Partial deficiency of 17α-hydroxylase/17,20-lyase caused by a novel missense mutation in the canonical cytochrome heme-interacting motif

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Abstract

Background: Deficiency of 17α-hydroxylase/17,20-lyase is a rare cause of 46,XY disordered sex development.

Objective: We characterize in vitro and in vivo effects of two novel CYP17A1 gene mutations identified in a patient with a mild phenotype of CYP17A1 deficiency.

Subjects and methods: A 46,XY patient presented with ambiguous genitalia. CYP17A1 deficiency was suspected at 2 months on the basis of steroid analysis performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Mutational analysis of the CYP17A1 gene was performed by PCR and Sanger sequencing. To characterize the effect of CYP17A1 mutation on 17α-hydroxylase and 17,20-lyase activities in vitro, HEK293 cells were transiently transfected with CYP17A1 expression plasmids, incubated with progesterone or 17-OH-pregnenolone and concentrations of 17-OH-progesterone or DHEA were then measured in the cell culture medium by LC–MS/MS.

Results: Clinical and hormonal findings in the patient were consistent with partial combined deficiency of 17α-hydroxylase/17,20-lyase. The sequencing of the CYP17A1 gene in the patient revealed compound heterozygosity for two novel mutations: c.107delT p.R36fsX107 and p.W121R. After 6-h in vitro culture of transfected HEK293 cells in the presence of 1 μM progesterone, 17α-hydroxylase activity of p.W121R mutant was 60.5 ± 16.3%, while 17,20-lyase activity of mutant measured from the amount of DHEA produced in the presence of 1 μM of 17-OH-pregnenolone was 15.8 ± 2.6% compared with the WT.

Conclusions: p.W121R substitution, affecting the first residue in the conserved heme-interacting WXXXR motif of CYP17A1, is associated with partial combined deficiency of 17α-hydroxylase/17,20-lyase.

Introduction

Deficiency of cytochrome CYP17A1 is a rare disorder of steroid hormone biosynthesis affecting both adrenal and gonadal functions. CYP17A1 is a microsomal enzyme encoded by a single gene CYP17A1 located on chromosome 10q24.3 (1). The protein catalyzes both 17α-hydroxylase and 17,20-lyase activities required for conversion of pregnenolone to 17-OH-pregnenolone and progesterone to 17-OH-progesterone, and then for further conversion of the above 17-hydroxylated steroids to DHEA and androstenedione respectively (2). The impaired 17-hydroxylation leads to cortisol deficiency and, consequentially, adrenocorticotropic hormone (ACTH) overproduction, adrenal hyperplasia, and excessive synthesis of mineralocorticoid precursors (mainly corticosterone and 11-deoxycorticosterone), presenting clinically with low-renin hypertension. Defective sex steroid synthesis results in the lack of pubertal development in both sexes, whereas genetically males are born with

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undermasculinized external genitalia (46,XY disorder of sex development (DSD)) (3).

The majority of patients with 46,XY DSD due to CYP17A1 deficiency present clinically with female external genitalia, whereas the cases associated with partial loss of 17,20-lyase activity are far less common (4, 5). Herein, we present another case of CYP17A1 deficiency associated with partially preserved testicular function, which was attributed to the effect of a novel p.W121R mutation in the CYP17A1 gene. This substitution alters the first residue in the WXWR heme-interacting sequence presented in all cytochromes. We used in-house developed liquid chromatography–tandem mass spectrometry (LC–MS/MS) steroid analysis to illustrate the partial CYP17A1 deficiency both in the patient and the cell culture expressing the novel mutation.

Subjects and methods

LC–MS/MS analysis of steroids

The detection and quantitation of steroids were performed with the AB SCIEX QTRAP 5500 tandem mass spectrometer (AB SCIEX, Concord, ON, Canada) using the Perkin Elmer UPLC system (Waltham, MA, USA) for the chromatographic separation.

11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 21-deoxycortisol, androstenedione, DHEA, cortisol, cortisone, corticosterone, pregnenolone, progesterone, and testosterone were purchased from Steraloids, Inc. (Newport, RI, USA). The deuterated internal standards (ISs): 17-hydroxyprogesterone-d8, progesterone-d8, 17-hydroxypregnenolone-d3, and DHEA-d2 were obtained from Cambridge Isotope Laboratories (MA, USA). MS-grade methanol, acetone, MTBE, water, and formic acid were purchased from Merck.

Steroid-free human serum was prepared using the activated charcoal. The procedure included the stirring of human serum three times for 6 h at 4 °C with charcoal and subsequent filtration. Stock solutions of steroid hormones and ISs were prepared in ethanol at concentrations of 1 mg/ml and were stored at −20 °C until use. The substock solution contained all steroid hormones at a concentration 100 times higher than the highest working calibrator. Working calibrators were prepared before the analysis by serially diluting the standard substock in ethanol and then by adding 10 ml of each dilution to 990 ml of steroid-free serum. The working calibrators covered the ranges 0.1–100 ng/ml for all steroids, except for cortisol (1–1000 ng/ml), and pregnenolone (0.25–250 ng/ml). Seven levels of calibrators were used for all the analytes. The concentrations of each IS steroid in the IS substock were 100 times higher than those in the working IS solution. The working IS solution was prepared by diluting the IS substock with methanol. Quality controls (QCs) were prepared in steroid-free serum at three levels (low, medium, and high) by spiking with the substock solution to the concentrations corresponding to 1, 10, and 50% of the highest working standard. All the solutions of standards, calibrators, and QCs were stored in glass 2.0 ml autosampler sample vials with screw caps at −20 °C.

For the current study, 10 μl of the working IS solution and 0.2 ml of acetone were added to 0.2 ml of patient serum/calibrator solution/culture medium in 10 ml borosilicate glass vials with disposable caps and the mixture was vortexed for 1 min. After that, it was extracted with 2 ml MTBE for 1 min with vigorous stirring. The vials were allowed to freeze for 30 min at −20 °C, and after that the organic layers were transferred to 10-ml borosilicate glass test tubes and evaporated to dryness in a stream of nitrogen at 35 °C. The residues were reconstituted in 0.2 μl of methanol–water mixture (1:1), and after centrifugation were transferred to 2.0 ml autosampler vials with 0.25 ml glass inserts.

Following extraction, 20 μl of the reconstituted sample were injected into a reverse phase column (Acquity UPLC BEH C18, 1.7 μ, 2.1 × 50 mm, and 0.2 μ in-line precolumn filter) using the Perkin Elmer UPLC system for the chromatographic separation. LC separation was performed using a gradient mobile phase: phase A (water +0.1% formic acid) and mobile phase B (methanol +0.1% formic acid) at a flow rate of 0.25 ml/min. The column temperature was maintained at 45 °C throughout the separation. The detection and quantitation were achieved by MS/MS using an AB SCIEX QTRAP 5500 tandem mass spectrometer (AB SCIEX) fitted with an atmospheric pressure chemical ionization (APCI) source. The nebulizer current was set at 2 mA with a source temperature of 450 °C. Nitrogen and dry air were produced by a PEAK Scientific generator (Parker Balston, Haverhill, MA, USA) ABN-2ZA. The steroids were monitored in positive-ion mode using multiple-reaction monitoring (MRM).

DNA analysis

This study was approved by the institutional review board, and the parents gave informed consent for DNA analysis. Genomic DNA was extracted from peripheral leukocytes by standard procedure. The CYP17A1 gene was amplified by standard PCR with sense primer 5′-CTTCAAGCCATACGCTGCTC-3′ and antisense primer 5′-ATACACGAGAATCCAGGAA-3′. Genomic DNA was extracted from peripheral leukocytes by standard procedure. The CYP17A1 gene was amplified
by PCR in three fragments and the amplification products were purified and directly sequenced using an automated DNA sequencer (3130 Genetic Analyzer, Applied Biosystems). The following primers were used for PCR and sequencing: 1F, CTGGCTGGGTCCTCAGAGAACTC; 2F, CCAGTACCCTCGCTTCTC; 3R, TGGGGCTGGAGCAGGGGAAGTG; 4F, GTCAGGGGTGGAGTAGAATTC; 5R, GGAAGCAGGGCAGGCCTAGTCTTC; 6F, GGAAGGGACTGGAGAGGCTCTTC; 7F, CATGAGTGAGTGGGAATGAG; and 8R, GGCAGAGTGGGTTGGGAGTAG. GenBank cDNA entry with accession number M14564.1 was used as a reference sequence for analyses of mutations and numbering of nucleotides for the CYP17A1 gene.

Construction of mammalian expression plasmids

WT human CYP17A1 cDNA was amplified from total adrenal cDNA by PCR with CYP17-for (5'-CCTGCCGGCACCCCAGCA-3') and CYP17-rev (5'-GGCAGAGTTGGGTTGGGAGTAG-3') primers and cloned into mammalian expression vector pcDNA3.1(+) (Invitrogen). p.W121R mutation was introduced into the CYP17A1 sequence using two rounds of PCR with mutagenic primers CYP17-W121R-for (5'-TCTGGCGCACACCGGCACCTGCATC-3') and CYP17-W121R-rev (5'-GATGCAGCTGCCGGTGTGCGCCAGA-3').

Functional expression of the replacement mutant

In vitro functional analysis of p.W121R mutant was performed as described previously (6). Briefly, HEK293T cells were transfected with WT and mutant CYP17A1 expression vectors using Lipofectamine 2000 (Invitrogen) and cultured in 24-well plates. Forty hours after transfection, various concentrations of progesterone (0.2, 1.0, and 2.0 μmol/l) or 17-hydroxypregnenolone (0.2, 1.0, and 2.0 μmol/l) (Sigma–Aldrich) were added and incubated for another 6 h. The medium was collected and frozen at −20 °C until the concentrations of 17-hydroxyprogesterone and DHEA were measured.

Molecular modeling

To highlight the relative three-dimensional positions of heme and the residues W121 and R125 in the highly conserved WXXXR motif, we used the crystal structure of the human CYP17A1 (PDB accession code 3SWZ, chain A). Image was generated using the Swiss-PdbViewer 4.1.0 software (www.expasy.org) (7).

Results

Case report

The proband was the first child born to a healthy nonconsanguineous couple. The birth weight of the proband was 2.89 kg, and the length was 49 cm. At birth, he presented with bilateral cryptorchidism with inguinal location of the testes and scrotal hypospadias, and was assigned as a boy. His karyotype was 46,XY. The basal serum sample was initially sent to our center at the age of 7 days and showed no abnormalities in steroid profile (Table 1).

At his first admission at the age of 2 months his body length was 58 cm (0.6 S.D.), the weight 5.0 kg (−0.3 S.D.), and the physical findings were unremarkable. The right testis was in the scrotum, the left testis in the inguinal

<table>
<thead>
<tr>
<th>Case Report</th>
<th>P Rubtsov and others</th>
<th>Case of partial CYP17A1 deficiency</th>
<th>172:5</th>
<th>K21</th>
</tr>
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</table>

Table 1  Serum steroid hormone concentrations at different ages (all values in nmol/l for basal samples, if not indicated otherwise).

<table>
<thead>
<tr>
<th></th>
<th>7 days</th>
<th>2 months</th>
<th>6 months</th>
<th>6 months, ACTH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>8 months</th>
<th>8 months, hCG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-F</td>
<td>2.7 (2.0–12.5)</td>
<td>0.2 (2.0–12.5)</td>
<td>0.3 (3.5–16)</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>11-DOC</td>
<td>0.5 (1.1–5.0)</td>
<td>3.8 (1.1–5.0)</td>
<td>0.2 (0.1–10.5)</td>
<td>2.1</td>
<td>&lt;0.08</td>
<td>0</td>
</tr>
<tr>
<td>17-OHPr</td>
<td>1.9 (0.6–19.2)</td>
<td>10.2 (0.3–4.1)</td>
<td>0.7 (0.3–9.1)</td>
<td>0.4</td>
<td>1.64</td>
<td>13.5</td>
</tr>
<tr>
<td>17-OPG</td>
<td>4.8 (0.3–4.1)</td>
<td>0.2 (0.1–10.5)</td>
<td>2.1</td>
<td>&lt;0.08</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4-A</td>
<td>0.07 (0.2–9.0)</td>
<td>0.2 (1.4–7.9)</td>
<td>0.2 (1.4–7.9)</td>
<td>0.5 (1.0–12.1)</td>
<td>7.4</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>DHEA</td>
<td>3.4 (2.5–23.5)</td>
<td>8.8 (1.0–18.3)</td>
<td>0.5 (1.0–12.1)</td>
<td>&lt;0.03</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>F</td>
<td>54.5 (30.0–370)</td>
<td>251.1 (150–650)</td>
<td>91.5 (150–650)</td>
<td>137.8</td>
<td>160.5</td>
<td>112.2</td>
</tr>
<tr>
<td>B</td>
<td>38.6 (2.7–42.7)</td>
<td>629.2 (3.8–66.5)</td>
<td>411.9 (3.8–66.5)</td>
<td>881.8</td>
<td>403.8</td>
<td>189.4</td>
</tr>
<tr>
<td>Prog</td>
<td>2.4 (0.5–2.6)</td>
<td>7.0 (0.5–2.6)</td>
<td>2.5 (0.8–3.9)</td>
<td>1.6</td>
<td>3.5</td>
<td>6.6</td>
</tr>
<tr>
<td>T</td>
<td>2.3 (0.8–4.2)</td>
<td>3.7 (0.8–4.2)</td>
<td>0.1 (0.3–0.6)</td>
<td>0.4</td>
<td>0.5</td>
<td>6.1</td>
</tr>
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<sup>a</sup>60 min after cosyntropin (i.v., 200 μg/m<sup>2</sup>).
<sup>b</sup>hCG test (1000 IU i.m., daily for 3 days, blood sample on day 4).
area, volume of testes 1 ml, and the penis was 2 cm in length. The serum potassium and sodium levels were normal. DHEAS was 442 nmol/l (reference range, 500–2600), LH was 5.6 U/l, FSH, 1.7 U/l. His basal steroid profile showed a significantly elevated level of corticosterone (Table 1), on the basis of which CYP17A1 deficiency was suspected.

At 6 months, a cosyntropin test showed elevated basal and stimulated levels of corticosterone, and blunted cortisol response. At the follow-up visit at the age of 8 months, his height was 75 cm (1.3 s.d.), weight 9.9 kg (0.1 s.d.), and blood pressure 90/50 mmHg. The plasma renin activity was normal (3.2 ng/ml per h, reference range, 1.6–6.1), and ACTH was slightly elevated (99.5 pg/ml, reference range, 7–66). After human chorionic gonadotrophin (hCG) stimulation (1000 IU, 3 days), T rose from 0.49 nmol/l (basal level) to 6.1 nmol/l (day 5).

Sequencing of CYP17A1 gene

By sequencing the CYP17A1 gene of the proband, we found two novel heterozygous mutations: in exon 1, deletion of thymine at position c.107 resulting in a frameshift and premature stop codon at amino acid position 107 (c.107delT p.R36fsX107); and c.361T>C transition in exon 2, which resulted in p.W121R substitution (Fig. 1). The former mutation was inherited from the father, and the latter from the mother.

Figure 2

Enzymatic activity assay by in vitro expression. (A) The production of 17-hydroxyprogesterone in the presence of various concentrations of progesterone (0.2, 1.0, and 2.0 mM) is shown as a measure of 17-hydroxylase activity in HEK293T cells transfected with WT and W121R mutant. (B) The production of DHEA in the presence of various concentrations of 17-hydroxypregnenolone (0.2, 1.0, and 2.0 mM) is shown as a measure of 17,20-lyase activity in HEK293T cells transfected with WT and W121R mutant. Data are expressed as mean ± s.d. (n = 4 in A and B).

CYP17A1 activity of p.W121R mutant

From the data shown in Fig. 2, we have calculated that, after 6-h culture of transfected HEK293 cells in the presence of 1 μM progesterone, the amount of 17-OH-progesterone produced by p.W121R mutant is 60.5 ± 16.3% of that produced by WT enzyme. The amount of DHEA synthesized by p.W121R mutant in the presence of 1 μM of 17-OH-pregnenolone was 15.8 ± 2.6% of that produced by the WT enzyme.
Deficiency of CYP17A1 is a rare cause of 46,XY DSD. Typically, the diagnosis is made in a patient with unambiguously female genitalia presented at puberty with primary amenorrhea and/or hypertension or, less frequently, earlier in life after incidental finding of inguinal gonads resembling and often being considered as androgen insensitivity syndrome. Cases of CYP17A1 deficiency in infancy are rarely reported (5, 8, 9). The present report illustrates the value of LC–MS/MS for simultaneous analysis of multiple steroid hormones in diagnostic evaluation of patients with DSD. Alternatively, gas chromatography–mass spectrometry urinary steroid profiles have been used successfully to the same extent by others (10, 11). The elevation of serum corticosterone in our case clearly pointed to 17α-hydroxylase deficiency. It has to be noted, however, that the basal steroid profile at the age of 8 days was not remarkable and the increase in corticosterone became evident only in the second profile at the age of 2 months. It is not clear whether cosyntropin test at 8 days would have produced a more distinct profile. In our experience with other forms of CAH (including non-classical variants), basal LC–MS/MS steroid profiles in the first 2 weeks of life are discriminative (T Ionova, A Nizhnik, N Kalinchenko and A Tiulpakov, unpublished observations). Stimulation with cosyntropin at the age of 6 months did not show any additional diagnostic advantage compared with basal values. Interestingly, in spite of elevated corticosterone, all steroid profiles (including the one after cosyntropin stimulation) showed normal 11-deoxycorticoosterone, which was in line with normal blood pressure, normal serum potassium, and unsuppressed plasma renin activity.

The follow-up will demonstrate whether the mineralocorticoid excess develops later. It has been well documented that hypertension may occur in the second decade of life even in the cases that were initially considered to have normal 17α-hydroxylase activity (5, 12). As to gonadal function in our patient, both the degree of masculinization and hormonal data were consistent with partial 17,20-lyase deficiency. Although levels of gonadotropins were not elevated, testosterone response to hCG stimulation was subnormal (13).

One of the two CYP17A1 gene mutations found in our patient, c.107delT p.R36fsX107, is predicted to result in the protein truncation and the complete loss of the enzymatic activity. Therefore, the phenotype of the partial CYP17A1 deficiency observed in the patient is most likely to be attributed to the effect of the p.W121R mutation. Our in vitro functional studies in HEK293 cells transiently transfected with the vector expressing p.W121R mutation were consistent with partial deficiency of the enzyme, with 17α-hydroxylase activity being ~60% and 17,20-lyase activity ~16% compared with the WT CYP17A1 expressing vector. Although it is not entirely accurate to compare results of different transfection experiments, the degree of residual activity of 17α-hydroxylase was in the same range as those shown for cases with isolated

**Discussion**

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**Figure 3**

Partial sequences with WXXXR motifs of CYP17A1 in vertebrates and closely related human cytochromes P450. The gray-shaded areas indicate the identical (black box) or similar (grey box) amino acids. Number of the first amino acid in the sequence is given in parentheses.

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**Figure 4**

The relative positions of W121 (magenta) and R125 (blue) residues and heme on the crystal structure of the human CYP17A1. The protein backbone is shown in yellow ribbon with heme in green. Both W121 and R125 residues appear to form hydrogen bonds (dotted green lines) with heme.
17,20-lyase deficiency (5, 14). Hormonal findings in our patient (elevated corticosterone and lack of cortisol response to cosyntropin stimulation), however, corresponded to partial 17α-hydroxylase deficiency. It is not clear whether the partial loss of activity, which was also noted in some patients diagnosed with isolated 17,20-lyase deficiency (5, 11), is sufficient to show these subclinical changes. It is also possible that the other mutation (c.107delT p.R36fsX107) contributed to this partial 17α-hydroxylase deficiency in vivo. The in vitro reduction of 17,20-lyase activity was comparable with the results for P342T and R496H mutations associated with the similar clinical manifestation of CYP17A1 deficiency (4, 5, 8).

W121 is highly conserved in CYP17A1 across species, and this aromatic residue is also located at corresponding positions of closely related human cytochromes P450, e.g., CYP21A2 (W116), CYP11A1 (W147), CYP19A1 (W141), and CYP27A1 (W154) (Fig. 3). In CYP17A1, W121 resides at the amino-terminus of the C helix, as was shown by the analysis of crystallized molecules of structurally related bacterial cytochromes (15). This was confirmed by detailed modeling of human CYP17A1 (16) and crystal structure of the enzyme with bound inhibitors (17). The residue is in the first position in the canonical WXXXR motif, in which the two conserved amino acids (tryptophan and arginine) are involved in coordination of propionate side chain of the heme, the former representing a conserved hydrogen-bond donor and the latter being a conserved basic residue that forms a salt bridge to the D-ring propionate (15) (Fig. 4). Interestingly, the only other known mutation in that region of CYP17A1, R125Q, affecting the other critical residue of the WXXXR sequence, completely abolished 17α-hydroxylase/17,20-lyase activities (18). It can be speculated that W121 substitution for another potential hydrogen-bond donor, e.g., arginine, led to a less significant structural change than replacement of the basic residue in that second WXXXR mutation, consequently contributing to a milder phenotype in our case.

In summary, we present a case of partial combined deficiency of 17α-hydroxylase/17,20-lyase caused by compound heterozygosity for two novel mutations in the CYP17A1 gene. One of the two mutations, p.W121R, resides in the heme-interacting motif conserved in all cytochromes. Expression of W121R CYP17A1 mutant in HEK293 cells showed partial loss of function with predominant reduction of 17,20-lyase activity, which was consistent with clinical and hormonal findings in the patient. In addition, we demonstrated the value of LC–MS/MS steroid profiling that was used both for early clinical diagnostics and for in vitro analysis of steroiogenesis in cells expressing mutated CYP17A1.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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