutation in CAH patients. The genetic characteristics of Costa Rican patients differ from what has been documented worldwide and could respond to a founder effect.

1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of enzymatic defects in cortisol biosynthesis. Steroid 21-hydroxylase deficiency is responsible for 95% of cases, and the severity of the disease is related to the degree of residual enzymatic activity [1–4]. Cortisol deficiency may lead to the loss of the negative feedback on the hypothalamic-pituitary axis, leading to high levels of ACTH, steroidal precursors accumulation, and an increased synthesis of sex steroids, which are responsible for some of the clinical features of the disease [1,3].

The classical form of CAH has a greatly impaired enzymatic activity, resulting in the most sever phenotypes. Around 75% of these patients may present with a salt wasting phenotype and can develop a salt wasting crisis, which is potentially fatal if left untreated [1,3,5]. The remaining patients with the classical form can have a residual enzymatic activity of 1–2%, which is enough to maintain adequate mineralocorticoid levels. These patients develop a simple virilizing phenotype, characterized by accelerated postnatal growth and androgen excess [6]. Female patients with the classical form may have various degrees of sexual ambiguity, due to prenatal exposure to high levels of fetal adrenal androgens since early stages [1,3,6]. Male patients usually have normal genitalia [1,5].

The non-classical form of the disease exhibits greater residual enzymatic activity, allowing for adequate cortisol and aldosterone production at the expense of steroid precursors accumulation [5,7]. Thus, female patients may develop signs of androgen excess, such as acne, hirsutism, irregular menstrual cycles, and infertility [5,7,8]. Due to prolonged exposure to high levels of androgens, central precocious

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puberty may follow [1,7]. Excess androgen may affect the hypothalamic response to progesterone, resulting in an increased production of luteinizing hormone and a higher androgen secretion by the ovarian Theca cells that may contribute to the clinical manifestations [9].

Patients with the classical form have a lifetime risk of presenting adrenal crisis, especially during periods of physiologic stress [10,11]. They also may have higher mortality rates related to adrenal crisis and cardiovascular disease than the general population [12–14].

Classic CAH has an incidence of about 1:10000 to 1:20000 newborns worldwide, but there is high variability on the basis of ethnic and geographical backgrounds [1,3,5]. In Costa Rica, the incidence of CAH is estimated to be about 1.1/10000 newborns [15].

Diagnosis of CAH is based on determination of 17-hydroxyprogesterone (17-OHP) levels in serum. Patients with the classical form have levels higher than 1000 ng/dL (30 nmol/L) [1,5]. In the non-classical form, a corticotropin stimulation test is usually required to establish the diagnosis [1,5]. Newborn screening has allowed for early identification of CAH patients with a high degree of sensibility; its main goal is to reduce the morbidity and mortality associated with adrenal crisis during the newborn period, especially in male patients that may have more subtle clinical findings and may therefore go undiagnosed in early stages [16–18]. False positive results may be related to maternal hypertension, prematurity, sepsis, seizures, jaundice, dehydration, and perinatal asphyxia [19–21]. In Costa Rica, newborn screening for CAH has been available since 2002 and it is routinely performed on every newborn as part of the national newborn screening program [15]. Corticotropin stimulation test however, is not available in our country.

Molecular analysis for CAH may detect between 90 and 95% of allelic variants, with an accurate genotype-phenotype correlation [22–24]. In Costa Rica, molecular analysis is routinely performed to CAH patients by the National Newborn Screening Laboratory (CCSS) using Sanger sequencing and MLPA. In this study, we present the results of the mutations identified in our patients and highlight the identification of a rare genetic variant that has a high frequency in our population.

2. Materials and methods

This was an observational, retrospective, descriptive study. Clinical records of all the patients with diagnosis of CAH due to 21-OH deficiency who had genetic test performed by Sanger sequencing or MLPA and were in control at the Endocrinology Department of the National Children's Hospital during the period between January 1st, 2006 to January 31st, 2018 were reviewed. This represents the complete population during this period since the National Children's Hospital (CCSS) is the reference center for pediatric patients in Costa Rica. A total of 58 patients were included. Clinical, demographic, and genetic test results were collected for all the patients.

Genomic DNA was isolated from peripheral blood leukocytes using standards and automated procedures (EZ1 Advanced, QIAGEN). DNA quality and quantity were measured by Nanodrop 2000c (Thermo Scientific™). Dosage analysis and gene sequencing were performed for each sample.

CYP21A2 exons and its intronic flanking regions were amplified by polymerase chain reactions (PCR) with specific primers. PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems®) and analyzed in an Applied Biosystems™ 3500 Series instrument. Sequences were aligned with the reference *CYP21A2* sequence (NM_000500.9) with the Basic Local Alignment Search Tool of the U.S. National Center for Biotechnology Information (BLAST, https://blast.ncbi.nlm.nih.gov). The interpretation was based on published papers. Dosage analysis was carried out with SALSA® MLPA® Probemix P050-C1 CAH according to the MRC-Holland procedure.

Descriptive statistics were used to present the data, and result of chi-squared test for the phenotype-genotype association is presented. The report of the variants was based on the nomenclature guidelines dictated by the "Human Genome Variation Society" [25].

Informed consent was not requested since no additional interventions were undertaken aside from the standard evaluations routinely performed to all the patients with CAH in our center. The study was approved by the local bioethics committee with the number CEC-HNN-025-2017.

3. Results

A total of 31/58 patients (53%) in our sample were male. The mean age at diagnosis was 16.7 ± 36.3 months, and 75% of the patients were younger than 2.5 months old at diagnosis. All the classic CAH patients were younger than 4 months at diagnosis, and the non-classic CAH patients age at diagnosis ranged between 5 and 11 years.

Gestational age in our patients ranged between 33.3 and 42.5 weeks, with a mean of 38.6 ± 2.0 weeks, and 80% of the patients were born full term, while 19% were born preterm. Birth weight ranged between 1765.0 and 4730.0 g, with a mean of 3208.6 ± 593.6.

A total of 42/58 patients (72%) were classified by the attending endocrinologist as salt wasting on the basis of clinical and laboratory findings; 5/58 (8%) were classified as simple virilizing and 11/58 (19%) had non-classical phenotype. The clinical manifestations of the patients according to phenotype are presented in Table 1. None of the patients presented with a salt wasting crisis.

Table 2 presents a list of the variants identified in the patients. The most frequent variants identified in our population were c.292+5G>A in 15/58 patients (26%) and c.844G>T in 15/58 patients (26%), followed by Del/Del in 12/58 patients (21%), and c.332_339delGAGACTAC in 8/58 patients (14%). In 4 patients (patients 55 to 58) no mutations could be identified. Association between the genotypes and phenotypes could be demonstrated for the patients with CAH, with a likelihood ratio chi-square of 53.2377 ($p = 0.000$). The main variants identified were c.292+5G>A, Del/Del and c.332_339delGAGACTAC, and they were related with a salt wasting phenotype.

4. Discussion

The majority of our patients were diagnosed with CAH before 2.5 months of age, probably because of an early detection of the cases with classical phenotype thanks to the newborn screening, which is routinely performed on every newborn as part of the national newborn screening program in Costa Rica. All of the classic CAH patients were detected by newborn screening and due to this early detection, the clinical manifestations at diagnosis were few and, in most cases, not severe, with none of the patients presenting with a salt wasting crisis at the time of diagnosis. Description of the clinical manifestations at diagnosis could be under-reported in some patient records due to the early diagnosis and mild symptoms, and could thus explain the absence of clinical symptoms reported in simple virilizing patients. Diagnosis and phenotype classification were made by the attending endocrinologist and is reported as stated in the patient files.

The most frequent variants identified in our patients differ from what has been reported in previous studies. One of the most frequent variants in our patients was the c.292+5G>A, which was found in 26% (15/58)

Table 1
Clinical findings at diagnosis.

Phenotype	Clinical manifestation	Patients
Salt wasting ($N = 42$)	Hiperpigmentation	Female: 10 / Male: 17
	Genital ambiguity	Female: 16 / Male: 1
	Vomiting	Female: 2 / Male: 7
	Failure to thrive	Female: 1 / Male: 6
Simple virilizing (N = 5)	Hiperpigmentation	Female: 1 / Male: 0
Non-classical ($N = 11$)	Peripheral precocious puberty	Female: 8 / Male: 0
	Accelerated growth	Female: 5 / Male: 1
	Hirsutism	Female: 1 / Male: 0

Table 2
Variants identified and Genotype/Phenotype association ($N = 58$).

Patient	Variants	Phenotype
1	c.292+5G>A homo[a]	SW
2	c.292+5G>A homo[a]	SW
3	c.292+5G>A hemi	SW
4	c.292+5G>A hetero; del/con hetero	SW
5	c.292+5G>A hetero; del/con hetero	SW
6	c.292+5G>A hetero; del/con hetero	SW
7	c.292+5G>A hetero; del/con hetero	SW
8	c.292+5G>A hetero; del/con hetero	SW
9	c.292+5G>A hetero; del/con hetero	SW
10	c.292+5G>A hetero; del/con hetero	SW
11	c.292+5G>A hetero; del/con hetero	SW
12	c.292+5G>A hemi; c.844G>T hetero; c.1439G>T homo	SW
13	c.292+5G>A homo; c.844G>T homo	SW
14	c.292+5G>A homo; c.844G>T homo	SW
15	c.292+5G>A hetero; c.844G>T hetero; c.955C>T hetero	SW
16	Del/Del	SW
17	Del/Del	SW
18	Del/Del	SW
19	Del/Del	SW
20	Del/Del	SW
21	Del/Del	SW
22	Del/Del	SW
23	Del/Del	SW
24	Del/Del	SW
25	Del/Del	SW
26	Del/Del	SW
27	Del/Del	SW
28	c.332_339delGAGACTAC hetero; del/con hetero	SW
29	c.332_339delGAGACTAC hetero; del/con hetero	SW
30	c.332_339delGAGACTAC hetero; del/con hetero	SW
31	c.332_339delGAGACTAC hetero; del/con hetero	SW
32	c.332_339delGAGACTAC hetero; del/con hetero	SW
33	c.332_339delGAGACTAC hetero; del/con hetero	SW
34	c.332_339delGAGACTAC hemi; c.844G > T hemi	SW
35	c.332_339delGAGACTAC hetero; c.844G > T homo	SW
36	c.844G>T homo[a]	NC
37	c.844G>T hemi	SV
38	c.844G>T homo	NC
39	c.844G>T homo	NC
40	c.844G>T hetero; del/con hetero	NC
41	c.844G>T hetero; del/con hetero	NC
42	c.844G>T hetero; del/con hetero	NC
43	c.844G>T hetero; del/con hetero	NC
44	c.293-13C>G homo; c.844G>T homo	SW
45	c.293-13C>G hemi	SW
46	c.293-13C>G hemi	SW
47	c.518T>A hemi	SV
48	c.518T>A hemi	SV
49	c.518T>A hemi	SW
50	c. [-126C>T;-113G>A;-110T>C] hetero; del/con hetero	NC
51	c. [-126C>T;-113G>A;-110T>C] hetero; del/con hetero	NC
52	c.323T>A hemi	NC
53	c.1069C>T hetero; Del hetero	SW
54	c. [*52C>T, c.*440C>T, c.*443T>C]	NC
55	Negative	SW
56	Negative	SW
57	Negative	SV
58	Negative	SV

SW = salt wasting; SV = simple virilizing; NC = non classical; hetero = heterozygous; homo = homozygous; hemi = hemizygous.
[a] No MLPA report was available.

of the cases. Interestingly, this is a rare variant that has not been described in most of the largest studies worldwide [22,23,26–33]. Friaes *et al* were the first to report this mutation in their series of 56 Portuguese patients, in which they identified only one patient with the genotype c.292+5G>A;c.844G>T and salt wasting phenotype [34]. Later, Ezquieta *et al* reported in their study nine patients with this same variant associated with salt wasting phenotype. Given the high number of cases with this rare variant, they suggested a founder effect and recommended including the variant in the panels for patients from Mediterranean descent [35]. Finally, Marino *et al* described the variant in three patients from Argentinian descent; two had salt wasting phenotype and one had simple virilizing phenotype [36]. The high prevalence of this mutation in our population could respond to a founder effect.

The c.292+5G>A variant (commonly known as IVS2+5G>A) is an intronic variant (Fig. 1). To date, only 17 intronic variants have been reported in the *CYP21A2* gene [37]. The mutation is suggested to suppress the wild type intron 2 splice donor site, leading to an aberrant splicing with exon skipping or activation of an alternative splice donor site [37].

Another interesting finding is the relatively low proportion of cases with the c.293-13C>G variant in our patients (3/58 patients), as this is one of the most frequent mutations in most other studies [22,23,27,28,30–35]. Thus, the genotypes identified in our population seem to differ from what has been reported elsewhere.

Different studies have demonstrated genotype-phenotype correlation in CAH. Knowing the genotype of these patients can be used to predict the severity of the disease, especially in the salt wasting and non-classical phenotypes [22,24]. In the present study, association between genotypes and phenotypes could be demonstrated.

Clinical sensitivity for full *CYP21A2* screening is >95%, but pitfalls and false negative results can occur during *CYP21A2* analysis due to different reasons [38]. Four of our patients with CAH had negative molecular test results, two of which had salt wasting phenotype and two had simple virilizing phenotype. Patient number 55 was one of such patients with negative molecular test result. Interestingly, he was the male patient who initially presented with a salt wasting phenotype and genital ambiguity. He was found to have karyotype 47XXY, and a *post hoc* molecular analysis showed that a pathogenic variant in *HSD3B2* and not *CYP21A2* was the cause of the disease. The association of Klinefelter syndrome and CAH is extremely rare, with very few cases reported in the literature [39–41]. More details of this case will be presented in a future manuscript. The reason no mutations were identified in the other 3 patients could be due to the limitations related with molecular testing, including disease-causing variants lying in a different gene, as was the case of the aforementioned patient [38]. Also, previous studies have described mutations in non-coding regions of the gene that have clinical manifestations of disease [42]. Such is the case of mutations in the promoter region that have been associated with non-classical phenotype [42]. In our study, we identified two patients (patients 50 and 51) who initially were reported c.844G>T heterozygote but later promoter sequencing was performed and the variant c. [-126C>T;-113G>A;-110T>C] hetero; del/con hetero was identified. This variant has been previously associated with non-classical phenotype [42]. Patient 54 was also reported initially with a negative molecular test, but *post hoc* molecular analysis showed a mutation in 3`UTR c. [*52C>T, c.*440C>T, c.*443T>C] to be the cause of the disease. Sequence variations in 3`UTR of the gene have previously been associated with a non-classical phenotype [43,44]. Non-coding regions however, were not routinely examined in all of our patients.

5. Limitations

Due to the retrospective design of the study, data such as subtle clinical manifestations at diagnosis in the case of patients with simple virilizing phenotype could have been under-reported in the patient records. Also, even though all patients had gene sequencing, three patients did not have MLPA results available. Non-coding regions of the gene were not routinely explored in the patients with a negative molecular test.

6. Conclusion

This is, to the best of our knowledge, the first study in the region to report the genotypes of patients with CAH. One of the most frequent variants in our study population was the c.292+5G>A, which was found in 15/58 patients. This rare variant has only been reported in three other

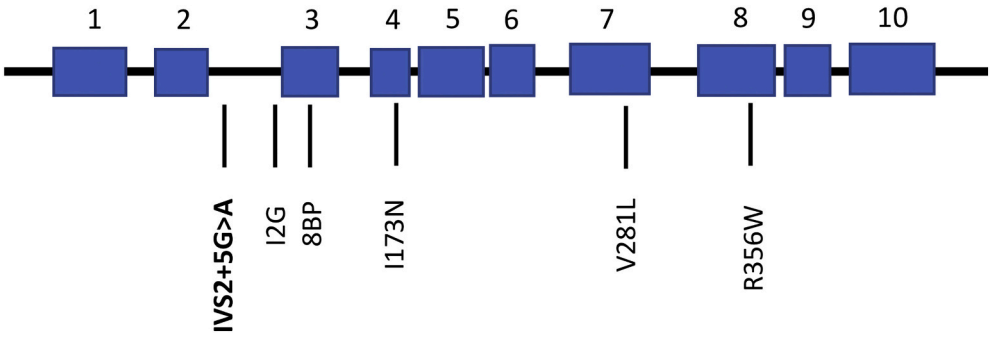

Fig. 1. *CYP21A2* mutations identified in the present study. In bold is shown the intronic mutation IVS2+5G>A, which was the main variant identified in our study population.

studies so far but as an infrequent mutation in CAH patients. Moreover, it is rarely included in most genetic panels for CAH. The genetic characteristics of Costa Rican patients differ from what has been documented worldwide and could respond to a founder effect. Given the high number of cases with this mutation, we support the suggestion of including the c.292+5G>A variant in the genetic tests of CAH patients, particularly those of Mediterranean descent. In cases with no identified mutations, sequencing of other non-coding regions should also be considered.

Conflicts of interest and financial disclosure

A donation for the statistical work was granted by the National Association for the Study of Diabetes, Endocrinology and Metabolism of Costa Rica (ANPEDEM). ANPEDEM had no involvement in the study design, the collection, analysis and interpretation of data, nor in the writing of the report or in the decision to submit this article for publication.

Author statement

The authors of this publication have no conflicts of interest to declare.

CRediT authorship contribution statement

Andrés Umaña Calderón: Conceptualization, Data Curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Writing-original draft, Writing-reviewing & editing. María José Acuña Navas: Investigation, Investigation, Writing-reviewing & editing. Danny Alvarado: Conceptualization, Data Curation, Formal analysis, Investigation, Software, Validation, Writing-reviewing & editing. Mildred Jiménez: Conceptualization, Investigation, Software. Fred Cavallo Aita: Conceptualization, Methodology, Resources, Supervision.

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