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A rational, non-radioactive strategy for the molecular diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

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ABSTRACT

Context: Molecular diagnosis of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) has not been straightforward.
Objective: To conduct a comprehensive genetic analysis by Multiplex Ligation dependent Probe Amplification (MLPA) and evaluate its reliability for the molecular CAH-21OHD diagnosis.
Patients and methods: We studied 99 patients from 90 families with salt-wasting (SW; n = 32), simple-virilizing (SV; n = 29), and non-classical (NC; n = 29) CAH-21OHD. Molecular analysis was sequentially performed by detecting the most frequent point mutations by allele-specific oligonucleotide polymerase chain reaction (ASO-PCR), large rearrangements by MLPA, and rare mutations by direct sequencing. Parental segregation was evaluated.
Results: ASO-PCR detected microconversions in 164 alleles (91.1%). MLPA identified *CYP21A1P* large conversions to *CYP21A2* in 7 of the remaining 16 (43.7%), 30-kb deletions including the 3′-end of *CYP21A1P*, *C4B*, and the 5′-end of *CYP21A2* in 3 of the 16 (18.7%), and a complete *CYP21A2* deletion in one (6.3%). Five alleles (2.7%) required direct sequencing; three mutations located in the *CYP21A2* gene and two derived from *CYP21A1P* were found. No parental segregation was observed in patients with the c.329_336del and/or the CL6 cluster mutations. These cases were not diagnosed by ASO-PCR, but MLPA detected deletions in the promoter region of the *CYP21A2* gene, explaining the genotype/phenotype dissociation.
Conclusion: Using the proposed algorithm, all alleles were elucidated. False-positive results in MLPA occurred when mutations or polymorphisms were located close to the probe-binding regions. These difficulties were overcome by the association of MLPA with ASO-PCR and paternal segregation. Using these approaches, we can successfully use MLPA in a cost-effective laboratory routine for the molecular diagnosis of CAH-21OHD.

1. Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is an autosomal recessive disorder caused by an inborn error of steroid metabolism and accounts for 90–95% of all CAH cases (White and Speiser, 2000). The 21-hydroxylase enzyme is encoded by the *CYP21A2* gene located on chromosome 6 in a region known as the RCCX module, which comprises the *RP*, *C4*, *CYP21* and *TNX* genes. There are three pseudogenes in the RCCX module, *CYP21A1P*, *TNXA*, *RP2*, which are located between the two C4 loci. Because of the high homology and tandem-repeat organization of the RCCX module, this region of the genome is subjected to unequal crossover events leading to large rearrangements including duplications, deletions, and fusions of the RCCX module (Yang et al., 1999).

Diminished or absent 21-hydroxylase activity leads to the reduction or elimination of cortisol and aldosterone synthesis, accumulation of progesterone and 17-hydroxyprogesterone and shunting through the adrenal androgen biosynthetic pathway, which leads to overproduction of adrenal androgens (White and Speiser, 2000). 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 the external genitalia in female fetuses and postnatal virilization in both sexes. There is also a mild, non-classical form (NC),

Abbreviations: CAH, congenital adrenal hyperplasia; 21OHD, 21-hydroxylase deficiency; RCCX module, *RP*, *C4*, *CYP21* and *TNX* genes; SW, salt-wasting; SV, simple-virilizing; NC, non-classical; ACTH, Adrenocorticotropic Hormone; 17-OHP, 17-hydroxyprogesterone; DHEAS, Dehydroepiandrosterone Sulfate; ASO-PCR, allele specific oligonucleotide polymerase chain reaction; MLPA, Multiplex Ligation dependent Probe Amplification; RFLP, restriction fragment length polymorphism; 5′UTR, 5′ untranslated; 3′UTR, 3′ untranslated; 30-kb deletion, deletion of 30-kb including 3′-end *CYP21A1P*, *C4B*, and 5′-end *CYP21A2*.

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in which patients remain asymptomatic or develop symptoms during childhood or puberty (Speiser et al., 2010; White and Speiser, 2000).

The establishment of genetic diagnosis of patients with CAH due to 21OHD (CAH-21OHD) has not been straightforward. Screening programs have indicated that the genetic diagnosis is a potentially useful adjunct to hormonal measurements for the genetic counseling of parents at the birth of a CAH child and of adolescents at the transition to adult care (Speiser et al., 2010). Finally, the molecular diagnosis of *CYP21A2* mutations can be used in patients with the NC form of CAH-21OHD whose ACTH-stimulated 17-OHP levels are in the borderline range of the heterozygote group (Speiser et al., 2010).

A complete characterization of the *locus*, including the number of RCCX modules and the identification of chimerical genes, has required Southern blotting with probes for the different genes combined with other techniques, such as the use of restriction endonucleases (Chang and Lee, 2011). However, because this technique is labor intensive and usually uses radioactive probes, alternative methods are being suggested to substitute for Southern blotting in the molecular diagnosis of CAH-21OHD. Multiplex Ligation dependent Probe Amplification (MLPA) has been used to assess gene copy number and to help identify chimerical genes in patients with CAH-21OHD in a single experiment without using radioactive probes (Chan et al., 2011; Chen et al., 2012; Choi et al., 2012; Coeli et al., 2010; Concolino et al., 2009; Jang et al., 2011; Kleinle et al., 2009; Schouten et al., 2002; Skordis et al., 2011). However, the substitution of a methodological approach should not hamper the reliability and cost effectiveness of the current tests.

In the present study, we conducted a comprehensive genetic analysis to assess whether MLPA combined with allele-specific PCR (ASO-PCR) and direct sequencing, in a sequential strategy, would provide reliable genetic diagnoses.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board for Human Research, and written informed consent was obtained from all the patients or their parents (Protocol no. 701/2010).

We studied 99 patients (90 families) with CAH-21OHD, representing 180 unrelated affected alleles; we also studied all the available parents to assess genetic segregation. In 82 families, there was 1 affected subject, 2 affected subjects in 7 families, and 3 affected subjects in 1 family. Families were divided into salt-wasting (SW; n = 32), simple-virilizing (SV; n = 29), and non-classical (NC; n = 29) forms defined according to standard criteria (White and Speiser, 2000).

Table 1 shows the median and the confidence interval (CI95%) of age, sodium and potassium, 17OHP, androstenedione, testosterone, and DHEAS plasma levels at the diagnosis of patients with SW, SV, and NC forms of CAH-21OH. All patients with the NC form of CAH-21OH showed ACTH-stimulated 17OHP levels greater than 1200 ng/dl or 36.4 nmol/L.

Table 1
Clinical and biochemical findings in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

	SW (n = 32)	SV (n = 29)	NC (n = 29)
Median age	40 days (1 to 180)	7 years (0.3 to 17)	17 years (3 months to 20.8 years)
Plasma Na (mEq/L)	130.5 (125.9–132.7)	139.0 (135.2–141.0)	140.0 (135.0–145.0)
Plasma K (mEq/L)	5.9 (5.6–6.5)	4.3 (3.7–5.0)	4.0 (3.5–4.5)
Plasma 17-OHP (nmol/L)	584 (463.8–1135.8)	462.4 (335.5–774.7)	31.9 20.7–45.9
Plasma androstenedione (nmol/L)	43.5 (41.4–140.8)	442 (257–1222)	4.8 (3.6–7.2)
Plasma testosterone (nmol/L)	9.4 (7.9–14.8)	122 (83.6–252.3)	2.1 (1.8–3.7)
Plasma DHEAS (nmol/L)	2.2 (1.6–3.4)	2.2 (1.5–3.6)	2.9 (2.0–5.2)
Prader classification	III to IV	I to III	Normal genitalia

All clinical and biochemical findings were evaluated at diagnosis. Median (CI 95%). To convert from ng/mL multiply 3.026 for 17-OHP; 3.492 for Δ4-A; 3.576 for testosterone; and 0.002714 for DHEAS.

2.2. Methods

2.2.1. Steroid determination

Blood samples were collected in heparinized tubes and immediately centrifuged at 4 °C. Plasma was kept frozen at −20 °C until assayed. Plasma steroids were measured by specific radioimmunoassay as previously described (Mermejo et al., 2005).

2.2.2. Molecular analysis

Genomic DNA was obtained from peripheral blood by QIAamp DNA blood kit (Qiagen, CA, USA). Molecular analysis was performed in three sequential steps.

2.2.2.1. Allele-specific PCR (ASO-PCR). ASO-PCR was performed using eight primer pairs derived from the most frequent *CYP21A1P* mutations (p.P30L, IVS2-13 A/C>G, c.329_336del, p.I172N, CL6, p.V281L, p.R356W), as described (Wilson et al., 1995). The p.Q318X mutation analysis was carried out by the PCR/RFLP (restriction fragment length polymorphism) method (Dardis et al., 1997). Positive and negative control DNAs were used in all reactions.

2.2.2.2. Multiplex Ligation dependent Probe Amplification (MLPA). MLPA was performed as recommended by the manufacturers using SALSA MLPA P050B2 kits (MRC Holland, Amsterdam, Netherlands). The probe mix comprises 33 different probes with amplification products between 130 and 391 bp. These probes include: five specific probes for *CYP21A2*, one located 18 nucleotides before exon 1 that recognizes the 5′UTR, four probes recognizing the wild-type sequences for the c.329_336del, p.I172N, cluster 6, and p.Q318X mutations positioned in the exons 3, 4, 6 and 8, respectively; three specific probes for *CYP21A1P*, three for *TNXB*, one for *C4A*, one for *C4B*, three located on chromosome 6p21.3, one Y-chromosome specific (UTY-gene) probe, and 16 probes as references. Fragment analysis was performed on an ABI 3130 Genetic Analyzer (ABI PRISM/PE Biosystems, Foster City, CA, USA), and the results were initially analyzed using Genemapper v4.0 software (Applied Biosystems, Foster City, CA, USA). Thereafter, data were normalized and analyzed using the software Coffalyser v9.4 (MRC Holland, Amsterdam, Netherlands). This program identifies a sample peak as "normal" when the ratio of the peak height of the sample to that of the control is between 0.8 and 1.2, as "deleted" when the ratio is <0.8, and as "duplicated" when the ratio is >1.2.

2.2.2.3. Direct sequencing. Two fragments of *CYP21A2*, one of 600 bp from the 5′UTR to exon 6 (CL6N) and the other from exon 6 (CL6N) to the 3′ UTR, were amplified. The fragments were directly sequenced with internal primers using Big Dye™ Terminator Cycle Sequencing Kits V3.1 Ready Reaction (ABI PRISM/PE Biosystems, Foster City, CA, USA). The sequences obtained with an ABI 3130 Genetic Analyzer (ABI PRISM/PE Biosystems) were compared to *CYP21A2* and *CYP21A1P* sequences (Ensembl-ENSG00000231852 and ENSG00000204338, respectively) using the software CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA).

2.2.3. Genotype categories

Patients were divided into four different genotype groups, according to the enzymatic activity impairment, as described by Speiser et al. (1992) and modified by others (Krone and Arlt, 2009). The Null group included patients who were homozygous for mutations that predict

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