A rare CYP 21 mutation (p.E431K) induced deactivation of CYP 21A2 and resulted in congenital adrenal hyperplasia

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Abstract. Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is caused by mutations in the CYP21A2 gene. The residual enzyme activity is strongly associated with the phenotype. We describe a rare case of CAH with a rare CYP21A2 mutation. The patient was a one-year-old Japanese boy. At 16 days old, he was referred to our hospital because of elevated serum 17-OH-progesterone (17-OHP) levels in neonatal screening. The compound heterozygous mutations (IVS2-13 A/C>G, and p.E431K) in CYP21A2 were identified at 2 months old, and we diagnosed non-classical CAH, since he did not have significant physical signs (pigmentation and salt-wasting). However, his body weight decreased, and his serum 17-OHP level (99.5 ng/mL) was elevated at 3 months old. Steroid replacement therapy was started at 3 months old. Our patient’s clinical course resembled simple virilizing (SV) CAH, but classification was difficult because the patient showed increased renin activity indicating an aldosterone deficiency, and late onset of symptoms. While the IVS 2-13 A/C>G mutation is common in the classical form of CAH, p.E431K is a rare point mutation. Functional analysis revealed that the residual enzyme activity of p.E431L was 5.08±2.55% for 17-OHP and 4.12±2.37% for progesterone, which is consistent with SV CAH. p.E431 is localized in the L-helix near the heme-binding site. The mutation might interfere with heme binding, leading to deactivation of CYP21A2. This report showed that CYP21A2 p.E431 has an important effect on enzyme activity.

Key words: Congenital adrenal hyperplasia, 21-hydroxylase deficiency, CYP21A2

DEFICIENCY of 21-hydroxylase due to an abnormality in the 21-hydroxylase gene (CYP21A2) is the most frequent cause of congenital adrenal hyperplasia (CAH). It results in impaired secretion of adrenal cortisol and aldosterone production with increased production of androgen [1, 2].

The 21-hydroxylase locus has an active form of the gene (CYP21A2) and an inactive pseudogene (CYP21A1P) [1-4]. The genes are located 3′ of each of the genes encoding the fourth component of complement, C4A and C4B. One 21-hydroxylase gene and one C4 gene form a unit; these units are tandemly repeated in the HLA class III gene region on chromosome 6p21 [3]. Accordingly, most disease-causing mutations in CYP21A2 have arisen through interaction with CYP21A1P, and are either deletions of CYP21A2 resulting from misalignment followed by unequal crossing over, or aberrations that represent deleterious sequences that have been transferred from CYP21A1P into CYP21A2 [2, 3, 5]. However, several rare, pseudogene-independent mutations of CYP21A2 were found in a specific CAH case.

CAH is a broad spectrum disorder. The classical form involves complete corticoid deficiency and severe prenatal androgen stimulation with ambiguous genitalia in females with or without neonatal salt-wasting. The non-classical (NC) form involves moderate androgen excess with growth acceleration in childhood and precocious puberty. Other mild forms present only as hirsutism and anovulatory amenorrhea in adulthood. A previous study revealed that disease severity depends on the residual activity of 21-hydroxylase [1, 5, 6]. For
instance, complete loss of the residual enzyme activity causes the salt-wasting (SW) type, 2–5% residual activity causes the simple virilizing (SV) type, and 20–30% residual activity causes the NC type [7]. Although the mutations underlying the NC or SV type exhibit wide phenotypic variability, there is a good genotype–phenotype correlation for CAH, especially the SW type [1]. That means that the residual activity of the mutated CYP21A2 is very important for the patients, their families, and the physician to predict the prognosis and clinical condition for the next baby.

We describe a patient with a rare clinical form of CAH eventually identified to carry novel compound heterozygous mutations that arose independently of CYP21A1P. We also performed a functional analysis of the rare mutation.

Case Report

The patient was a one-year-old Japanese boy, the first child of his parents. At 16 days old, he was referred to our hospital because of elevated serum 17-OH-progesterone (17-OHP) levels (16.6 ng/mL) in neonatal screening. He did not have significant physical signs (virilization, pigmentation, and salt-wasting), and his body weight gain was good. His laboratory data at 16 days old showed normal sodium, potassium, and chloride levels (Na 136 mEq/L, K 4.1 mEq/L, Cl 104 mEq/L). The cortisol level was 2.7 μg/dL and the ACTH level was 41.3 pg/dL, and plasma renin activity was 24 ng/mL per hour.

The urine steroid hormone profile at 28 days showed increased levels of pregnanetriolone (3.632 mg/g cr) and 11β-hydroxyandrostosterone (0.589 mg/g cr) with normal cortisol and aldosterone metabolite; thus, 21-hydroxylase deficiency was strongly suspected. As compound heterozygous mutations (IVS2-13 A/C>G and p.E431K) in CYP21A2 were identified at 2 months old (Fig. 1), we diagnosed NC CAH. However, the boy’s body weight decreased, and his serum ACTH, plasma renin activity, and 17 OHP levels were elevated (ACTH: 80.1 pg/dL; plasma renin activity: 22 ng/mL per hour; 17OHP: 99.5 ng/mL with normal sodium, potassium, and chloride levels at 3 months old (Fig. 2). We started steroid replacement therapy at 3 months old. The steroid therapy improved his general condition, and normalized the serum 17 OHP level. He showed appropriate height and weight growth, and normal motor and neurological development.

![Fig. 1](image1.png)
**Fig. 1** The chromatogram of the mutations in intron 2 or exon 10 on CYP21A2 in the proband and his parents. This chromatogram, obtained by direct sequencing of PCR products, reveals heterozygous IVS2-13 A/C-G and a heterozygous G to A mutation at position 1400 (NM_000500.7, c. 1401 G>A) that resulted in a glutamine (GAG) to lysine (AAG) substitution at residue 431 (mature peptide numbering) in CYP21A2. The heterozygous IVS2-13 A/C>G mutation was detected in his mother, and a heterozygous E431K mutation was detected in his father.

![Fig. 2](image2.png)
**Fig. 2** The growth curve of the patient. Body weight is plotted on the cross-sectional growth chart for Japanese boys (0–12 months) in 2000. The steroid treatment (hydrocortisone and fludrocortisone) period and the elevated 17 OHP level are indicated.
Materials and Methods

Sequence analysis of the CYP21A2 gene
Genomic DNA was isolated from peripheral blood lymphocytes of the patient and his parents, and amplified by PCR as described [8].

Plasmid construction
Human CYP21A2 vector (p-Receiver –Mo2) (WT) was purchased from GeneCopoeia, Inc. A mutated CYP21A2 vector (p.E431K) was constructed by a 1400 G to A (NM_000500.7, c. 1401 G>A) point mutation using primers 5′CGCGTGTGCTGGCATTAAGCCGCTGGCGCGC 3′, where A is the mutation site. The mutations were generated by Dragon Genomics Centre, TAKARA BIO Inc. (Yokkaichi, Japan) and confirmed by DNA sequencing. The mutated CYP21A2 vector (p.Q318X) was constructed by a 1062 C to T (NM_000500.7, c. 1062C>T) point mutation using primers 5′TTTCAGCAGCGACTGTAGGAGGAGCTAGACC 3′, where T is the mutation site, and the QuikChange site-directed mutagenesis kit (Stratagene, Agilent technology, La Jolla, CA). The mutation was confirmed by DNA sequencing.

The ethics review board of Tottori University Faculty of Medicine approved this study.

Enzyme activity assay
COS-7 cells were transiently transfected with wild-type (WT) or mutant CYP21A2 vector using Lipofectamine 2000 (Invitrogen, Life Technology) according to the manufacturer’s protocol. The preparation of cell lysates and immunoblotting were performed as described [8, 11]. We used antibodies against CYP21A2 (Abcam, Cambridge, UK), and β actin (Cell Signaling, Beverly, MA, USA) for blotting.

Statistical analysis
Statistical analysis was performed using Microsoft Excel software to compare groups using Student’s t test. The results are shown as mean ± SE. P < 0.05 was considered statistically significant.

Result

Misssense mutation (IVS2-13 A/C>G and p.E431K) of the CYP21A2 gene
In the patient, we identified a heterozygous IVS2-13 A/C>G and a heterozygous G to A mutation at position 1400 (NM_000500.7, c. 1401 G>A) that resulted in a glutamine (GAG) to lysine (AAG) substitution at residue 431 (mature peptide numbering) in CYP21A2 (Fig. 1). Since the heterozygous IVS2-13 A/C>G mutation was detected in our patient’s mother, and the heterozygous E431K mutation was detected in his father (Fig. 1), the patient is considered to have compound heterozygous CYP21A2 mutations (IVS2-13 A/C>G/p.E431K). While the IVS2-13 A/C>G mutation is known as a common mutation in patients with the SW type [12, 13], p.E431K is a rare point mutation that arises independently of the pseudogene. Although Minutolo et al. reported a patient with NC CAH who had p.V281L/p.D322G-p.E431K [14, 15], the mutation (NM_000500.7, c. 1401 G>A) has not been reported in the literature or in the single nucleotide polymorphism (SNP) database of the dbSNP-polymorphism repository (http://www.ncbi.nlm.nih.gov/SNP/) apart from their report. This is the first report of a CYP21A2 p.E431K mutation with no accompanying mutation on the same allele. Furthermore, the p.E431K was assigned as “probably damaging,” with a score of 1.000 from in silico analysis with Poly-phen 2.

Enzyme activity
As shown in Table 1, the E431K mutation reduced enzyme activity to 5.08% ± 2.55% for 17-OHP and 4.12% ± 2.37% for progesterone. On the other hand, the Q318X mutation abolished enzyme activity, like
Kawashima et al. and identified novel compound heterozygous mutations that arose independently of CYP21A1P. We also evaluated the function of the rare mutation. This is a report of a rare case and the first report of a CYP21A2 p.E431K mutation with no accompanying mutation on the same allele.

There are three major phenotypes of CAH: SW, SV, and NC [2]. The SW type is most common and severe. In this form, both cortisol and aldosterone are severely impaired, and adrenal overproduction of androgen precursors leads to pre- and postnatal virilization. Patients present with life-threatening conditions (failure to thrive, vomiting, hyponatremia, hyperkalemia, acidosis, and hypovolemia), which usually appear at 1–4 weeks of age. The SV type is of moderate severity; cortisol synthesis is impaired, while aldosterone synthesis is normal. Patients present with only simple virilizing, and most patients present as SV type at 1–4 weeks of age. On the other hand, the NC type is least severe, and patients present with premature pubarche, hirsutism, acne, menstrual irregularities, and infertility.

**Immunoblotting analysis**

Fig. 3 shows a representative western blot analysis. Our data show that cells transfected with WT and p.E431K CYP21A2 expressed significantly more of the 53-kDa band, which is the CYP21A2 site, than MOCK and p.Q318X, and there were no significant differences between cells expressing WT and mutated CYP21A2. Although the 53-kDa band was also detected in cells transfected with MOCK, there was no enzyme activity in these cells. Higashi et al. have previously reported that COS cells produce CYP21A1P mRNA, but do not produce CYP21A2 mRNA [16], accordingly, the band on MOCK is strongly suspected to be CYP21A1P. These findings suggest that p.E431K is expressed at least as well as WT.

**Discussion**

In this case report, we described a patient with a rare clinical form of CAH, and identified novel compound heterozygous mutations that arose independently of CYP21A1P. We also evaluated the function of the rare mutation. This is a report of a rare case and the first report of a CYP21A2 p.E431K mutation with no accompanying mutation on the same allele.

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**Table 1** Residual 21-hydroxylase activity of the CYP21 mutants in transiently transfected intact COS-7 cells

<table>
<thead>
<tr>
<th></th>
<th>17OHP (%WT activity)</th>
<th>Progesterone (%WT activity)</th>
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<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E431K</td>
<td>5.08±2.55</td>
<td>4.12±2.37</td>
</tr>
<tr>
<td>Q318X</td>
<td>0.35±0.35</td>
<td>0.017±0.013</td>
</tr>
<tr>
<td>Mock</td>
<td>0.43±0.22</td>
<td>0.053±0.053</td>
</tr>
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</table>

The activities of the mutants are expressed as percentage of wild-type (WT) activity, which is defined as 100%. Cells were incubated for 2 hours in DMEM with 0.2 μmol/L progesterone and 0.2 μmol/L 17-OHP. The supernatant was analyzed using an LC-MS/MS method by ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan). Conversion values are shown for the two natural substrates (17-OHP to 11-deoxycortisol, and progesterone to 11-deoxycorticosterone). Results are shown as the mean ± SE of three samples.
Our patient had no electrolyte abnormality, but showed high renin activity, indicating impaired aldosterone synthesis, from 14 days old, and had poor weight gain and high 17-OHP level at 3 months old. It is difficult to classify the form of CAH, and our case’s clinical course seems rare, although it is similar to the SV type.

The compound heterozygous mutations of CYP21A2 (IVS2-13 A/C>G and p.E431K) were identified in our patient. While the IVS 2-13 A/C>G mutation is common in the SW type of CAH [12] and results in almost complete loss of CYP21 enzyme activity [17], p.E431K is a rare mutation and its function has been uncertain. We suspected p.E431K was associated with our patient’s rare clinical course.

On the other hand, Taboas et al. recently described a patient with NC type with p.V281L/p.D322G-p.E431K, and reported that the p.E431K mutation produced about 20% residual enzyme activity and a reduced protein level of CYP21A2 [14, 15]. Although their patient did not have p.E431K on single allele and had a different clinical type and combination of mutations, their presented residual enzyme activity and protein data are not consist with our patient’s clinical course, or our functional analysis. Since the SV type usually shows 2–5% residual enzyme activity [7], the enzyme activity in our case (about 5%) for p.E431K is consistent with the clinical course.

Then, how does p.E431K affect the enzyme activity on CYP21A2? The p.E431 amino acid is well conserved in prokaryotic P450 enzymes (P450bm3, P450terp, P450cam, P450eryf, and P450nor)[5, 18], and in the CYP21 enzyme in different species [5]. This indicates that the p.E431 amino acid is important for enzyme activity. Moreover, p.E431 is localized in the L-helix and near the binding site for heme (Fig. 4), which is a cofactor of CYP21A2 [5, 19]. Krone et al. reported that CAH patients with compound heterozygous mutations of CYP21A2 (I77T /p.A434V) showed the SV form, and found that the p.A434V mutation, which affects the site 3 amino acid next to p.E431, results in about 14% enzyme activity and no abnormality of CYP21A2 protein expression or localization in the cell [5]. These findings are similar to ours. From comparative analysis of three crystal structures, they and Heider et al. suggested that p.A434V interferes sterically with the apolar part of the heme group [5, 19]. This suggests that the pE431K mutation might also interfere with heme binding and result in little enzyme activity. Heider et al. also analyzed p.E431K in NC CAH by comparative analysis. They reported that p.E431K induced a change in the salt-bridge formation with R435 in the L-helix, and prevented destabilization of the tertiary structure [19].

In conclusion, we have described a rare patient with an unusual clinical form of CAH. We identified novel compound heterozygous mutations (IVS2-13 A/C>G / p.E431K) in the patient. Our functional analysis of the p.E431K mutation and clinical data revealed that p.E431 is important for the enzyme activity.

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**Disclosures**

All authors have no disclosures to report.
References


