Molecular *CYP21A2* diagnosis in 480 Brazilian patients with congenital adrenal hyperplasia before newborn screening introduction

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Abstract

Background: Most congenital adrenal hyperplasia (CAH) patients carry *CYP21A2* mutations derived from conversion events involving the pseudogene, and the remaining carry new mutations.

Objective: To review causal mutations and genotype–phenotype correlation in 480 Brazilian patients.

Methods: DNA was extracted from 158 salt-wasters (SWs), 116 simple virilizing (SV), and 206 nonclassical (NC) patients. Fourteen point mutations were screened by allele-specific PCR, large rearrangements by Southern blotting/MLPA, and sequencing was performed in those with incomplete genotype. The gene founder effect was analyzed by microsatellite studies. Patients were divided into six genotypes (Null; A: <2%; B: 3–7%; C: >20% of residual enzymatic activity (EA); D: unknown EA; E: incomplete genotype).

Results: Targeted methodologies defined genotype in 87.6% of classical and in 80% of NC patients and the addition of sequencing in 100 and 83.5%, respectively. The most frequent mutations were p.V281L (26.6% of alleles), IVS2-13A/C>G (21.1%), and p.I172N (7.5%); seven rare mutations and one novel mutation (p.E351V) were identified. Gene founder effect was observed in all but one (p.W19X) mutation. Null, A, B, and C genotypes correlated with SW (88%), SW (70%), SV (98%), and NC forms (100%), respectively. In group D, the p.E351V mutation correlated with classical form and group E comprised exclusively NC-patients. ACTH-stimulated 17OHP level of 44.3 ng/mL was the best cutoff to identify NC-patients carrying severe mutations.

Conclusions: We identified a good genotype–phenotype correlation in CAH, providing useful data regarding prediction of disease's severity; moreover, we suggest that ACTH-stimulated 17OHP levels could predict carrier status for severe mutations. Sequencing is essential to optimize molecular diagnosis in Brazilian CAH patients.

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Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is the most common inborn metabolism error, which presents an autosomal recessive inheritance and comprises 90–95% of CAH cases. A spectrum of clinical manifestations is observed and, traditionally, it is divided into classical (simple virilizing – SV and salt wasting – SW) and nonclassical

(NC) forms. The SV form is characterized by prenatal external genitalia virilization in females and postnatal virilization in both sexes. In the SW form, in addition to the prior listed symptoms, patients also present with hyponatremic dehydration in the first weeks of life. In the NC form, postnatal hyperandrogenic manifestations initiate at infancy, adolescence, or even at adult life (1).

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The classical form frequency is approximately 1:10 000 to 1:15 000 live births, whereas the NC form is much more frequent and is estimated to have a frequency of 1 in 100–1000 in the general population (2, 3, 4, 5).

The 21-hydroxylase coding gene (*CYP21A2*) and its pseudogene (*CYP21A1P*) extend over a long region in the short arm of chromosome 6, alternating in *tandem* with C4A and C4B genes. Because of the high homology between *CYP21* genes and the tandem-repeat organization of the *CYP21/C4* locus, this genomic region is vulnerable to gene conversion events; thus, 90–95% of patients carry *CYP21A2* mutations derived from the pseudogene, instead of new casual mutagenic events (6, 7, 8, 9).

The great variability in phenotypic expression of CAH results from mutations leading to different degrees of enzymatic impairment, from mild to severe, and the phenotype correlates with the allele harboring the less severe mutation in a compound heterozygous patient (10, 11, 12).

A high genotype–phenotype correlation of up to 90–95% has been described in several studies (9, 13, 14, 15, 16, 17). Mutations resulting in up to 2% of residual enzymatic activity correlate with the SW form, 3–10% correlate with the SV form, and 20–50% correlate with the NC form (6, 7, 8, 9, 11, 16). These findings have emphasized the use of molecular diagnosis in clinical practice, especially in situations that require phenotype prediction. For example, molecular analysis could elucidate the diagnosis in male asymptomatic newborns with positive neonatal tests or in premature females in whom true clitoromegaly can be overestimated (4, 18). In Brazil, CAH newborn screening (CAH-NBS) is being implemented, and, considering the usefulness of molecular diagnosis to confirm/exclude CAH diagnosis, its use has been approved as a confirmatory test (19).

The aim of this paper is to review our 20 years of experience in diagnosis of CAH due to 21-hydroxylase deficiency. We determined the causing mutations in a cohort of 480 Brazilian patients to obtain a panel of the most prevalent mutations and evaluated their phenotype prediction. This information would be helpful for improving diagnostic accuracy and genetic counseling.

Subjects and methods

Patients

The study was approved by the Ethical Committee of Faculdade de Medicina da Universidade de São Paulo and written consent was obtained from all the participants.

Between 1993 and 2013, a total of 480 CAH patients from 429 families were diagnosed (158 SW, 116 SV, and 206 NC). All patients were followed at the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, most of them were from São Paulo state, but few were from other Brazilian States, especially from Northeast region. The cohort included 359 females and 121 males, and the female/male ratios in classical and nonclassical forms were 1.9/1.0 and 6.7/1.0, respectively.

Clinical and hormonal-criteria defined phenotypic classification is as follows: SW, SV, and NC forms (6). In the SV form, females were referred by atypical genitalia at birth according to Prader genital scores (PGSs) or by clitoris enlargement during early childhood; males were diagnosed by precocious pubarche and penis enlargement. In both genders, bone age was advanced. In the SW form, in addition to the virilizing signs, patients also failed to thrive and demonstrated hyponatremic dehydration in the first weeks of life. All classic patients presented with extremely increased basal 17OHP levels. The mean chronological age at diagnosis in the SW form was 66 ± 121 days, and in the SV form, it was 4.0 ± 4.5 years in girls and 4.2 ± 2.0 in boys. The mean bone age at diagnosis in SV girls was 4.2 ± 5.3 years and in SV boys was 8.5 ± 6.6 years. The NC form included female patients with late-onset hyperandrogenic manifestations such as precocious pubarche, menstrual abnormalities, hirsutism, and/or infertility. Nonclassical males were referred by precocious pubarche. All NC patients demonstrated basal or 1-h after ACTH-stimulated 17OHP levels clearly increased above 10 ng/mL (20). NC females were tested during the follicular phase of the menstrual cycle or with progesterone levels ≤ 3 ng/mL. The mean age at diagnosis in NC females was 18.3 ± 13.7 years and in NC males was 7.1 ± 3.6 years.

Hormone assays

Serum 17OHP and androstenedione levels and plasma renin activity were measured by radioimmunoassays, and the intra- and interassay coefficients of variation were 12%, 18%, and 18%, respectively. Serum cortisol and testosterone levels were analyzed by immunofluorometric assays, and the intra- and intervariation coefficients were $<8\%$ for both variables. The 17OHP values were expressed in ng/mL ($1 \text{ ng/mL} = 3.18 \text{ nmol/L}$). All steroid hormones were measured before treatment introduction.

Mutation analysis of the *CYP21A2* gene

DNA samples from patients and at least one parent were obtained from peripheral leukocytes. The *CYP21A2* gene was PCR-amplified, and the most frequent 14-point mutations were screened by allele-specific PCR (10, 11). Large gene rearrangements (LRs) were screened by Southern blot followed by *CYP21* probe hybridization or by multiplex ligation-dependent probe amplification (MLPA) (SALSA MLPA kit P050B CAH; MCR Holland, Amsterdam, the Netherlands) (21, 22).

When at least one allele remained with no mutation identified, entire *CYP21A2* sequencing was performed by dideoxynucleotide terminator methodology as described previously. The new allelic variant identified was also screened in at least 100 alleles of *CYP21A2* and *CYP21A1P* genes from normal Brazilian subjects (10, 23, 24), and also assessed in online known genome databases like ExAC (Exome Aggregation Consortium) and 1000 Genome. *In silico* prediction analysis of the novel p.E351V mutation was assessed using Mutation Taster (www.mutationtaster.org), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2>), and SIFT (Sorting Intolerant from Tolerant Human Protein) (http://sift.jcvi.org/www/SIFT_enst_submit.html) tools.

Two microsatellite loci (D6S273 and TAP 1), flanking the *CYP21A2* gene, were studied in all patients and their relatives who carried new mutations, as described previously (23, 24).

Genotype categories

After mutation segregation analysis, patients' genotypes were divided into six different groups. Group Null included patients who carried two mutations that predicted 0% overall activity. Group A included patients who were homozygous for the IVS2-13A/C>G mutation (<2%) or in compound heterozygosis with the group Null mutations. Group B included patients who were homozygous for the p.I172N mutation (3–7%) or in compound heterozygosis with mutations from groups Null or A. Group C included patients who were homozygous for the p.P30L, p.V281L, and p.P453S mutations (20–50%) or in compound heterozygosis with the former mutations (12). Finally, group D included patients with novel mutations for which enzymatic activity impairment had not been assessed and group E with at least one allele without identified mutations.

Statistical analysis

Data are presented as median and interquartile range of variables for which the normality test failed. The Fisher's test was used to analyze the association of mutations with clinical forms and also to verify the association of the new mutations with their respective haplotypes. Correlation between genotype groups and 17OHP levels was verified using either Mann–Whitney or Hodges–Lehmann nonparametric tests. Statistical significance was considered if $P < 0.05$. All of the statistical analyses were performed using SPSS software version 22.

Results

Disease-causing allele's distribution

Mutations were identified in both alleles using allele-specific PCR followed by Southern blot or MLPA methodologies in 88.6% of SW patients, 86.3% of SV, and 80% of NC patients. The addition of *CYP21A2* sequencing allowed for the identification of mutations in all of the classical patients and in 83.5% of NC patients. The rate of compound heterozygosis was 65.3%.

Mutations were observed in 95.7% of the 856 unrelated alleles; 86.7% had point mutations and 9% carried large gene rearrangements. The identified mutations were segregated in samples from at least one of the parents, and 6% of the alleles carried two or more point mutations. The most frequent point mutations were p.V281L (26.6% of alleles), IVS2-13A/C>G (21.1%), and p.I172N (7.5%) (Table 1).

Mutations not derived from gene conversion events

The previously described mutations p.G424S, p.R408C, IVS2-2A>G, p.Ser170fs, p.R426H, p.H365Y, and p.W19X were observed in 35 nonrelated patients (23, 24, 25, 26) (Table 2). Among them, the most frequent were the IVS2-2A>G, p.G424S, and p.R426H mutations. Microsatellite analysis was performed for six former mutations using markers flanking the *CYP21A2* gene and disclosed the same haplotype for each new mutation, suggesting a gene founder effect.

Additionally, a new allelic variant was observed: the p.E351V, in three nonrelated patients (1SW/2SV) (Table 2). This substitution was not found in 100 alleles of *CYP21A2* and *CYP21A1P* genes from normal subjects, and neither in Human Genome databases, ruling out the possibility

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Table 1 Frequency of *CYP21A2* mutations in Brazilian CAH patients and in other countries.

	Country or region (reference)									
	Brazil (this study)	Argentina (13)	Portugal (29)	Spain (17)	Chile (27, 28)	Germany (14)	Sweden (9)	USA (15)	Mexico (30)	Mid-Europe (35)
Number of unrelated alleles	856	866	112	354	164	310	186	3005	94	864
Mutations										
<i>CYP21A2</i> deletion or conversion	9.0	11.2	25.9	19.2	19.5	27.4	29.8	20.0	1.0	30.6
p.P30L	0.6	0.7	1.8			2.6	1.6	2.6	8.5	3.7
IVS2-13A/C>G	21.1	20.6	9.8	17.5	15.8	30.3	30.0	22.9	47.9	31.2
p.G110EfsX21 (E3Δ8bp)	1.8	0.8	2.7	3.3		1.6	1.2	2.1	2.1	1.0
p.I172N	7.5	8.2	9.8	3.7	7.3	19.7	20.3	8.2	11.7	14.5
p.I235N, p.V236E, p.M238K (E6 cluster)	1.2	2.0			1.8	1.0	1.1	2.1	0.0	0.3
p.V281L	26.6	26.2	25.9	33.9	2.4	2.9	7.0	23.9	8.5	3.4
p.G291S	0.1	*					0.5		0	*
p.Leu307fs (InsT)	2.2	*	4.4	1.4	0.0	0.3	0.5		1.1	1.6
p.Q318X	6.1	6.7	6.3	9.3	7.9	4.8	3.2	3.5	4.2	2.6
p.R356W	5.4	4.2	1.8		9.7	4.5	4.3	3.6	7.4	2.4
p.P453S	1.4	1.4				0.3	0.5		2.1	0.7
p.R483P	0.0									0.1
p.R483PfsX58	0.0	1.5								0.9
% of mutations involving events with pseudo-gene	82.9	82	88.4	93.9	64.4	98.1	100	88.9	94.5	92
% of alleles with no mutations identified	4.3	11.9	0.9	9.3	23.7	1.3			21.3	

*Not screened.

of polymorphism. All of the carriers disclosed the same haplotype, suggesting a gene founder effect. *In silico* analysis by Polyphen and Mutation Taster revealed scores of 0.811 and 121, respectively.

Genotype–phenotype correlation

The overall genotype–phenotype correlation was 97.3%. Genotype accurately predicted phenotype in 88.2% of the

patients in group Null, 70% of the patients in group A, 98% of the patients in group B, and 100% of the patients in group C. The Null and A genotypes comprised mainly SW patients; the B genotype was mainly associated with the SV form, but one patient presented with the NC form (LR, p.P30L/IVS2-2A>G). The C genotype was exclusively associated with the NC form. The D genotype comprised four classical patients. Finally, the E genotype was observed in 34 NC patients (Table 3).

Table 2 *CYP21A2* mutations not derived from pseudogene events: mutations with gene founder effect and a novel mutation.

ENSP00000408860	ENST00000418967	Frequency (%)	Patients [§] (n)	Founder effect	Microsatellites	
					D6S273	TAP-1
p.G424S	c.1273G>A	0.6	6	+	1	5
p.Ser170fs	c.511_512insA	0.5	4	+	3	6
p.R408C	c.1225C>T	0.5	4	+	5	5
IVS2-2A>G		1.2	10	+	3	1
p.H365Y	c.1096C>T	0.2	2	+	2	5
p.R426H	c.1280G>A	0.8	7	+	6	3
p.W19X	c.59G>A	0.2	2	–	–	–
p.E351V	c.1055A>T	0.3	3	+	4	5

[§]Number of unrelated patients.

In the SW form, the most frequent mutation was IVS2-13A/C>G (33.2% of alleles), in the SV form the p.I172N (32.0%) followed by IVS2-13A/C>G (27.8%), and in the NC form the p.V281L (61.4%).

Prader genital score was available in 101 classical females. The most common PGS in Null, A, and B genotypes was Prader III (49.5%). Severe virilization (Prader IV and V) was observed more frequently in genotypes Null and A than in B ($P=0.03$), although there was no difference between groups Null and A (Fig. 1). The PGSs were similar in all classical female siblings (5 families). Interestingly, 8% of the NC females had a slight clitoromegaly (Prader I).

Median basal 17OHP levels were significantly different among genotype groups Null, A, B, and C. The lowest basal 17OHP level in a classical patient was 38 ng/mL. Among NC patients from group C, median basal 17OHP levels varied from normal (7.2%) to those that overlap with the classical form. The median basal 17OHP level was significantly higher in genotype A/C (median 17.5 ng/mL, range 0.4–80 ng/mL) than in C/C (median 7.6 ng/mL, range 0.3–58 ng/mL) as was ACTH-stimulated 17OHP levels ($P=0.005$) (Table 4). Data of basal and stimulated-17OHP levels from pre- and postpubertal NC patients were analyzed together, since their values did not significantly differed ($P>0.05$). The lowest ACTH-stimulated 17OHP level in an NC patient from group C was 11 ng/mL. ACTH-stimulated serum 17OHP level of 44.3 ng/mL was the cutoff with higher sensitivity (0.674) and specificity (0.702) to identify NC patients with compound heterozygosity for severe mutations (area under ROC curve = 0.701 (0.597–0.804) ($P=0.001$).

Even after *CYP21A2* sequencing, 16.5% of the NC patients persisted with an incomplete genotype (group E). Among these, 8 patients presented basal 17OHP level above 15 ng/mL, 8 presented ACTH-stimulated 17OHP level between 10 and 15 ng/mL, and 20 presented ACTH-stimulated 17OHP level above 15 ng/mL. These patients had their *CYP21A2* sequencing revised though a new PCR product and allelic drop out was ruled out.

Discussion

In the present study, we explored the clinical and molecular features of one of the largest Latin American CAH cohorts. We identified the nine previously described *CYP21A2* point mutations, derived from gene conversion events, in 74% of unrelated alleles and large gene

rearrangements in 9% of the alleles. Compared with other ethnic groups, the overall results were similar, especially concerning the high frequency of IVS2-13A/C>G mutation. Our lower frequency of LR was probably because many SW patients died without precocious neonatal diagnosis (6, 13, 15, 16, 17, 26, 27, 28, 29). The lower SW:SV ratio (1.3:1.0) than that found in countries where CAH neonatal screening is routinely performed (3-4:1), in addition to the lower proportion of males than females (1.0/1.9) among classical patients, illustrates the importance of introducing CAH newborn screening (4). In Argentina (13) and Mexico (30), countries in which CAH-NBS has been implemented more recently, the patient cohort characteristics are similar to ours.

The widely used site-directed mutation analysis and Southern blot/MLPA techniques failed to identify mutations more often than expected. Considering the significant proportion of mutations that were not derived from the pseudogene (4.4% of the alleles), we suggest the addition of sequencing to propose an accurate diagnostic molecular panel in our population. *CYP21A2* sequencing increased the identification of mutations to 100% of classical alleles, similar to what was described previously (9, 16).

Remarkably, analysis of the Brazilian cohort found eight rare/novel mutations. The gene founder effect was observed in all carriers of the p.R408C, p.Ser170fs, p.H365Y, IVS2-2A>G, and p.G424S, as described previously (23, 24, 26), and in the present work, we also observed in carriers of the p.E351V and p.R426H mutations. For instance, the p.H365Y, p.G424S, and p.R426H mutations were already described in Spanish and Portuguese patients, which are the two main parental populations responsible for Brazilian population constitution, suggesting a common origin (29, 31). Although the majority of our patients was from São Paulo state, we have no information about their ancestry, which may come from other places of Brazil. For instance, we also identified these mutations in screened patients from Goiás state, suggesting that they are present in Brazilian population overall (32).

The codon 351 is a highly conserved residue of P450 cytochromes and belongs to the E351/R354/R408 triad that stabilizes the three-dimensional protein structure (33). A missense mutation p.E351K was described in the same codon leading to severe enzyme activity impairment (34). Although we did not perform *in vitro* studies, *in silico* analysis suggested deleterious effect of this mutation; the p.E351V mutation was found in classical patients confirming the severity of the mutation *in vivo*.

Table 3 Genotype–phenotype correlation and gender distribution of patients with point mutations or large rearrangements.

Group	CYP21A2 genotype	CAH phenotype				Sex	
		SW	SV	NC	Total	F	M
Null	Del or conv./Del or conv.	9	1		10	6	4
	Del/p.Leu307fs	1			1	1	
	Del or conv./p.Q318X	5	1		6	1	5
	Del or conv./p.R356W	5			5	2	3
	Del/p.W19X	1			1	1	
	p.Leu307fs/E6 cluster	2			2	1	1
	E3 Δ8bp/E3 Δ8bp	3			3		3
	E3 Δ8bp/p.Q318X	3			3		1
	E3 Δ8bp/p.R356W	1			1	1	
	E6 cluster/E6 cluster	1			1		1
	E6 cluster/p.R356W	1			1	1	
	p.Q318X/p.Q318X	7			7	4	3
	p.Q318X/p.Leu307fs	1			1	1	
	p.R356W/p.R356W	5	1		6	5	1
	p.R356W/p.Q318X	4	1		5	1	4
	p.R356W/p.Leu307fs	5			5	3	2
	p.H365Y/IVS2-13A/C>G	1			1	1	
	p.Ser170fs/p.Leu307fs	1			1	1	
	p.Ser170fs/p.Q318X	1			1		1
	IVS2-2A>G/IVS2-2A>G	1			1	1	
	p.Ser170fs/p.R408C	1			1		1
	p.R408C/p.Q318X		1		1		1
	p.R408C, IVS2-13A/C>G/IVS2-13A/C>G	1			1	1	
A	IVS2-13A/C>G/Del or conv.	18	3		21	19	2
	IVS2-13A/C>G/IVS2-13A/C>G	23	15		38	25	13
	IVS2-13A/C>G/E3 Δ8bp	2	1		3	2	1
	IVS2-13A/C>G/E6 cluster	1			1	1	
	IVS2-13A/C>G/p.G291S	1			1	1	
	IVS2-13A/C>G/p.Leu307fs	4			4	2	2
	IVS2-13A/C>G/p.Q318X	9	4		14	10	4
	IVS2-13A/C>G/p.R356W	3	3		6	3	3
	IVS2-13A/C>G/IVS2-2A>G	3			3	2	1
	IVS2-13A/C>G/p.W19X, p.V281L	1			1	1	
	IVS2-13A/C>G/p.Ser170fs	1			1	1	
B	p.I172N/Del or conv.		4		4	3	1
	p.I172N/IVS2-13A/C>G		14		14	12	2
	p.I172N/E6 cluster		3		3	2	1
	p.I172N/p.I172N		16		16	12	4
	p.I172N/p.Q318X		5		5	4	1
	p.I172N/p.R356W		4		4	3	1
	p.I172N/p.Leu307fs		1		1	1	
	p.I172N/Conv P30L		2		2	2	
	p.I172N/IVS2-2A>G		2		2	1	1
	p.I172N/p.G424S		1		1		1
	p.I172N/p.R408C		1		1	1	
	p.G424S/IVS2-13A/C>G		2		2	2	
	p.G424S/p.R356W		2		2	1	1
	p.R426H/Del		1		1	1	
	p.R426H/p.Q318X		2		2	1	1
	p.R426H/p.H365Y		1		1		1
	p.R426H/p.R426H		1		1	1	
	Conv P30L/IVS2-13A/C>G		1		1	1	
	Conv P30L/p.R356W		1		1	1	
	Conv P30L/IVS2-2A>G			1	1	1	
C	p.V281L/Del or conv.			13	13	11	2
	p.V281L/IVS2-13A/C>G			21	21	19	2
	p.V281L/p.I172N			7	7	5	2

Continued

Table 3 (Continued).

Group	CYP21A2 genotype	CAH phenotype				Sex	
		SW	SV	NC	Total	F	M
	p.V281L/p.V281L			73	73	64	9
	p.V281L/p.P30L			1	1	1	
	p.V281L/E3 Δ8bp			3	3	2	1
	p.V281L/E6 cluster			1	1		1
	p.V281L/p.Leu307fs			3	3	3	
	p.V281L/p.Q318X			3	3	3	
	p.V281L/p.R356W			8	8	7	1
	p.V281L/Conv P30L			1	1	1	
	p.V281L/p.R426H			1	1		1
	p.V281L/p.G424S			1	1	1	
	p.V281L/IVS2-2A>G			1	1	1	
	p.V281L/p.P453S			8	8	6	2
	p.P30L/IVS2-13A/C>G			2	2	1	1
	p.P30L/p.P30L			1	1	1	
	p.P453S/E3 Δ8bp			1	1	1	
	p.P453S/IVS2-13A/C>G			2	2	2	
D	p.E351V/E6 cluster		1		1	1	
	p.E351V/IVS2-13A/C>G	2			2		2
	p.E351V/p.G424S		1		1		1
E	IVS2-13A/C>G/-			3	3	3	
	p.V281L/-			20	20	19	1
	-/-			9	9	9	

A strong positive genotype–phenotype association was verified, similar to that found in other ethnic groups (9, 13, 14, 15, 16, 17, 27, 28, 29, 30, 35). The salt-waster frequency was higher in the Null group than in group A, which is also important for clinical decisions in asymptomatic patients (18) during the neonatal period. When the most frequent mutation IVS2-13A/C>G was present in the less severe allele (group A), carriers has an increased chance (71%) to develop the SW form. Its presence in some simple virilizing patients can be explained by alternative splicing, which leads to a minimal residual enzyme activity, thus avoiding salt loss (36).

High concordance between genotype and phenotype was also observed in group B. This was different from other studies, which demonstrated a wide phenotypic spectrum with the p.I172N mutation, varying from SW to SV forms (7, 13, 15, 16). Another positive genotype–phenotype correlation was observed in group C, where all patients presented with the NC form except for those carrying the p.P30L mutation, since some patients also presented the SV form. It is important to evaluate whether p.P30L mutation represents a microconversion event involving the exon 1 of the pseudogene or a macroconversion event involving the promoter region plus exon 1 of the pseudogene. For the latter cases, in our experience, the phenotypic expression of SV form prevails, since the promoter region of the pseudogene carries mutations that

reduce the transcriptional activity five-fold, resulting in a more severe phenotype (37).

Despite the above-mentioned positive genotype–phenotype correlation, we observed few cases with less severe phenotypes than what was predicted by the genotype. This divergence has been observed in some

Figure 1

Frequency of Prader genital scores (PGSs) according to genotype groups.

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Table 4 Serum basal and ACTH-stimulated 17OHP levels according to genotype in CAH patients.

Genotype group	Patients (n)	Basal 17OHP (ng/mL)			ACTH-stimulated 17OHP (ng/mL)		
		Mean	Median	Range	Mean	Median	Range
Null	85	227.5	188.3	50–801	–	–	–
A	115	225.8	180.1	50–1549	–	–	–
B	71	166.8	145.7	38–393	–	–	–
C	171	17.6	12.8	0.6–80	49.2	43.6	11–255
NC genotypes							
A/C	74	23.1	17.5	0.4–80	60.3	55	11–255
B/C	10	23.2	19	8–43	64.5	60	20–118
C/C	87	12	7.6	0.3–58	39.8	36	15–121
C/– or –/–	34	10.7	3.5	0.9–58.9	24	19	10.8–83.7

studies worldwide (14, 15); this may be due to a role of modifier genes involving 21-hydroxylase transcription/translation or even the presence of extra-adrenal 21-hydroxylation (21, 38).

Data available regarding genotype correlation with the degree of external genitalia virilization reveal conflicting results (9, 14, 39). We observed severe genitalia virilization more frequently in genotypes Null and A than in B; however, due to the wide overlap of this manifestation, genotype could not be used as a predictor.

Median basal 17OHP levels were significantly different among genotypes Null, A, B, and C; however, a great overlap among genotype groups has been observed (7, 11). Basal and ACTH-stimulated 17OHP levels were significantly higher in NC genotype A/C than C/C. These findings are in accordance with other series that included a significant number of NC patients (2, 20, 40).

Different ACTH-stimulated 17OHP levels (10–15 ng/mL) have been used for NC diagnosis (2, 3). Most of our NC patients presented with highly increased stimulated 17OHP levels, but 3.3% of them presented with levels lower than 15 ng/mL. Among these latter patients with incomplete genotype, we cannot rule out the polycystic ovary syndrome diagnosis. In this way, it was observed that some obligate heterozygotes for severe mutations presented with ACTH-stimulated 17OHP levels that overlapped with those of NC diagnosis (41). All these data indicate that hormonal nonclassical diagnosis criteria should be revised taking into account molecular data. However, we also observed that most patients with incomplete genotype (group E) presented ACTH-stimulated 17OHP levels higher than 15 ng/mL. Nevertheless, this finding also points out to the possibility of mutations in other sites like distal regulatory regions (42).

Due to our large number of NC patients, we investigated the peak 17OHP cutoff that better predicts the risk of compound heterozygosis with severe mutations

(genotype A/C), which is important for genetic counseling purposes. The ACTH-stimulated 17OHP value of 44.3 ng/mL presented the highest sensitivity and specificity to identify this subgroup of patients; a previous study found a similar value (49.8 ng/mL) (43).

CAH molecular diagnosis is one of the most complicated of all monogenic disorders. Through our large CAH cohort, we were able to designate the complexities in their genotypes. We provided mutation prevalence in Brazil, which enables the development of a mutation panel for molecular diagnosis in our population in the clinical practice, such as newborn screening.

The CAH-NBS is not universally adopted, mainly because of its high false-positive results rate and of the good healthcare professionals' performance in detecting CAH during neonatal period in developed countries (44, 45). Unfortunately, the scenario is different in emergent nations. Data of this unscreened Brazilian cohort highlight the pitfalls of clinical diagnosis, illustrating the importance of CAH-NBS introduction nationwide. Recent data from NBS in São Paulo indicated that it is highly efficient to promote early diagnosis, preventing neonatal deaths in our state (32). In NBS, the molecular study is particularly useful to set the precise diagnosis, avoiding unnecessary treatment and follow-up of oligo/asymptomatic patients with persistently increased 17OHP levels.

In conclusion, we demonstrated a good genotype-phenotype correlation and that *CYP21A2* sequencing is essential to achieve adequate CAH molecular diagnosis, although 16.5% of the NC patients persisted with an incomplete genotype. We also suggest that stimulated 17OHP levels could be predictive of carrier status for severe mutations, which is important for genetic counseling. Genotyping is a useful complementary tool for confirming or excluding CAH diagnosis and predicting the clinical manifestations in patients who are diagnosed before the symptomatic phase.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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