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p.H282N and p.Y191H: 2 novel *CYP21A2* mutations in Italian congenital adrenal hyperplasia patients

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ABSTRACT

More than 90% of all cases of congenital adrenal hyperplasia (CAH) result from steroid 21-hydroxylase gene (*CYP21A2*) mutations. The *CYP21A2* gene is located in the human leukocyte antigen (HLA) class III region on the short arm of chromosome 6p21.3, along with an inactive pseudogene, *CYP21A1P*, that is 98% homologous in its coding sequence with *CYP21A2*. Most *CYP21A2* mutations result from intergenic recombinations between *CYP21A2* and the closely linked *CYP21A1P* pseudogene. Rare mutations not generated by gene conversion account for only 5% to 10% of 21-hydroxylase deficiency alleles. However, detection of these rare and spontaneous mutations has continued to expand worldwide. We identified 2 novel *CYP21A2* missense mutations (p.H282N and p.Y191H) in 2 Italian patients with simple-virilizing and nonclassic CAH forms. Functional analysis of these *CYP21A2* mutations was performed. Functional in vitro assay for mutagenized CYP21A2 enzymes was performed in transiently transfected mammalian cells to test the residual enzyme activity and the apparent kinetic values. The residual activities obtained allowed us to classify the p.H282N and p.Y191H variants as simple-virilizing and nonclassic CAH associated mutations, respectively. These results correlate with the rate of severity of the patients' disease. This finding provides a further contribution for assisting in the diagnosis of CAH patients.

1. Introduction

Congenital adrenal hyperplasia (CAH) is one of the most frequent inborn metabolic errors inherited in an autosomal recessive manner. More than 90% of cases of CAH are due to 21-hydroxylase deficiency [1].

In the adrenal cortex, steroid 21-hydroxylase normally converts 17-hydroxyprogesterone (17-OHP) into 11-deoxycortisol (11-DOF) and progesterone into 11-deoxycorticosterone (11-DOC). These steroids are subsequently converted into cortisol and aldosterone, respectively. Deficiency of the enzyme 21-hydroxylase leads to impaired adrenal steroid hormone synthesis. The result is an increased secretion of the adrenal corticotropic hormone from the pituitary gland with subsequent adrenal hyperplasia and overproduction of androgen [1,2].

Authors' contributions: Paola Concolino: carried out the molecular genetics studies, participated in the sequence alignment, and drafted the manuscript. Enrica Mello: participated in the molecular genetics study and in the sequence alignment. Maria Cristina Patrosso: performed clinical studies. Silvana Penco: performed clinical studies. Cecilia Zuppi: participated in the design of the study and in its coordination. Ettore Capoluongo: participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

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21-Hydroxylase deficiency includes a wide spectrum of clinical manifestations [3]. Milder forms are referred to as *nonclassic* (NC) or *late-onset CAH*. Symptoms of NC-CAH include premature pubarche in children and acne, hirsutism, and menstrual irregularities in women. The severity of hyperandrogenic symptoms is variable in these patients, and males are usually asymptomatic [1,3]. Patients with the severe classic disease form are classified as either salt-wasting or simple-virilizing (SV) depending on whether or not synthesis of the salt-retaining hormone, aldosterone, is affected [1,3].

The *CYP21A2* gene is located in the human leukocyte antigen (HLA) class III region on the short arm of chromosome 6p21.3, along with an inactive pseudogene, *CYP21A1P*, that is 98% homologous in its coding sequence with *CYP21A2* [4,5].

Most of the mutations found in the *CYP21A2* gene are normally present in the pseudogene, implying that recombination events (microconversion) between these 2 genes may lead to the transfer of mutations from the pseudogene to the functional gene [6]. Only around 5% of all disease-causing *CYP21A2* alleles harbor rare mutations not originating from the pseudogene [1,7]. Detection of these rare and spontaneous mutations has continued to expand worldwide [8-10].

In this study, we report the characterization of 2 novel *CYP21A2* missense mutations (p.H282N and p.Y191H) found in 2 different CAH patients of Italian origin. These mutations are not apparently gene conversions and have not been described before.

2. Material and methods

2.1. Patients

Both patients were referred to our laboratory for molecular screening of the *CYP21A2* gene after endocrine evaluation and general clinical examination. Informed consent for the study was obtained from patients and patients' relatives.

Patient 1 was a 37-year-old Italian woman. Clinical diagnosis of SV-CAH was made at birth due to genital virilization (Prader stage III) and elevated 17-OHP serum concentration. No salt-wasting crisis was noted. Substitutive therapy was administered and clitorido-vulvoplasty was performed at 4 years of age.

Patient 2 was a 39-year-old Italian woman who had hirsutism (Ferriman index = 15) and polycystic ovarian syndrome with regular menses from the age of 12 years. The serum basal level of 17-OHP was 13.7 nmol/L (reference range at follicular phase, 0.6-3.6 nmol/L), increasing to 54.6 nmol/L after administration of 250 μg of adrenal corticotropic hormone. All other serum hormonal levels were within the reference ranges. The patient, who had the NC form of CAH, was treated with dexamethasone (0.25 mg/d); and her compliance is currently good.

2.2. Molecular analysis of *CYP21A2* gene

To perform genetic analysis of CAH, genomic DNA was isolated from peripheral blood samples using High Pure PCR Template Preparation Kits (Roche Diagnostic, USA), quantified by spectrophotometer at 260 nm, and stored at –20°C until use.

Molecular diagnosis of 21-hydroxylase deficiency, including multiplex ligation-dependent probe amplification (MLPA) analysis, was performed as previously described [11,12].

The *CYP21A2* reference sequence was NCBI-M13936.

2.3. Site-directed mutagenesis

The p.H282N and p.Y191H mutations were introduced using the pALTER-CYP21A2 mutagenesis vector (kindly provided by Mrs M Barbaro, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden) and the Altered Sites II in vitro Mutagenesis System (Promega Italy, Milano, Italy) according to the manufacturer's specifications. Mutagenesis sequences primers, covering the specific point mutations, are available on request. BglII-KpnI fragments of pALTER-CYP21A2 (p.H282N) and pALTER-CYP21A2 (p.Y191H) were transferred into pCMV4 vector, thereby generating pCMV4-CYP21A2 (p.H282N) and pCMV4-CYP21A2 (p.Y191H) constructs. The complete *CYP21A2* complementary DNA was sequenced to verify the correct mutation incorporation and to exclude additional sequence aberrations.

2.4. Transient expression of *CYP21A2* in COS-1 cells and assay of enzyme activity

The experiments were performed as previously described [13,14]. Briefly, after COS-1 cells transfection, 3H-labeled substrate (17-hydroxyprogesterone or progesterone) was added to each well of cells together with unlabeled steroid (Sigma Diagnostic) and NADPH cofactor (Sigma Diagnostic, USA). After conversion of the substrates to the corresponding products, 11-DOF or 11-DOC, the medium was collected in duplicate samples; and steroids were extracted and separated by thin layer chromatography.

Radioactivity was measured by liquid scintillation spectrophotometry. Enzyme activities were expressed as a percentage of wild-type CYP21A2 activity (arbitrarily defined as 100%).

Finally, different unlabeled steroid amounts (0.5, 1.0, 2.0, 3.0, 4.0, or 6.0 μmol/L) were used to determine the following apparent kinetic constants: the Michaelis-Menten constant (Km) and the maximum velocity (Vmax) [13,14]. Graph Pad Prism software (version 5.0; GraphPad Software, San Diego, CA) was used for kinetic constants calculation.

2.5. Western blot analysis

Western blot analysis was used to determine the level of expression of each 21-hydroxylase/pCMV4 construct. These assays were performed using anti-human CYP21A2 (C-17) polyclonal antibodies (1:800 sc-48466; Santa Cruz Biotechnology, Santa Cruz, CA) raised in goat as primary antibody and an anti-goat IgG-HRP (1:10000 sc-2020; Santa Cruz Biotechnology) as secondary antibody.

The amount of the applied samples was 18 μg per lane.

3. Results

3.1. Genetic analysis

Subject 1: The subject proved to be heterozygote for 2 mutations: the salt-wasting CAH g.868A/C>G mutation in intron 2 and the new p.H282N (g.1901C>A) in exon 7 of the

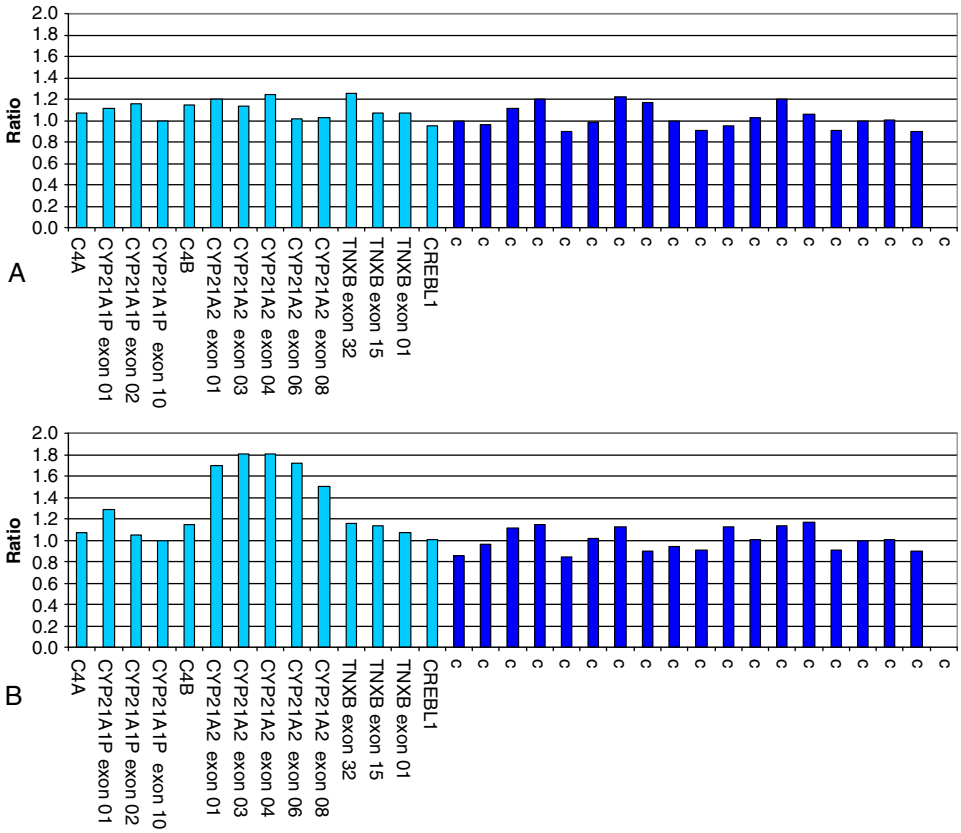

Fig. 1 – A, The MLPA data from a female control subject: relative peak ratio of all probes was close to 1. B, The MLPA data from a patient carrying a duplicated copy of *CYP21A2* on paternal chromosome 6: relative peak ratio of *CYP21A2* probes was greater than 1.3. RPR indicates relative peak ratio.

CYP21A2 gene. The MLPA analysis, confirmed by Southern blot test, showed that the woman carried 3 *CYP21A2* gene copies (Fig. 1).

The patient's family was enrolled for *CYP21A2* molecular study. The segregation analysis showed that the proband inherited the g.868A/C>G mutation from the father and the novel p.H282N mutation from the mother. In addition, MLPA results revealed that whereas the father carried 3 *CYP21A2* alleles, the mother was heterozygote at the investigated locus.

The patient's sister, a young woman with the same clinical diagnosis and clinical phenotype, showed an identical proband genotype.

The 2 sisters' clinical phenotype and a careful analysis of electropherogram peaks at g.868 position (the mutated base peak was 2-fold higher than the wild-type base peak) suggest that both *CYP21A2* copies inherited in tandem by the father carried the intron 2 g.868A/C>G mutation.

Subject 2: The MLPA analysis showed that the patient carried 2 copies of the *CYP21A2* gene (data not shown). Complete DNA sequencing of the *CYP21A2* gene was performed. The woman was heterozygous for the following 2 mutations: the NC-CAH p.V281L mutation in exon 7 and the novel p.Y191H (g.1358T>C) in exon 5 of the gene. We were only able to extend the *CYP21A2* genetic analysis to the patient's daughter, an asymptomatic 10-year-old girl who presented only the p.Y191H mutation in heterozygous status.

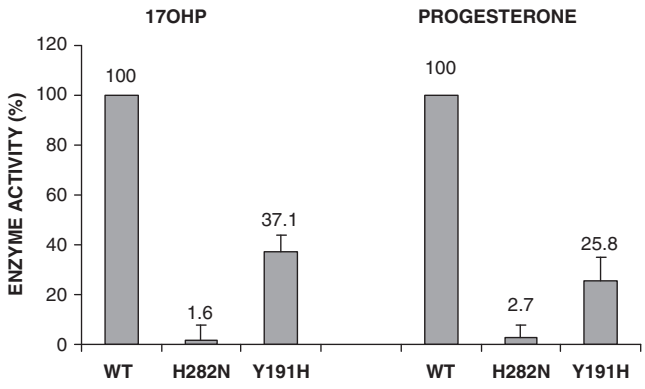

Fig. 2 – Enzymatic activities of P450c21 mutant in intact COS-1 cells. Activity is expressed as a percentage of wild-type activity, which is arbitrarily defined as 100%. Values are depicted for the conversion of 17-OHP to 11-DOF and progesterone to 11-DOC at a substrate concentration of 2 μmol/L of unlabeled steroid. The bars represent the mean ± 1 SD of 3 independent experiments, each one performed in triplicate. The mean values obtained are the following: (p.H282N) 17-OHP 1.6% ± 6%, progesterone 2.7% ±5%; (p.Y191H) 17-OHP 37.1% ± 7%, progesterone 25.8% ± 9%.

Table 1 – Kinetic constants of both wild-type and mutated enzymes

	WT	H282N	Y191H
17-OHP			
Km (μmol/L)	1.59 ± 0.20	4.83 ± 0.16	1.61 ± 0.21
Vmax (nmol/[min mg])	5.80 ± 0.17	1.52 ± 0.14	2.63 ± 0.18
Vmax/Km	3.65	0.31	1.63
Progesterone			
Km (μmol/L)	1.05 ± 0.07	2.62 ± 0.1	0.89 ± 0.09
Vmax (nmol/[min mg])	3.73 ± 0.05	1.56 ± 0.08	1.92 ± 0.05
Vmax/Km	3.55	0.60	2.16

Values are shown as the mean ± 1 SD of 3 independent experiments. There is a significant difference between Vmax values for the wild-type and mutant proteins for both substrates. No variations could be detected for p.Y191H Km values, whereas significant differences were observed for p.H282N mutation (by analysis of variance, Student-Newman-Keuls test, $P < .05$). WT indicates wild type.

The p.H282N and p.Y191H variants were not detected upon analysis of 50 normal controls (100 unrelated alleles). Thus, we can exclude polymorphisms occurring in the general population.

Finally, the presence of p.H282N and p.Y191H mutations in the pseudogene was investigated in CAH patients, in their family members, and in 30 healthy subjects. Neither of the 2 mutations was present in any of *CYP21A1P* alleles analyzed.

3.2. *Enzyme activity of mutants*

The effects of 2 different amino acid substitutions (p.H282N and p.Y191H) on 21-hydroxylase activity are shown in Fig. 2, with wild-type enzyme activity defined as 100%. The p.H282N mutation reduced enzyme activity to 1.6% for 17-OHP and 2.7% for progesterone, whereas the p.Y191H reduced activity to 37.1% and 25.8%, respectively (Fig. 2).

Finally, we investigated the kinetic constants of both wild-type and mutated enzymes. As reported in Table 1, the p.H282N variant showed an increased Km for both substrates, whereas Vmax was clearly reduced. In contrast, the p.Y191H mutation exhibited a normal Km but a low Vmax (Fig. 3).

3.3. *Expression of normal and mutant CYP21A2 in COS-1 cells*

To investigate whether the new *CYP21A2* mutations affected the levels of protein expression, Western blot analysis was performed on cells transfected with the different constructs. The data of wild-type and mutant proteins expressed in vitro demonstrate that neither mutation affected translation efficiency or stability (data not shown).

4. Discussion

We have assessed the impact on CYP21A2 enzyme function of 2 missense mutations by performing in vitro site-directed mutagenesis and enzyme activity assay. The 2 p.H282N and p.Y191H mutations, not previously described, were found in different patients investigated for 21-hydroxylase deficiency.

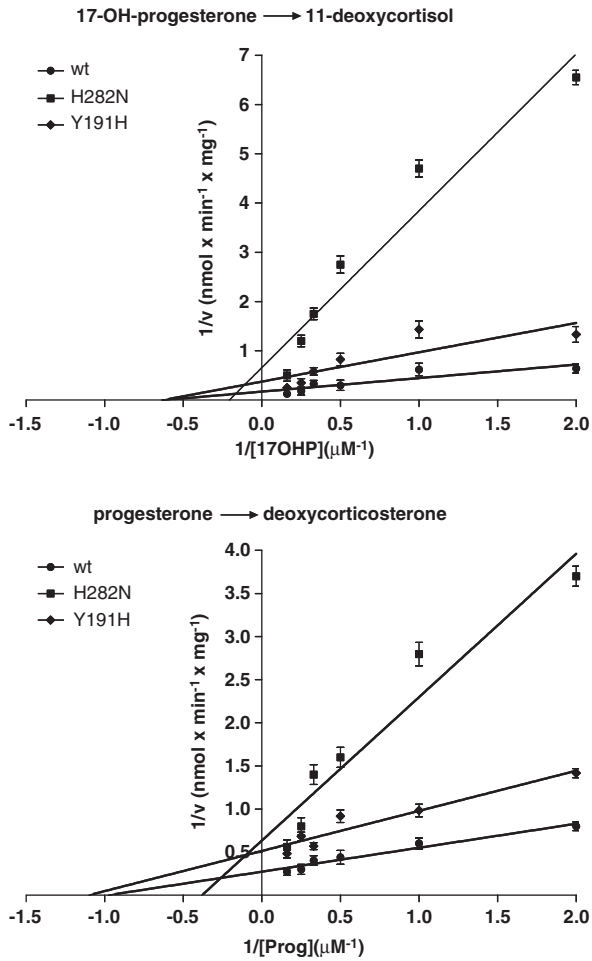

Fig. 3 – Apparent kinetics of wild-type and mutant proteins. The graphs show Lineweaver-Burk plots of enzymatic activity measured in intact COS-1 cells expressing CYP21A2 enzymes.

The first patient, clinically diagnosed for SV-CAH form, was a young woman carrying 3 *CYP21A2* gene copies (Fig. 1). A duplicated g.868A/C>G mutated allele was inherited from her father, whereas the novel p.H282N mutation was present on the maternal chromosome.

The effects of amino acid substitution on 21-hydroxylase activity are shown in Fig. 2, with wild-type enzyme activity defined as 100%. The mutation reduced enzyme activity to 1.6% for 17-OHP and 2.7% for progesterone (Fig. 2).

In addition, we investigated the kinetic constants of both wild-type and mutated enzymes. As reported in Table 1, the variant showed an increased Km for both substrates, whereas Vmax was clearly reduced (Fig. 3). The Western blot data of wild-type and mutant protein expressed in vitro showed that p.H282N mutation did not affect the translation efficiency. In fact, comparable amounts of proteins were detected (data not shown).

Based on these results and on the literature data [14], we can classify the p.H282N mutation as an SV mutation; and we can confirm that the clinical phenotype of our patient is determined by the allele carrying this less severe p.H282N mutation [15].

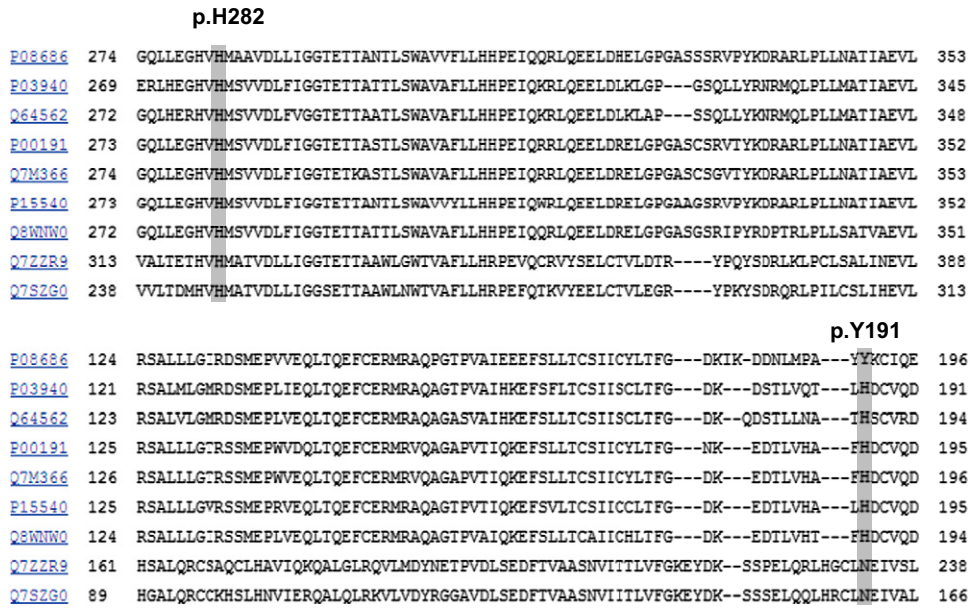

Fig. 4 – Multiple amino acid alignment of CYP21A2 orthologs from the Uniprot database including sequences from species in the following order: human (P08686), mouse (P03940), rat (Q64562), bovine (P00191), sheep (Q7M366), pig (P15540), dog (Q8WNW0), Japanese eel (Q7ZZR9), and Japanese puffer fish (Q7SZG0). The mutant amino acids of human CYP21A2 and corresponding residues of aligned sequences are highlighted.

The novel p.H282N mutation maps in a region of *CYP21A2* exon 7 that could be considered a mutational hot spot. In fact, at 281 and 283 amino acidic position, there are 2 well-known NC mutations: p.V281L and p.M283L [16].

Although histidine and asparagine are both polar amino acids, the lack of imidazolic ring of asparagine could modify the structure of the protein and, consequently, its enzymatic activity. In addition, homology alignments show that the 282 position is a strongly conserved amino acid position between different species (Fig. 4).

The p.Y191H mutation was found in a female patient with the NC form of CAH. She was compound heterozygote because she also carried the very common NC-CAH p.V281L mutation on the other *CYP21A2* allele. The p.V281L is a most common NC-CAH mutation and results in an enzyme with 50% of normal activity when 17-OHP is the substrate and with 20% of normal activity for conversion of progesterone to 11-DOC [17].

Because the residual enzymatic activity was 37.1% (conversion of 17-OHP to 11-DOF) and 25.8% (progesterone to 11-DOC) of the wild-type enzyme, the p.Y191H variant may be also considered an NC-CAH mutation. These results properly correlate with the severity of the patients' disease. The mutant enzyme did not show any change in substrate recognition; in fact, the Km was not affected, whereas Vmax was clearly reduced (Table 1).

Finally, the full activity of the CYP21A2 enzyme is achieved in primates with a tyrosine at 191 position, whereas alignments with CYP21A2 orthologs show that, at this position in mouse, rat, bovine, sheep, pig, and dog species, there is a histidine residue (Fig. 4). A compensatory substitution might exist somewhere in the 21-hydroxylase molecule to accommodate the histidine to tyrosine change in primates.

In conclusion, we described 2 novel *CYP21A2* missense mutations performing in vitro protein expression and testing residual enzyme activity and kinetics. Our results allow for the classification of the p. H282N and p.Y191H variants as SV and NC-CAH mutations, respectively.

This finding provides a further contribution for molecular diagnosis of CAH patients [7].

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Acknowledgment

Written consent was obtained from the patients and their relatives for publication of study.

Conflict of Interest

The authors declare that they have no competing interests.

REFERENCES

[1] White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev 2000;21:245-91.

[2] Nimkarn S, Lin-Su K, New MI. Steroid 21 hydroxylase deficiency congenital adrenal hyperplasia. Endocrinol Metab Clin North Am 2009;38:699-718.
[3] New MI. An update of congenital adrenal hyperplasia. Ann N Y Acad Sci 2004;1038:14-43.
[4] White PC, Grossberger D, Onufer BJ, et al. Two genes encoding steroid 21-hydroxylase are located near the genes encoding the fourth component of complement in man. Proc Natl Acad Sci U S A 1985;82:1089-93.
[5] White PC, New MI, Dupont B. Adrenal 21-hydroxylase cytochrome P-450 genes within the MHC class III region. Immunol Rev 1985;87:123-50.
[6] Torres N, Mello MP, Germano CM, et al. Phenotype and genotype correlation of the microconversion from the CYP21A1P to the CYP21A2 gene in congenital adrenal hyperplasia. Braz J Med Biol Res 2003;36:1311-8.
[7] Balsamo A, Baldazzi L, Menabò S, et al. Impact of molecular genetics on congenital adrenal hyperplasia management. Sex Dev 2010;4:233-48.
[8] Database of CY21A2 by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee: http://www.imm.ki.se/CYPalleles/cyp21.htm.
[9] Concolino P, Mello E, Zuppi C, et al. Molecular diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency: an update of new CYP21A2 mutations. Clin Chem Lab Med 2010;48:1057-62.
[10] Katsumata N, Shinagawa T, Horikawa R, Fujikura K. Novel intronic CYP21A2 mutation in a Japanese patient with classic salt-wasting steroid 21-hydroxylase deficiency. Metabolism 2010;59:1628-32.
[11] Concolino P, Mello E, Toscano V, et al. Multiplex ligation-dependent probe amplification (MLPA) assay for the detection of CYP21A2 gene deletions/duplications in congenital adrenal hyperplasia: first technical report. Clin Chim Acta 2009;402:164-70.
[12] Concolino P, Mello E, Minucci A, et al. A new CYP21A1P/CYP21A2 chimeric gene identified in an Italian woman suffering from classical congenital adrenal hyperplasia form. BMC Med Genet 2009;10:72.
[13] Concolino P, Vendittelli F, Mello E, et al. Functional analysis of two rare CYP21A2 mutations detected in Italian patients with a mildest form of congenital adrenal hyperplasia. Clin Endocrinol (Oxf) 2009;71:470-6.
[14] Concolino P, Vendittelli F, Mello E, et al. Two novel CYP21A2 missense mutations in Italian patients with 21-hydroxylase deficiency: identification and functional characterisation. IUBMB Life 2009;61:229-35.
[15] Speiser PW, Dupont J, Zhu D, et al. Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Invest 1992;90:584-95.
[16] Ezquieta B, Cueva E, Varela J, et al. Non-classical 21-hydroxylase deficiency in children: association of adrenocorticotropic hormone-stimulated 17-hydroxyprogesterone with the risk of compound heterozygosity with severe mutations. Acta Paediatr 2002;91:892-8.
[17] Tusie-Luna MT, Traktman P, White PC. Determination of functional effects of mutations in the steroid 21-hydroxylase gene (CYP21) using recombinant vaccinia virus. J Biol Chem 1990;265:20916-22.