High frequency of Q318X mutation in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency in Northeast Brazil

Alta freqüência da mutação Q318X em pacientes com hiperplasia adrenal congênita por deficiência da 21-hidroxilase no nordeste do Brasil

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ABSTRACT

Objetives: Deficiency of 21-hydroxylase is the most common form of congenital adrenal hyperplasia (CAH-21OH). The aim of this study was to determine, by allele-specific PCR, the frequency of microconversions of the *CYP21A2*, in sixteen patients with the classical forms and in 5 patients with the nonclassical (NC) form of CAH-21OH and correlate genotype with phenotype. **Methods:** Genotypes were classified into 3 mutation groups (A, B and C), based on the degree of enzymatic activity. Screening for 7 microconversions by allele-specific PCR diagnosed 74.3% (n=26) of the 35 unrelated alleles. **Results:** The most frequent mutations were Q318X (25.7%), V281L (17.1%), I2 Splice (14.3%), I172N (14.3%), and R356W (14.3%). Genotype was identified in 57.1% of the patients. We observed correlation between genotype and phenotype in 91.7% of the cases. **Conclusion:** The highest frequency for Q318X (25.7%) when compared to other studies may reflect individual sample variations in this Northeastern population. Arq Bras Endocrinol Metab. 2009;53(1):40-46.

Keywords
21-hydroxylase deficiency; adrenal hyperplasia; *CYP21A* microconversions.

RESUMO

Objetivos: Deficiência de 21-hidroxilase é a forma mais comum de hiperplasia adrenal congênita (CAH-21OH). O objetivo deste estudo foi determinar, por PCR alelo-específica, a freqüência de microconversões no *CYP21A2*, em 16 pacientes com a forma clássica e em cinco pacientes com a forma não-clássica (NC) de CAH-21OH e correlacionar o genótipo com o fenótipo. **Métodos:** Genótipo foi classificado em três grupos de mutações (A, B e C), baseado no grau de atividade enzimática. A técnica de PCR alelo-específico diagnosticou 74,3% (n = 26) dos 35 alelos não relacionados. **Resultados:** As mutações mais freqüentes foram Q318X (25,7%), V281L (17,1%), I2 Splice (14,3%), I172N (14,3%) e R356W (14,3%). O genótipo foi identificado em 57,1% dos pacientes. Houve correlação genótipo-fenótipo em 91,7% dos casos. **Conclusão:** A mais alta freqüência da mutação Q318X (25,7%) comparada a outros estudos pode refletir variações individuais desta população do nordeste. Arq Bras Endocrinol Metab. 2009;53(1):40-46.

Descritores
Deficiência de 21-hidroxilase; hiperplasia adrenal; microconversões; *CYP21A*.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) due to steroid 21-hydroxylase deficiency (CAH-21OHD) is one of the most common inborn endocrine disorders and is inherited in an autosomal recessive manner (1). This disease occurs due to molecular defects in the steroid 21-hydroxylase gene (*CYP21A2*), which encodes the 21-hydroxylase enzyme (2-5). The *CYP21A2* gene, as well as the pseudogene (*CYP21A1P*), is located in the HLA class III region on the short arm of chromosome 6 (6p21.3). Both

genes consist of 10 exons and show a high homology with a nucleotide identity of 98% in their exon and 96% in their intron sequences (6). Because of the high homology and tandem-repeat organization of *CYP21A2*, *CYP21A1P*, and *C4* genes, this region of the genome is subject to unequal crossover events and gene conversions, which give rise to mutations in *CYP21A2* (2-6).

There are three major phenotypes depending on the degree of impairment of enzyme activity caused by the specific mutation in the *CYP21A2* (3,4). In the classic salt-wasting (SW) form, the most severe form, patients suffer from renal salt loss due to the lack of aldosterone as well as pre- and postnatal virilization, due to accumulated adrenal androgen. Therefore, affecting female newborn with ambiguous external genitalia. In the classic simple virilizing (SV) form, patients also present pre- and postnatal virilization, but they do not have renal salt loss. In the NC form, patients lack the neonatal symptoms and present with late-onset symptoms and diagnosis (3).

The most common source of mutations, involving around 95% of the alleles, is the result of recombination events between the homologous pseudogene (*CYP21A1P*) and the active *CYP21A2* gene. In addition, more than 100 point mutations have been described around the world (7). There is usually a good correlation between phenotype and genotype in patients with 21-hydroxylase deficiency (2,3,8,9). Genotypes with total or near-total impairment of enzymatic activity are associated with the SW form; the SV form is associated with severe impairment and the nonclassical form with moderate impairment of enzymatic activity. However, some discrepancies in genotype/phenotype correlation have been reported, and are associated with alternative splicing or the presence of additional mutations not detected in the screening studies. In addition, recent reports have also demonstrated that microconversions between *CYP21A2* and *CYP21A1P* promoters could be involved in the classical and nonclassical phenotype (10,11).

The establishment of genotype in complete families with at least one CAH-21OHD-affected individual is useful for genetic counseling, prenatal diagnosis and prenatal treatment strategies, and also for differentiating between heterozygous and late onset form as well as an adjunct to hormonal measurements in screening programs (12,13). Therefore, in the present study, we report the frequency of seven *CYP21A2* microconversions, P30L, I2Splice, 706_713del8, I172N, V281L, Q318X, and R356W, using the allele-specific polymerase chain reaction (PCR) based approach. We studied 21 Brazilian families with CAH-21OH originating from a state in Northeast Brazil, Sergipe State, in order to amplify the data on the ethnic specific frequency of *CYP21A2* gene mutations in the Brazilian population. In addition, we have also correlated genotype with phenotype in these CAH -21OHD patients.

PATIENTS AND METHODS

Fifteen families and 1 isolated case with CAH-21OHD followed at the Division of Endocrinology, University Hospital of Federal University of Sergipe were studied. There was one affected subject in 12 families, two affected subjects in 2 families and four affected subjects in another family. There were no consanguineous families. Their parents and normal siblings were also studied. Among the 21 patients, 14 (66.7%) were females and 7 (33.3%) males. Seven patients presented with the SW form (6 females and 1 male); 9 with the SV form (4 females and 5 males), and 5 with the NC form (4 females and 1 male).

The SW form was characterized by elevated concentrations of 17OHP (911.6 ± 806.5 (mean ± standard deviation); ranging from 32 to 1615 ng/mL), ambiguous genitalia in females, hyperkalemia (7.4 ± 0.8; ranging from 6.2 to 8.3 mEq/L), hyponatremia (125.4 ± 12.0; ranging from 105 to 134 mEq/L), and dehydration in the first months of life. The SV form was characterized by elevated 17OHP (151.8 ± 124.7; ranging from 10.2 to 410.3 ng/mL), ambiguous genitalia in females, sexual precocity in both genders, high stature and advanced bone age and no history of salt wasting. The NC form was characterized in girls by normal external genitalia or mild clitoral enlargement and, in both sexes, by precocious pubarche and/or other signs of elevated androgens. Basal 17OHP levels in the NC form ranged from 3.5 to 22.3 ng/mL (13 ± 6.8 ng/mL) and 4 patients were submitted to exogenous ACTH (17OHP post ACTH test ranging from 24 to 46 ng/mL). The 17OHP cut-off value after the exogenous ACTH stimulation test was of 17OHP levels greater than 15 ng/mL. Levels of testosterone and androstenedione were divided by the upper limit of normality of the ages, to correct the different ages of the patients.

Families originated from two regions in Sergipe State: North (5 families, 10 patients) and East (9 families and the isolated case, 10 patients). One family (1 patient) originated from Bahia State. The study was approved by the Research Ethics Committees at the Federal University of Sergipe (UFS). All families gave their informed consent for the genetic study.

GENOTYPING OF MUTATIONS IN *CYP21A2*

DNA samples were obtained from peripheral blood leukocytes by standard procedures. Allele-specific PCR was used for the determination of 7 microconversions (P30L, I2Splice, 706_713del8, I172N, V281L, Q318X, and R356W) in 35 unrelated alleles, as previously described (8,14). Positive and negative control DNAs were used in all reactions.

GENOTYPE CATEGORIES

Patients were divided into three genotype groups, according to the impairment of enzymatic activity, as described by Speiser and cols. (2). Group A included patients who were homozygous or compound heterozygous for mutations that predict 0% overall activity. Subgroup A1 included 706_713del8, Q318X, and R356W mutations, and Subgroup A2 included patients who were homozygous for I2Splice or compound heterozygous for I2Splice with mutations from Subgroup A1. Subgroup A2 presented low, but measurable, enzymatic activity (<2%). Group B included patients who were homozygous for I172N (2% of enzymatic activity) or compound heterozygous with mutations from Group A. Group C included patients who were homozygous for P30L and V281L (20-50% enzymatic activity) or compound heterozygous with mutations from Groups A or B.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard deviation, unless otherwise explained. Chi-Square and Binomial tests were applied to compare the frequencies of the *CYP21A2* mutated alleles observed in the present study and other Brazilian studies. Non-parametric tests were used to compare clinical and hormonal data of patients. The Mann-Whitney test was used to compare data between two different groups and Kruskal-Wallis test was used to compare data between three groups. The Program Statistical Package for the Social Science (SPSS Base 8.0) was used for data analysis.

RESULTS

Table 1 shows clinical and hormonal data of patients with classical and NC form of CAH-21OHD, at diagnosis. 17OHP levels were higher in SW and SV compared to NC (p=0.008 and p=0.03, respectively). Values of testosterone were higher in SW and SV compared to NC (p=0.03 and p=0.04, respectively) and in SW compared to SV forms (p=0.04). Values of androstenedione were elevated in all forms.

Table 1. Clinical and hormonal data of patients with classical (salt wasting: SW and simple virilizing: SV), and late onset (LO) forms of 21-hydroxylase deficiency, at diagnosis.

	SW	SV	LO
Patients	7 (33.3%)	9 (42.9%)	5 (23.8%)
Age (years)	0.10 ± 0.15	2.0 ± 1.3 *	13.6 ± 10.4 *
Sex	M 1 (14.3%)	M 5 (55.6%)	M 1 (20%)
17OHP (ng/mL)	911.6 ± 806.5	151.8 ± 124.7 †	13 ± 6.8 *
Testosterone	15 ± 10.9	5.7 ± 3.4 * †	1.5 ± 1.7 *
Androstenedione	2.6 ± 2.6	6.2 ± 4.3	1.8 ± 1.0
Sodium (mEq/L)	125.4 ± 12.0	135.3 ± 1.5 *	138 ± 1.7 *
Potassium (mEq/L)	7.4 ± 0.8	4.1 ± 0.8 *	4.6 ± 0.21 *

17OHP = 17-hidroxyprogesterone; Androstenedione = androstenedione ng/mL/upper limit of normality of the ages; Testosterone = testosterone/upper limit of normality of the ages; M: Male; * $p < 0.05$ in comparison to SW; † $p < 0.05$ SV in comparison to LO.

The distribution of the *CYP21A2* mutated alleles is shown in Table 2, which also compares the frequencies observed in the present study and other Brazilian studies. In 35 unrelated alleles studied, the most frequent mutations were Q318X (25.7%), V281L (17.1%), I172N (14.3%), I2 Splice (14.3%), and R356W (14.3%). In the 12 unrelated alleles of the SW patients, 9 alleles (75%) were identified and the most frequent mutation was Q318X (58.3%), followed by R356W (16.7%), and I2Splice (16.7%). In the 14 alleles of the SV patients, 10 alleles (71.4%) were identified and the most frequent mutation was I172N (35.7%). In the 10 alleles of the NC patients, 8 alleles (80%) were identified and V281L was the most frequent mutation (60%).

Allele-specific PCR diagnosed 26 of the 35 unrelated affected alleles (74.3%), 4 of them (11.4%) presented 2 mutations (Q318X and R356W) and 1 allele presented 3 mutations (P30L, 706_713del8, and I2 Splice). When searching for the mutations in index case parents, we detected one asymptomatic affected father, who showed the V281L/I2Splice genotype, and had 2 affected daughters, one with SW form (I2Splice / Q318X, and R356W) and another with NC form (V281L/Q318X, and R356W). Another father presented homozygosity or hemizigosity for V281L, but his child, who presented the SW form, did not present identified mutations in her alleles. Paternity was not tested. In addition, we did not perform

Table 2. Frequency of *CYP21A2* microconversion in patients with classical (salt wasting: SW and simple virilizing: SV) and late onset (LO) forms of 21-hydroxylase deficiency.

Mutations	Present Study (2007)				Bachega and cols. (15)	Torales‡ (16)	Paulino and cols. (17) ‡	Torres and cols. (14)	Bachega and cols. (18)
	WS	SV	LO	Total					
Alleles		42			228	46	74	91	410
Q318X %	58.3	14.3	10	25.7	5.7*	13*	11.3*	1.1*	7.3*
I172N %		35.7		14.3	14	15.2	18.9	20.4	14
R356W %	16.7	14.3	20	14.3	7	6.5	8.2	6.5	11
V281L %			60	17.1	18	4.3 *	4.1 *	18.2	18
I2 sp %	16.7	21.4		14.3	20.6	21.7	24.7	14	34 *
Del8pb %		7.1		2.9	1.3	0	1.4	1.1	1.7
P30L† %		10	0	5	2.2	0	ND	1.1	1

I2Sp = I2 Splice; ND = not determined. *p<0,01. ‡, study with the classical forms. † P30L was analyzed in 20 unrelated alleles (10 LO and 10 SV).

Table 3. Genotype, phenotype, and 17OH progesterone levels in patients with salt wasting (SW), simple virilizing (SV) and late onset (LO) forms of 21-hydroxylase deficiency.

Genotype (father / mother allele)	Sex M / F	Clinical form	Age at diagnosis (years)	17OHP basal (ng/mL)	17OHP stimulated (ng/mL)	Region of Sergipe
Group A (subgroup A1)						
Q318X / Q318X	F	SW	0.42	1600	–	E
Q318X/ Q318X	F	SW	0.16	NA	–	E
Group A (subgroup A2)						
I2Splice/ Q318X	F	SW	0.03	1615	–	E
I2Splice / Q318X, R356W†	F	SW	0.02	NA	–	N
Group B						
I2 Splice / I172N*	M	SV	4.0	93.7	–	N
I2 Splice / I172N*	F	SV	2.0	410.3	–	N
Q318X, R356W / I172N	F	SV	2.0	NA	–	N
Q318X, R356W / I172N	M	SV	3.0	13	–	N
Group C						
V281L / V281L	F	LO	20.8	3.5	24.6	E
V281L / Q318X, R356W†	F	LO	13.0	4.8	43.9	N
R356W / V281L	F	LO (A)	26.4	22.3	24.0	B
I2 Splice / del 8pb,	M	SV	2.0	44.6	–	E
I2 Splice, P30L						

A = asymptomatic; F = female; M = male; NA = not available; E = East region of Sergipe; N = North region of Sergipe; B = Bahia; Groups A, B and C, as described by Speiser and cols. (2); * and †, brothers.

Southern Blotting to verify the presence of deletion or macroconversion in father and/or child alleles. Twelve patients (57.1%) had their genotype identified (Table 3); 4 presented the Group A genotype (all of them with the SW form), 4 patients presented the Group B genotype (all of them with the SV form) and 4 patients presented the Group C genotype (3 NC and 1 SV). There was a prevalence of 18.2 cases of CAH-21OHD/100.000 inhabitants in the North region and 1.65 cases of CAH-21OHD/100.000 inhabitants in the East region of Sergipe State (Table 4).

Table 4. Prevalence of congenital adrenal hyperplasia due to 21-hydroxylase deficiency in the North and in the East region of Sergipe State.

	North	East
Cases	10	10
Estimated Population	54.843	605.661
Estimated Prevalence	18.2 / 100.000	1.65 / 100.000 *

North = Cedro de São João (5.538 inhabitants), Neópolis (20.483 inhabitants) and Propria (28.822 inhabitants) (19). East: Santo Amaro das Brotas (10.699 inhabitants), Barra dos Coqueiros (20.990 inhabitants), São Cristóvão (75.353 inhabitants) and Aracaju (498.619 inhabitants) (19). * p<0.0001

DISCUSSION

In the present study, we report the frequencies of seven microconversions described in *CYP21A2* (P30L, I2Splice, 706_713del8, I172N, V281L, Q318X, and R356W) in a new Brazilian cohort, a population from Sergipe, located in the Northeast region of Brazil. The ethnic origin of the Brazilian population is extremely heterogeneous, which justifies the current study. All other studies on the genetic characteristics of *CYP21A2* available in the Brazilian population are restricted to the Southeast region of Brazil (10,11,14,15,17,18,20-26), especially from Sao Paulo State. We found only one unpublished study performed in Bahia State, which evaluated 30 individuals with the classical forms of CAH-21OHD, where allele-specific PCR was used for the determination of 14 mutations. In 48 unrelated alleles, 16 SW and 32 SV forms, 75% of alleles were identified. I2Splice was observed in 25%, I172N in 20% and Q318X in 13% of the alleles (16).

We studied 15 families and one isolated case of CAH-21OHD using allele-specific PCR. *CYP21A2* mutation was detected in 74.3% of the disease-causing alleles. Torres and cols. (14), using the same methodology as the present study, identified 76% of mutated alleles. Other Brazilian studies, which besides screening the most frequent microconversions, also screened gene deletion and large gene conversion, identified about 80-85% affected alleles (15-17). Therefore, the slightly lower frequency of affected alleles (74.3%) observed in this study can be ascribed to the lack of studies on macroconversion and deletions, which were not evaluated systematically, since Southern Blotting and gene sequencing were not performed in the present study.

In the present study, genotype was identified in 57.1% of the patients, similar to other studies using the same diagnostic approach (14). Other studies, which analyzed microconversion, large gene conversion and deletion, genotype has been identified in up to 81% of the patients (15,16).

Our series presented a higher frequency for Q318X mutation (25.7%) compared to other worldwide studies (2,14-18,21,26-31). In studies from the Southeast region of Brazil, Q318X mutation frequency ranged from 1.1% (14) to 13% - 16% (16,26). The frequency observed in this study was even higher when compared to other countries, except for one study in Tunisian patients, in which Q318X mutation was found in 35.3% of the alleles (32). Kharrat and cols. (32) detected linkage disequilibrium between the Q318X mutation and a *CYP21A2* gene polymorphism (601C→G at intron 2) in 83.3% of alleles, a loci probably due to the antiquity of the founder chromosomes. Moreover, the authors found the highest rate of consanguinity (60.8%) described in the literature, which probably allowed the dissemination of the Q318X mutation among Tunisian population. It is difficult to suppose the same descendant from Tunisian and Brazilian patients, since six out of 11 alleles with the Q318X mutation in Sergipe´s patients had also the R356W mutation, which was found in only 2.9% of the Tunisian patients. These mutations (Q318X and R356W) in the same allele might have been transferred in a single event of gene conversion, since they are neighboring in pseudogene.

Despite no recent history of consanguinity in our patients, the molecular study of the *CYP21A2* gene in Bahia State also detected a higher frequency of Q318X mutation (13%) than the observed in previous Brazilian studies (16). As Bahia and Sergipe States are neighboring, a founder effect can be responsible for the high frequency of Q138X mutation in the Northeastern region of Brazil. Further studies in these families harboring the Q318X mutation, using microsatelites or polymorphism (SNPs) markers, will be essential to clarify the reason of the high frequency of Q318X mutation in patients with CAH-21OHD from Sergipe. In addition, it is important to point out that the allelic frequency of any *CYP21A2* point mutation, including Q318X mutation, varies according to the number of patients with late onset CAH-21OHD included in the study. Indeed, our study as well as the Witchel and cols. study (26) present a small number of LO form of CAH-21OHD and high frequency of Q318X mutation.

The second most frequent mutation was V281L (17.1%). This result was similar to those obtained in previous Brazilian reports, where the three clinical forms of the CAH-21OHD were analyzed (14,15,18). On the other hand, V281L mutation frequency was smaller in series in which only classical forms of CAH-21OHD were studied (16,17).

I172N was present in 14.3% of unrelated alleles. This result is similar to those obtained in previous Brazilian reports (14-18). In the present study, all patients harboring the I172N mutation presented the SV form of CAH-21OHD. Some series showed discrepancy between genotype and phenotype associated with the I172N mutation, with some patients presenting the SW form, probably because of the low enzymatic activity predict by this mutation cannot be sufficient to prevent the salt wasting (31,33).

In our series the R356W mutation was found in 14.3% of the studied alleles, similar to the frequency obtained in other Brazilian studies (13-17).

I2Splice mutation was present in 14.3% of the studied alleles. In all Brazilian studies, it has been the most frequent mutation associated with the SW form (13-15,17,26), suggesting the diversity in Brazilian ethnic background.

P30L, I2Splice, and del 8pb were found in one of the studied alleles. In this patient, allele–specific PCR could have amplified a hybrid gene, which results from the *CYP21A2* deletion or large gene conversion, presenting pseudogene´s sequences in the 5´ extremity and sequences of the active gene in the 3´ extremity. Indeed, in 92% of the hybrid genes, del 8 pb is present (17,22). Similarly, other studies (14,22) showed patients harboring the del 8pb mutation in association with P30L and I2 Splice in the same allele, also suggesting a deletion or large gene conversion in *CYP21A2*. To establish the actual genotype of this patient further studies using Southern Blotting or gene sequencing should be done.

In the present study, we found that 14.3% of the alleles presented more than one mutation. One asymptomatic affected father was diagnosed with two different mutations in his alleles; one of these mutations has been associated with severe reduction of enzymatic activity. He had a daughter with SW and another with NC form of CAH-21OHD. Indeed, it is well established that asymptomatic parents with different mutations in their alleles can generate children with different clinical forms of CAH-21OHD. The segregation analysis in this family is important to identify the genotype and genetic counseling.

In one patient, we did not differentiate homozygosis or hemizygosity. Unfortunately, her parents were not available to confirm her familial segregation. In another patient, homozygosity was not confirmed by segregation analysis studies in the family, because the mutated allele was not identified in the father. The patient might be hemizygous or might have a *de novo* mutation. So, to clarify these points it will be necessary to perform Southern Blotting studies in these families.

We observed a good correlation between genotype and phenotype. Most patients showed compound heterozygous mutations and the clinical form of CAH-21OHD was correlated with the mutated allele with higher enzymatic activity (3,33). Among the twelve patients who had mutations identified in both alleles, only one patient had genotype and phenotype discordance. This patient with the SV form of CAH-21OHD presented I2 Splice in one allele and P30L, I2 Splice, and del 8pb in another. In this case, I2 Splice can be associated with variation of phenotype, because a small amount of normally spliced mRNA can be detected and a small amount of normal enzyme can be synthesized (3,13,17,31,33).

There was a higher prevalence of CAH-21OHD in the North region of Sergipe State. We did not find history of recent consanguinity among these families that could justify the high prevalence of cases. It is unlikely there was a bias in selection, since the University's Hospital is the single center of reference to Sergipe State. Therefore, we believe that there is a higher number of undiagnosed patients in the other regions. In conclusion, our molecular analysis approach allowed the identification of *CYP21A2* mutations in 74.3% of the studied alleles and identified the genotype in 57.1% of patients. There was a good correlation between genotype and phenotype. However, the frequency of *CYP21A2* mutations in the Northeast of Brazil differed from other studies from Southeast of Brazil. Our dada amplify the knowledge on ethnic specific frequency of *CYP21A2* mutations in the Brazilian population.

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