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Research Article

CYP21 gene mutations in Brazilian patients with 21-hydroxylase deficiency from the Amazon region

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Abstract

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (P450c21, CYP21) accounts for about 95% of all CAH cases. The incidence of *CYP21* gene mutations has been extensively studied in the last years, but in Brazil it has been investigated only in Southeast Brazilian patients. This study is the first report on the distribution of *CYP21* mutations in patients from the Amazon region. Direct sequencing of the *CYP21* gene identified at least one mutation in 96% of the studied chromosomes. The most common mutations found were IVS2-13A/C > G (36%), Q318X (12%), V281L (12%), 1760_1761insT (9%), Cluster E6 (7%), and P30L (7%). The worldwide most common mutations were identified among patients from the Amazon region at frequencies that may be expected for a population resulting from the admixture of Europeans (predominantly Portuguese), African Blacks and Amerindians, in proportions that differ from those estimated for South Brazilian populations. Interethnic mixture may explain the differences in the frequencies of some mutations between Brazilian patients from the Amazon and from the Southeast of the country. However, the differences found may also be due to variation in the number of patients with the different clinical forms of 21-hydroxylase deficiency in the studies carried out so far.

Key words: Amazon region, 21-hydroxylase deficiency, DNA sequencing.

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Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OH, P450c21, CYP21) is a disorder of the adrenal cortex characterized by cortisol deficiency, with or without aldosterone deficiency, and excess of androgen. It accounts for about 95% of all CAH cases.

Reduced levels of cortisol promote an overproduction of adrenocorticotrophic hormone (ACTH) and the consequent increase in the secretion of androgen, resulting in clinical manifestations that include a severe type (classical form) and a milder type (nonclassical form, NC), whose carriers remain either asymptomatic or develop symptoms during childhood or at puberty. The classical form is subdivided into the simple virilizing (SV) form, with prenatal virilization of the external genitalia in the female fetus, and the salt-wasting (SW) form that consists in the virilization of the female fetus and can lead to a salt-wasting crisis in both genders (New *et al.*, 1989; Morel and Miller, 1991). The classical form occurs at a rate between 1:5,000 and 1:15,000 among live North American and European neonates, while the nonclassical form occurs in approximately 0.2% of the general white population. This rate is especially high in Ashkenazi Jews and a part of the Mediterranean population (*i.e.*, Italians, Hispanics) (Trakakis *et al.*, 2005).

The gene encoding human 21-OH (*CYP21*) is located at 6p21.3, within HLA class III, approximately 30 kb apart from its pseudogene (*CYP21P*), which is inactive due to the presence of several inactivating mutations (White *et al.*, 1985; Higashi *et al.*, 1986; Lee, 2001). *CYP21* and *CYP21P*, both with 10 exons, share about 98% homology in their exons and about 96% in their introns. These genes are included in the RCCX module, composed of four tandem genes: *RP1-C4A-CYP21P-TNXA-RP2-C4B-CYP21--*

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TNXB (Lee *et al.*, 2004). Because of the high homology and tandem-repeat organization, this gene cluster is subject to a high frequency of recombination events, which can lead to two distinct situations: (i) unequal crossing-over during meiosis, which can produce a wide variety of rearrangements depending on the breakpoints, such as gene duplications and gross gene deletions encompassing *C4* and *CYP21*; (ii) large and short gene conversion events that transfer deleterious mutations present in the *CYP21P* gene to *CYP21* (Higashi *et al.*, 1988; Tusie-Luna and White, 1995; White *et al.*, 1986). About 95% of the mutant alleles are generated by the conversion of DNA sequences from the *CYP21P* pseudogene to the active *CYP21* gene, while the remainder are new mutations not found in the pseudogene (White and Speiser, 2000).

To date, according to the Human Gene Mutation Database (HGMD-2007), 103 mutations of the *CYP21* gene have been reported, most of them nonsense/missense (64), splicing (7), regulatory (1), small deletions (11), small insertions (8), small indels (3), gross deletions (4), gross insertions (2), and complex rearrangements (3). However, apart from gene deletions and large gene conversions, nine mutations are reported with a higher frequency, namely P30L, IVS2-13A/C > G, 707_714del8 (exon 3), I172N, V281L, 1760_1761insT, Q318X, R356W, and P453S (Morel and Miller, 1991).

Most patients are compound heterozygotes (*i.e.*, they have different mutations on the two chromosomes), and the clinical phenotype is generally related to the less severely mutated allele and, consequently, to the residual 21-OH activity (Speiser *et al.*, 1992; Wilson *et al.*, 1995; Jaaskelainen *et al.*, 1997; Krone *et al.*, 2000).

The distribution of *CYP21* gene mutations in patients of Southeast Brazil has been investigated previously, and the results obtained reveal that the mutation frequencies did not differ from those found worldwide, but at least 13 new mutations were identified (Bachega *et al.*, 1998; Paulino *et al.*, 1999; Witchel *et al.*, 2000; Torres *et al.*, 2003). However, there are no genetic reports of studies conducted on patients of other areas, especially of the North of the country, where the population results from intense miscegenation of Europeans, Africans and Amerindians.

This is the first report on the prevalence of point mutations and short insertions/deletions in the *CYP21* gene of patients with 21-OH deficiency from the State of Pará, in the Brazilian Amazon region.

Materials and Methods

Subjects and DNA sample preparation

CYP21 gene analysis was performed in 46 unrelated patients with clinical symptoms of 21-OH deficiency, seen at Foundation Santa Casa de Misericórdia do Pará and University Hospitals Bettina Ferro de Souza and João de Barros Barreto. The patients were referred to the Human and Medical Genetics Laboratory for karyotyping and investigation of gene *CYP21* mutations. Either the patients or their legal guardians signed an informed consent for the participation in the research. The patients' age varied from 15 days to 84 years, and 61% of them were classified as genetic females. Thirty-four patients presented the classical form of the disease (19 simple virilization and 15 salt-wasting cases), and 12 had the non-classical form. The clinical diagnosis of the form of the disease (SV, SW or NC) was made by pediatric endocrinologists and gynecologists at the different hospitals from where the patients were referred. SW CAH was characterized by the onset of hyperkalemia, hyponatremia, dehydration, and/or shock in the first month of life. Female patients had ambiguous genitalia. The SV form of 21-OH deficiency was diagnosed based on the presence of ambiguous genitalia along with no evidence of sodium depletion. The NC form was characterized by late-onset signs of androgen excess, including growth acceleration and advancement of bone age in children, or menstrual disturbances, infertility and hirsutism in adult women. None of the patients had consanguineous parents.

Peripheral blood was collected and the leukocytes were used for DNA extraction, following the phenol/chloroform DNA extraction protocol of Sambrook *et al.* (1989).

Selective amplification of the CYP21 gene

Selective PCR amplification of the *CYP21* gene was performed in two segments, using primers P1 (5'-TCGG TGGGAGGGTACCTGAAGGT-3') and P2 (5'-GCATCT CCACGATGTGAT-3'), and P3 (5'-CCGGACCTGTCCT TGGGAGACTACT-3') and P4 (5'-CTGAGCGGCTGGG TGAAATGGAAC-3'), respectively. Fragment 1 represents a 1339-bp segment extending from exon 1 to exon 6. Fragment 2 is a 2220-pb fragment extending from the 8-bp deletion in exon 3 to beyond exon 10, according to Oriola *et al.* (1997). These two overlapping segments amplify the whole coding region of the *CYP21* gene using the Elongase kit (Invitrogen). The 5'UTR and the 3'UTR region were not included in this study.

DNA sequencing

Each *CYP21* exon and its flanking sequences were amplified by nested-PCR using the P1-P2 and P3-P4 reaction products (diluted 1:100) as templates, and internal primers designed according to gene sequence. Primer sequences are available upon request. After purification with an EXOSAP kit (USB), the PCR products were directly sequenced with the forward and reverse primers used for nested-PCR, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The reaction products were electrophoresed in an automated ABI Prism 3130 sequencer (Applied Biosystems).

Results

Mutations were identified in 45 of the 46 patients, corresponding to a spectrum of 22 different mutations: 17 missense or nonsense mutations, two short insertions, two short deletions and one splicing mutation. The frequencies of the different mutations found are shown in Table 1. The most common mutations found were IVS2-13A/C > G (35%), Q318X (12%), and V281L (12%). Mutations 1760_1761insT, Cluster E6, and P30L showed somewhat lower frequencies: 9.0%, 7.0%, and 7.0%, respectively. These six mutations account for 82% of all mutations found.

Regarding the distribution of the mutations by clinical forms of the disease, IVS2-13A/C > G was the most frequent among patients with the classical forms, accounting for 51% of the mutations found in the salt-wasting form and 30% in the simple virilizing form, while in patients with the nonclassical form, mutations Q318X and V281L were the most common (25% and 20%, respectively), followed by mutation IVS2-13A/C > G (15%).

Six patients (13%) were homozygous for a single mutation, 13 (28%) were homozygous for one mutation and heterozygous for one or more additional mutations, 14 (30%) were compound heterozygotes with different mutations on each chromosome, 12 (26%) patients were simple heterozygotes, and one patient (2%) had no mutation at all.

Table 1 - Distribution of the most common mutations found in Brazilian patients from the Amazon region for the different clinical forms of 21-hydroxylase deficiency.

Mutation	Clinical form (%)			Total (%)
	SW	SV	NC	
P30L		6 (0.14)	1 (0.05)	7 (0.07)
IVS2-13A/C > G	20 (0.51)	13 (0.30)	3 (0.15)	36 (0.35)
707_714del8	1 (0.03)			1 (0.01)
992_993insA		2 (0.05)	1 (0.05)	3 (0.03)
I172N		1 (0.02)	1 (0.05)	2 (0.02)
L226Q			1 (0.05)	1 (0.01)
Cluster E6	3 (0.08)	3 (0.07)	1 (0.05)	7 (0.07)
E238V		1 (0.02)		1 (0.01)
A265V		1 (0.02)		1 (0.01)
G274E	1 (0.03)			1 (0.01)
V281L	3 (0.08)	5 (0.12)	4 (0.20)	12 (0.12)
M283K		1 (0.02)		1 (0.01)
1760_1761insT	3 (0.08)	4 (0.09)	2 (0.10)	9 (0.09)
Q318X	3 (0.08)	4 (0.09)	5 (0.25)	12 (0.12)
R339H	1 (0.03)	1 (0.02)		2 (0.02)
R356W		1 (0.02)		1 (0.01)
A362S	2 (0.05)			2 (0.02)
G375S	1 (0.03)			1 (0.01)
R479L	1 (0.03)			1 (0.01)
2669delG			1 (0.05)	1 (0.01)
Total	39 (1)	43 (1)	20 (1)	102 (1)

The presence of at least one polymorphism was detected in six of the 11 heterozygous patients, and more than one polymorphism was identified in one of two patients who did not present mutations for 21-OH deficiency.

De novo mutations were found in two patients: mutation IVS2-13A/C > G, of maternal origin, and mutation 1760_1761insT, which could not be traced to any of the parents. In both cases parenthood was confirmed by multiple polymorphic markers, thus excluding the possibility of uniparental disomy.

Sequence changes that might represent novel substitutions were also found in this study: L226Q (1350T > A) in exon 6, A265V (1636C > T) and G274E (1663G > A) in exon 7. Mutation 1636C > T causes the substitution of alanine by valine, amino acids with similar properties, therefore being a neutral mutation that probably does not affect enzyme activity. On the other hand, the 1663G > A transition that results in substitution of the amino acid glycine by glutamic acid at position 274 (G274E), and the 1350T > A transversion that changes codon 226 from leucine to glutamine, are missense mutations which could cause a potentially pathogenic enzyme structural variation.

Discussion

Molecular analysis of the *CYP21* gene was performed, to determine genetic aspects of 46 unrelated Brazilian patients with 21-OH deficiency. This is the first reported study on the distribution of mutations causing 21-OH deficiency in the Brazilian population of the Amazon region.

Direct sequencing of *CYP21* exons allowed the identification of at least one mutation in 98% of the patients studied (46), corroborating previous findings that both large gene conversions and gene deletions are uncommon among Brazilian patients with 21-OH deficiency (Bachega *et al.*, 2004).

The most frequent point mutation in our patient sample, IVS2-13A/C > G, is a severe mutation, categorized as group A, based on about 2% residual enzyme activity, established *in vitro* (Higashi *et al.*, 1988). It is commonly associated to the SW form. This mutation is also the most common worldwide, except in some groups of patients of South Europe (Portugal, Italy and Spain), where it is less common and where mutation V281L has been identified as the most prevalent. Mutation V281L, which confers more than 20% enzymatic activity, is the most common variant associated to the nonclassical form of 21-OH deficiency in South European countries, reaching 34% in Spain (Ezquieta *et al.*, 2002), 28% in Portugal (Friaes *et al.*, 2006) and 24% in Italy (Balsamo *et al.*, 2000). In North Europe, the most frequent mutation in NC alleles is P30L (Table 2).

This mutation is also common in Argentine patients (25%) (Dain *et al.*, 2002).

Mutation Q318X, the second most common mutation in our patient sample along with mutation V281L (frequency of 12%), is a severe mutation (null group) that abolishes the activity of the 21-OH enzyme. It has been found at relatively low frequencies (3% to 10%) in most of the studied populations, except in a sample of Japanese patients in which its frequency was 20% (Tajima *et al.*, 1993). In our sample, the observed frequency of mutation Q318X was somewhat higher than those found in patients from Southeastern Brazil (6% and 7%) by Paulino *et al.* (1999) and Bachega *et al.* (2004), respectively.

Three other relatively common mutations found in our patients (1760_1761insT, Cluster E6, and P30L) also exhibited somewhat higher frequencies than those reported for patients from Southeast Brazil and for most of the populations studied worldwide (Table 2). Mutations 1760_1761insT and Cluster E6 are classified as null mutations, while P30L is considered a mild mutation (group C).

In our patient sample, the nine most common mutations found worldwide (Table 2) represented 80% of the total of mutations, 30% corresponding to null mutations (Q318X, 1760_1761insT, and Cluster E6); 34% to group A, with some residual enzyme activity (IVS2-13A/C > G); 2% to group B, with less than 10% residual enzyme activity (I172N); and 19% to group C mutations, with 10%-20% residual activity (V281L and P30L).

It is important to point out that the prevalence of null mutations in our sample is one of the highest ever reported (30%), higher than the frequencies reported for Brazilian patients from the Southeast region (23% by Bachega *et al.*, 2004) and also for patients from Southern Europe (Portugal, Italy and Spain). These differences were due mainly to the high frequencies of the Q318X and 1760_1761insT mutations found in these patients from the Amazon region. On the other hand, our data are comparable to those found in Argentine patients (Dain *et al.*, 2002). When the null and A group (IVS2-13A/C > G, predominantly) mutations are grouped together, the prevalence of mutations commonly associated to the SW and SV forms corresponds to 64% of the total in our patient sample, a value that is higher than those found in most of the previously studied populations, except in Japanese (64%-67%) and Mexican (62%) patients. In contrast, the frequencies found for group C mutations, both in our sample (19%) and in patients from South Brazil (20%), are lower than those reported for South European countries, particularly Spain (34%), Portugal (30%), and Italy (25%), as well as for Argentine, owing mainly to

Table 2 - Frequency (%) of the nine most common mutations found worldwide and in Brazilian patients from the Amazon region with classical and nonclassical forms of 21-hydroxylase deficiency.

Country	N	Mutation									Reference
		P30L	IVS2-13 A/C > G	707_714 del8	I172N	Cl E6	V281L	1760_1761 insT	Q318X	R356W	
Brazil	92	7	36	1	2	7	12	9	12	1	This study
Brazil	410	1	34	2	14	0	18	3	7	11	Bachega *et al.*, 2004
Brazil	74	2	21	1	14	1	18	3	6	7	Paulino *et al.*, 1999
Brazil	142	4	36	15	10	-	9	1	18	12	Witchel *et al.*, 2000
Argentina	73	0	10	8	6	3	25	1	8	10	Dain *et al.*, 2002
Mexico	94	9	48	2	12	0	9	1	4	7	Ordoñez-Sánchez *et al.*, 1998
U.S.A.	92	0	28	0	8	5	0	3	7	8	Speiser *et al.*, 1992
U.S.A.	394	3	27	3	8	0	9	1	4	4	Wilson *et al.*, 1995
U.S.A.	96	0	13	0	5	4	4	0	3	0	Olney *et al.*, 2002
Portugal	112	2	11	3	10	2	28	1	6	2	Friaes *et al.*, 2006
Italy	146	8	30	0	10	0	16	1	10	0	Carrera *et al.*, 1996
Italy	122	0	21	0	8	0	24	0	0	0	Balsamo *et al.*, 2000
Spain	76	3	25	4	1	0	18	1	4	4	Ezquieta *et al.*, 1995
Spain	354	0	18	2	4	0	34	1	9	0	Ezquieta *et al.*, 2002
Germany	124	2	24	0	21	1	6	1	6	3	Kapelari *et al.*, 1999
Germany	182	4	33	2	20	6	3	6	4	3	Krone *et al.*, 1998
Hungary	334	0	40	0	18	0	8	5	6	6	Ferenczi *et al.*, 1999
Romania	34	15	35	12	15	0	0	0	0	4	Grigorescu-Sido *et al.*, 2002
Japan	98	4	40	4	14	5	0	0	3	15	Koyama *et al.*, 2002
Japan	46	0	37	0	24	0	0	0	20	7	Tajima *et al.*, 1993

the high frequency of mutation V281L in the latter populations (Table 2).

The patient in which no mutations were found was clinically classified as presenting simple virilizing 21-hydroxylase deficiency, with ambiguous genitalia and undefined gender at birth. We suggest that this patient may carry mutations in regions which were not investigated, such as the promoter region, or in other genes involved in the cholesterol pathway, like 3β-hydroxysteroid dehydrogenase or 17α-hydroxylase, whose deficiency accounts for 5% of all cases of congenital adrenal hyperplasia (White and Speiser, 2000).

In conclusion, the most common mutations found worldwide were identified in patients from the Brazilian Amazon region, but the prevalence of null mutations was one of the highest in the world (30%), whereas the frequencies of group C mutation were lower than those reported for South European countries. However, overall, the mutations were found at frequencies that can be considered as the expected ones for a population resulting from the admixture of Europeans (predominantly Portuguese), African Blacks and Amerindians in proportions that differ from those estimated for South Brazilian populations, mainly regarding the contribution of Europeans, which is higher and more diversified in the Southeast, where the presence of Italian descendants besides the Portuguese is significant, mainly in the State of São Paulo. The available data also indicate a greater contribution of African Blacks in the Southeast, but a much lower Amerindian influence (Salzano, 2004).

Estimates of interethnic admixture for urban populations of the Amazon region based on blood group plus protein markers show average values of 47% European contribution, 12% African contribution, and 41% Amerindian contribution, with an increase of the Amerindian contribution from Belém towards Manaus and a decrease of the African contribution in the same direction. The average values found using nuclear DNA polymorphisms (STRs) were somewhat different from those obtained with the so-called classical polymorphisms, showing a larger European contribution (68% *vs.* 41%), similar values of African (14% *vs.* 12%), and a lower Amerindian contribution (18% *vs.* 41%). On the other hand, the two sets of results show values which are consistent with the Brazilian history, pointing to a higher Amerindian influence in the North (18%, 48%) and Midwest (11%, 17%) regions. Also according to history, the blood group + protein results suggest higher African contributions in the Northeast (26%) and Southeast (44%) regions (Salzano, 2004).

We suggest that interethnic mixture can explain the differences in the frequencies of some mutations between patients of the Brazilian Amazon and the Southeast regions. However, the differences found may also be due to the number of patients with the different clinical forms of 21-hydroxilase deficiency, which vary among the studies published so far.

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Internet Resources

Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/ac/gene.php?gene=CYP21A2.

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