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Phenotype and genotype correlation of the microconversion from the *CYP21A1P* to the *CYP21A2* gene in congenital adrenal hyperplasia

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Abstract

Deficiency of 21-hydroxylase is the most common form of congenital adrenal hyperplasia (CAH-21OH). We determined by allele-specific PCR the frequency of microconversion in the *CYP21A2* gene in 50 Brazilian patients with the classical (salt wasting: SW and simple virilizing: SV) forms and nonclassical (NC) form of CAH-21OH and correlated genotype with phenotype. Genotypes were classified into three mutation groups (A, B, and C) based on the amount of enzymatic activity in *in vitro* studies using adrenal cells. In 94 unrelated alleles, we diagnosed 76% of the affected alleles after screening for 7 microconversions. The most frequent point mutations observed in this series were I172N (19%), V281L (18%), and IVS2,A/C>G,-12 (15%). In the SW form, the most frequent mutation was IVS2,A/C>G,-12 (38%), in the SV form it was I172N (53%), and in the NC form it was V281L (57.7%). We observed a good correlation between genotype and phenotype. Discordance between genotype and phenotype was found in one SV patient with a mild mutation in one of the alleles (R356W/V281L). However, we cannot rule out the presence of an additional mutation in these alleles. We also observed a good correlation of genotype with 17α-hydroxyprogesterone, testosterone, and androstenedione levels. The severity of external genitalia virilization correlated with the severity of mutation. In conclusion, the frequencies described in the present study did not differ from worldwide studies, including the Brazilian population. The few differences observed may reflect individual sample variations. This new Brazilian cohort study suggests the presence of new mutations in Brazilian patients with different forms of CAH-21OH.

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Introduction

Congenital adrenal hyperplasia (CAH) is a monogenic autosomal recessive disorder and the deficiency of 21-hydroxylase (21OH) accounts for 90-95% of the cases (1). Steroid 21OH is a cytochrome P450 enzyme that catalyzes the hydroxylation of 17α-hydroxyprogesterone (17OHP) and progesterone during cortisol and aldosterone biosynthesis in the adrenal cortex, respectively (2). The spectrum of clinical manifestations includes a severe classical form with two phenotypic classifications, salt wasting (SW) and simple virilizing (SV), both with prenatal virilization of external genitalia in the female fetus and postnatal virilization in both sexes, and a mild nonclassical form (NC), in which patients remain asymptomatic or develop symptoms during childhood or puberty (1).

The structural gene (*CYP21A2*) for 21OH is located in the HLA class III region on the short arm of chromosome 6 (6p21.3), as also is the pseudogene (*CYP21A1P*). They present 98% identity in exons and are located adjacent to the genes for the complement system, *C4A* and *C4B* (3,4). Because of the high homology and tandem-repeat organization of the *CYP21A2* and *C4* genes, this region of the genome is subject to unequal crossover events and gene conversions (5,6). Therefore, the mutations that account for 21OH deficiency can be *CYP21A2* deletions, large gene conversion, or point mutations. Furthermore, other rearrangements such as duplications of either *CYP21A2* + *C4B* or *CYP21A1P* + *C4B* and deletion of *CYP21A2P* + *C4B* may also occur. Although these rearrangements are not responsible for the disease they determine different haplotypes (7,8).

The frequency of *CYP21A2* deletion and large gene conversion varies from 20 to 33% in several studies on Caucasian populations (9,10). Deleterious mutations usually present in *CYP21A1P* are transferred to *CYP21A2* probably by microconversion events. Microconversions are responsible for approximately two thirds of the affected alleles, and approximately 40 point mutations have been described (10,11). Furthermore, rare mutations have recently been described occurring only in *CYP21A2* alleles (12-16). Most of them are mutations found in only one chromosome and are considered sporadic. Thirteen new different mutations have been identified in the Brazilian population (17-21). There are codons considered to be hotspots, such as W22, P30, G291, R356 and R483 (19).

In the present study we report the frequency of 7 microconversions: P30L, a C to T base change in exon 1; IVS2,A/C>G,-12, an A/C to G base change in intron 2 causing abnormal RNA splicing generating a stop codon downstream; 706-713del8, an 8-bp deletion in exon 3 that generates a stop codon at position 130; I172N, a missense mutation at residue 172 in exon 4; V281L, a G to C base change in exon 7; Q318X, a base substitution determining a stop codon in exon 8, and R356W, a missense mutation in exon 8. We used an allele-specific polymerase chain reaction (PCR)-based approach in 50 Brazilian families with the classical and NC forms of CAH-21OH deficiency and correlated genotype with phenotype.

Subjects and Methods

Patients

The study was approved by the University Hospital Ethics Committee and all families gave informed consent to participate in the genetic study. We studied 5 isolated cases and 45 families including patients with CAH-21OH, representing 94 unrelated affected alleles. Among the 45 families, there was only one affected subject in 42 and 2 affected subjects in 3 families. Four of these families were consanguineous. Patients were assigned to one of the three clinical forms of CAH-21OH, defined according to standard criteria (10). Among the 50 patients, 18 pre-

sented the SW form, 19 the SV form, and 13 the NC form. The SW form was characterized by extremely elevated concentrations of 17OHP and plasma renin activity, hyperkalemia, hyponatremia, and dehydration in the first months of life. All females had ambiguous genitalia, graded according to Prader staging. The SV form was characterized by ambiguous genitalia in females, sexual precocity in males without SW, and elevated plasma 17OHP and plasma renin activity levels. Height and bone age were advanced. The NC form was considered to be present in girls with normal external genitalia or mild clitoral enlargement and was characterized by precocious pubarche and stimulated 17OHP of more than 1200 ng/dl in both sexes.

Hormone assays

Serum hormones (17OHP, androstenedione and testosterone) were measured by radioimmunoassay, as previously described (17), using specific antibodies provided by Dr. José Gilberto Vieira (Fleury Laboratory). Tritiated hormones were purchased from Amersham Biosciences (São Paulo, SP, Brazil). Plasma samples were previously extracted with ether before the assays. The intra- and interassay coefficients of variation were 3.3 and 16% for 17OHP, 5.2 and 6.8% for androstenedione and 6.3 and 10% for testosterone. The sensitivity of each method was 16 ng/dl for 17OHP, 3.9 ng/dl for androstenedione, and 10 ng/dl for testosterone.

Genotyping of mutations in the *CYP21A2* gene

DNA samples were obtained from peripheral blood leukocytes by standard procedures. Allele-specific PCR, as described by Wilson et al. (22), was used for the determination of 7 microconversions (P30L, IVS2,A/C>G,-12, 706-713del8, I172N, V281L, Q318X, and R356W), as previously described (19). Positive and negative control DNA was used in all reactions.

Genotype categories

The patients were divided into three different genotype groups according to the impairment of plasma 21OH activity, as described by Speiser et al. (9). Group A included patients who were homozygous for a mutation that predicted 0% overall activity (IVS2,A/C>G,-12, 706-713del8, Q318X, R356W, gene deletion and macroconversion), group B included patients who were homozygous for I172N (2% of the enzymatic activity) or compound heterozygous with mutation from group A. Group C included mutations which preserve 10 to 20% of 21OH enzymatic activity (V281L and P30L) in homozygosity or in compound heterozygosity with mutations from group A or B.

Statistical analysis

The association of each mutation with a clinical form of the disease was determined. Differences in basal levels of 17OHP, testosterone and androstenedione among the three genotype groups were evaluated using the Wilcoxon-Mann-Whitney test.

Results

In the 94 unrelated alleles, the most frequent point mutations were I172N (19%), V281L (18%), and IVS2,A/C>G,-12 (15%). In the SW form, the most frequent mutation was IVS2,A/C>G,-12 (38%), in the SV form it was I172N (53%), and in the NC form it was V281L (57.7%). There was a significant association of IVS2,A/C>G,-12, I172N, and V281L with the SW, SV, and NC forms, respectively ($P < 0.0001$). A point mutation was present in 70 alleles, 11 of which presented 2 point mutations (Table 1). Thus, 76% of all alleles presented at least one of the 7 most frequent microconversions from *CYP21A1P* to *CYP21A2*. Among the 27 patients who had mutations identified in both

alleles (Table 2), 8 presented the group A genotype (all of them with the SW form), 11 patients presented the group B genotype (all of them with the SV form), and 8 patients presented the group C genotype (7 with NC and 1 with SV). Virilization of the external genitalia in group A was Prader III in all female patients and varied from Prader I to III in group B (Table 1). Basal 17OHP levels ranged from 18,300 to 37,780 (median 32,620) in group A, 1,888 to 36,250 (median 14,440) in group B, and 308 to 8,500 ng/dl (median 1224) in group C. There was a significant difference in 17OHP levels among the three groups. Androstenedione levels ranged from 144 to 7,225 (median 989) in group A, 58 to 3,102 (median 408) in group B, and 24 to 351 ng/dl (median 118) in group C. Testosterone levels ranged from 32 to 648 (median 263) in group A, 59 to 466 (median 98) in group B, and 12 to 121 ng/dl (median 32) in group C. Androstenedione and testosterone levels were similar in groups A and B, and these groups presented higher levels compared to group C.

Table 1. Genotype and clinical data of Brazilian patients with the salt wasting (SW), simple virilizing (SV) and nonclassical (NC) forms of 21-hydroxylase deficiency.

Genotype	Clinical form	Age at diagnosis	Prader stage of female genitalia
Group A			
Sp2/Sp2	SW	13 days	*
Sp2/Sp2	SW	at birth	III
Sp2/Sp2	SW	25 days	*
Q318X + R356W/Sp2	SW	1 month	*
Sp2/Sp2	SW	12 days	III
Q318X + R356W/Sp2	SW	20 days	III
Sp2 + V281L/R356W	SW	34 days	III
Sp2/Q318X	SW	7 months	*
Group B			
I172N/Sp2 + Q318X	SV	9 years	I
I172N/I172N	SV	8 years	*
I172N/R356W	SV	NA	II
I172N/I172N	SV	at birth	III
I172N/I172N	SV	at birth	*
I172N/I172N	SV	at birth	*
I172N + Q318X/I172N	SV	2 years and 7 months	*
I172N/I172N	SV	4 years	*
Q318X + R356W/I172N	SV	at birth	III
I172N/Sp2 + Q318X	SV	1 year and 1 month	I
I172N/I172N	SV	5 years and 7 months	*
Group C			
V281L/V281L	NC	at birth	N
R356W/V281L	NC	5 years	I
V281L/V281L	NC	6 years	N
V281L/V281L	NC	4 years and 9 months	N
R356W/V281L	NC	6 years	N
V281L/V281L	NC	6 years	N
V281L/V281L	NC	12 years	*
R356W/V281L	SV	4 years	I

*: male patients; N: normal genitalia; NA: not available; Sp2: the intronic mutation IVS2,A/C>G,-12.

Discussion

We report here the frequencies of some *CYP21A2* gene mutations (P30L, IVS2, A/C>G,-12, 706-713del8, I172N, V281L, Q318X, and R356W), which may be introduced into *CYP21A2* from *CYP21A1P* by microconversion events, in a new Brazilian cohort with classical and NC forms of CAH-21OH deficiency. We characterized affected alleles by analysis of the families and correlated genotype with phenotype. As duplication, deletion and gene macroconversion events generated from hybrid genes cannot be amplified using only PCR-based methods, future Southern blot analyses are required to identify these rearrangements in every individual involved in this study.

The ethnic origin of the Brazilian population is extremely heterogeneous. There are not many data available on the genetic characteristics of the *CYP21A2* gene in this country (17-21). In the present study we detected at least one mutation in 76% of the disease-causing alleles. The two previous Brazilian populations screened for the most frequent mutations, which included gene deletion, large gene conversion, and microconversions, presented an affected allele at a frequency of 80% (18) and 85% (19). Therefore, the results of the present study confirm that in the Brazilian population about 15-25% of the remaining disease alleles were not elucidated and might carry other rare or novel mutations. Reports on the general popula-

Table 2. Frequency of mutation in Brazilian patients with the classical (salt wasting: SW and simple virilizing: SV) and nonclassical (NC) forms of 21-hydroxylase deficiency.

	Present study				Paulino et al. (19)	Bachega et al. (18)
	SW	SV	NC	Total		
Alleles (n)	33	32	26	91	68	228
P30L	–	–	1 3.8%	1 1.1%	ND	5 2.2%
Sp2	12 48%	2 6%	–	14 14%	26%	47 20%
Δ8	1 3%	–	–	1 1.1%	1.4%	3 1.3%
I172N	–	18 56%	–	18 20.4%	20.5%	32 14%
V218L	1 3%	1 3%	15 57.7%	17 18.2%	4.5%	41 18%
Q318X	1 3%	–	–	1 1.1%	12%	5 5.7%
R356W	1 3%	3 9%	2 7.7%	6 6.5%	9%	16 7%
P30L + I172N	–	–	1 3.8%	1 1.1%	ND	ND
I172N + Q318X	–	1 3%	–	1 1.1%	ND	ND
I172N + V281L	–	–	1 3.8%	1 1.1%	ND	ND
Q318X + R356X	2 6%	1 3%	–	3 3.7%	ND	ND
Sp2 + Q318X	–	2 6%	–	2 2.2%	ND	ND
Sp2 + R356W	1 3%	–	–	1 1.1%	ND	ND
Sp2 + V281L	1 3%	–	–	1 1.1%	ND	ND
Sp2 + Δ8	1 3%	–	–	1 1.1%	ND	ND
Conv	–	–	–	ND	13.5%	7%
Del	–	–	–	ND	8.1%	5%

Conv: conversion; Del: deletion; Δ8: 706-713del8; ND: not determined; Sp2: the intronic mutation IVS2, A/C>G,-12.

tion diagnosed 77.6 to 95% of the alleles (10,18,19,23-25). The highest frequency of undiagnosed alleles was also found in other Latin-American studies (24,26). More recently, Billerbeck et al. (20), Bachega et al. (21), and Lau et al. (27) have demonstrated the presence of novel mutations in the *CYP21A2* gene in the Brazilian population. Further studies searching for these mutations described in previous Brazilian populations are required not only for the present series, but also for other populations in order to reveal if they are restricted to the Brazilian ethnic background. In addition to the alleles carrying one mutation, alleles with more than one mutation are not rare and have been observed by segregation analysis in family studies (13,18,28). In the present study we found that 15.8% of patients had alleles carrying more than one mutation; most of them were compound heterozygotes, and the clinical form was correlated with the mutated allele with higher enzymatic activity (10,26,29).

In the 94 unrelated alleles studied in the present series the most frequent mutations were I172N, V281L, and IVS2,A/C>G,-12. In the SW form, the most frequent mutation was IVS2,A/C>G,-12, in the SV form it was I172N, and in the NC form it was V281L, showing a significant association between these mutations and the clinical forms of the disease. These results are similar to those obtained in previous reports, in which the intron 2 mutation was the most common, with frequencies ranging from 18 to 36%, followed by I172N mutation (10). Our study showed the highest frequency for I172N when compared to others. This result might reflect differences in the proportion of SV cases in each sample. V281L is generally associated with the NC form of the disease (29,30) and was found at a high frequency (57.7%) in our sample, which also included NC patients. An association of V281L in the same allele with another mutation (R356W and P30L) was also observed and was associated with the classical form of the disease. In addition, it is important to point out that V281L can occur associated with duplication of *CYP21A1P*, as previously reported (30), and alleles carrying duplication of *CYP21A1P* seem to be in linkage disequilibrium with the NC form of CAH. In the 27 patients genotyped for both alleles, we found a high frequency (59%) of homozygosity for point mutations that has not been commonly reported in the literature. It is important to point out that some of these cases might be hemizygous and, more unlikely, they might present a *de novo* mutation. To clarify this point it is necessary to perform Southern blots and to sequence the entire *CYP21A2* gene.

We observed a good, although not absolute, correlation between genotype and phenotype (10,11). Twenty-seven patients had mutations identified in both alleles. From these patients, 8 presented the group A genotype (all of them with the SW form), 11 presented the group B genotype (all of them with the SV form), and 8 presented the group C genotype (7 with NC and 1 with SV). Therefore, a discrepancy between genotype and phenotype was found in only one patient having the SV form. We cannot rule out the possibility of presence of an additional new mutation in these alleles or abnormalities in the regulatory region of the gene. Because of the discrepancy between phenotype and genotype in some cases, a phenotype prediction should be made with caution in prenatal diagnosis based on molecular genetic analysis of fetal DNA. We also observed a good correlation of genotype with 17OHP, testosterone, and androstenedione levels, which reflects the extent of enzymatic activity impairment. The degree of hyperandrogenism also correlated with the severity of neonatal external genitalia virilization, mainly in group A.

Despite the mixture of diverse ethnic origins in the Brazilian population, the mutation frequencies described in the present study did not differ from previous world-

wide and Brazilian studies. The few differences observed may reflect subtle sample variation in the ethnic background of the studied population. This new Brazilian cohort study may suggest the presence of other mutations in Brazilian patients with different forms of CAH-21OH. The establishment of genotypes in complete families with at least one individual affected with CAH-21OH is useful for genetic counseling, prenatal diagnosis and prenatal treatment strategies.

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