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Novel intronic *CYP21A2* mutation in a Japanese patient with classic salt-wasting steroid 21-hydroxylase deficiency

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Abstract

Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency (21-OHD) is an autosomal recessive disorder caused by the defective *CYP21A2* gene that leads to various degrees of impaired secretion of both cortisol and aldosterone. In the present study, we analyzed the *CYP21A2* gene in a Japanese male patient with 21-OHD and functionally characterized the mutant *CYP21A2* gene. The patient presented with hypoglycemia and a salt-losing crisis during the neonatal period, and was diagnosed as having the salt-wasting form of 21-OHD based on the clinical and laboratory findings. Analysis of the *CYP21A2* gene revealed that the patient is homozygous for a novel C to A conversion at −9 position of intron 9 (IVS9-9C>A) and that his parents are heterozygous for the IVS9-9C>A mutation. Transient expression of the IVS9-9C>A mutant *CYP21A2* gene in COS-1 cells demonstrated that the mutation creates an aberrant splice acceptor site at −7 position of intron 9 and totally inactivates the authentic splice acceptor site of intron 9, which results in complete deficiency of 21-hydroxylase activity and loss of immunoreactive 21-hydroxylase protein. Clinical presentations of the patient as the severe salt-wasting form of 21-OHD are in good agreement with these results of the expression study. In conclusion, the patient is a homozygote for the novel intronic IVS9-9C>A mutation, which affects messenger RNA splicing and totally inactivates 21-hydroxylase to give rise to clinically manifest classic salt-wasting 21-OHD.

1. Introduction

Steroid 21-hydroxalase is a microsomal cytochrome P450 enzyme that catalyzes conversion of progesterone and 17α-hydroxyprogesterone to deoxycorticosterone and 11-deoxycortisol, respectively, in adrenal glands [1,2]. The *CYP21A2* gene encoding this enzyme is located adjacent to a highly homologous *CYP21A1P* pseudogene on chromosome 6p21.3 [3-5]. Steroid 21-hydroxylase deficiency (21-OHD) is an autosomal recessive disorder that accounts for more than 90% of congenital adrenal hyperplasia cases [1,2]. The deficiency of this enzyme leads to various degrees of impaired secretion of both cortisol and aldosterone. Consequently, the secretion of corticotropin by the pituitary gland is increased, resulting in hyperplasia of the adrenal cortex and excessive androgen production. Depending on the severity of the clinical manifestations, the disease phenotype is divided into 3 forms: the salt-wasting and simple virilizing forms, also known as the *classic forms*, and the nonclassic form. More than 100 mutations in the *CYP21A2* gene have been reported in 21-OHD patients [1,2,6,7]. Although most 21-OHD alleles result from the transfer of deleterious microsequences normally present in the neighboring *CYP21A1P* pseudogene, mutations that do not apparently originate from the pseudogene account for 5% to 10% of 21-OHD alleles; and mutations affecting splicing are rare among them [1,2,6,7].

In the present study, we report a novel intronic mutation in the *CYP21A2* gene leading to aberrant splicing of *CYP21A2* messenger RNA (mRNA) in a Japanese patient with the salt-wasting form of 21-OHD.

This genetic study was approved by the Institutional Ethical Review Board at the National Center for Child Health and Development, and informed written consent for genetic analyses was obtained from each subject.

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2. Materials and methods

2.1. Patient

The patient is a Japanese man and the only child of healthy parents who are first cousins. The patient was born in 1981 by vaginal aspiration delivery after an uneventful 40-week and 6-day gestation. At birth, the patient weighed 3850 g. He suffered from neonatal asphyxia, from which he recovered with oxygen administration. His milk intake was poor since birth. On the first day, the patient developed tonic-clonic convulsion due to hypoglycemia (13 mg/dL), from which he recovered with an intravenous glucose infusion. At 2 weeks of age, the patient developed vomiting, dehydration, and electrolyte imbalance (Na, 128 mEq/L; K, 9.4 mEq) and was admitted to a university hospital. On admission, he weighed 3450 g and had normal male external genitalia and marked skin pigmentation especially at nipples, axillae, and genitals. Blood pressure was 90/60 mm Hg. Based on these findings, the patient was provisionally diagnosed as having salt-wasting congenital adrenal hyperplasia and was successfully treated with hydrocortisone, fludrocortisone, and NaCl. At 4 months of age, he was hospitalized for acute respiratory tract infection and vomiting, when plasma corticotropin and 17α-hydroxyprogesterone levels were 158 pg/mL (reference range, 7.4-55.7 pg/mL) and 59.2 ng/mL (reference range, 0.1-1.7 ng/mL), respectively. Taken together, the patient was diagnosed as having classic salt-wasting 21-OHD.

2.2. Southern blot and sequencing analyses of CYP21A2 and CYP21A1P

The genetic study was approved by the Institutional Ethical Review Board at the National Center for Child Health and Development. Genomic DNA of the patient and his parents was isolated from whole blood after written informed consent for the genetic analysis was obtained from each subject.

Southern blot and sequencing analyses of the *CYP21A2* gene were performed as described previously [8]. DNA fragments spanning exons 3 through 10 of the *CYP21A1P* pseudogene were specifically amplified with a forward primer, 5′-ACCTGTCGTTGGTCTCTGCTC-3′ (the nucleotides specific for *CYP21A1P* are underlined, and those missing in *CYP21A1P* are not shown), corresponding to nucleotides 695 to 723 and a reverse primer, 5′-ATCGGTCCTGCCCCATCACTGGTT-3′, corresponding to the reverse complement of nucleotides 2698 to 2721 of the *CYP21A2* gene (nucleotides are numbered according to the report by Higashi et al [5]). The amplified polymerase chain reaction (PCR) products were fractionated and isolated on a 1% agarose gel (Bio-Rad Laboratories, Richmond, CA), and directly sequenced using a Thermo Sequenase kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

2.3. In vitro expression study of the wild-type and mutant CYP21A2

The wild-type and mutant *CYP21A2* genes ranging from nucleotide −122 to 3167 [5] were amplified as described previously [8], ligated into a pCR 2.1 plasmid using a TA cloning kit (Invitrogen, San Diego, CA), and sequenced to confirm the validity. The cloned wild-type and mutant *CYP21A2* genes were cleaved from the plasmid by digestion with restriction enzymes, *Bam*HI and *Xba*I (New England Biolabs, Beverly, MA), and inserted between the corresponding restriction sites of a mammalian expression plasmid pcDNA3 (Invitrogen). The resultant constructs were designated *pCYP21A2* and *pIVS9-9C>A*, respectively. pCYP21A2 had exactly the same nucleotide sequence as pIVS9-9C>A except for the −9 nucleotide of intron 9.

COS-1 cells (RIKEN Cell Bank, Tsukuba, Japan) were transfected by electroporation (Gene Pulser II; Bio-Rad Laboratories, Hercules, CA) with 2 μg of pCYP21A2, pIVS9-9C>A, or empty pcDNA3, and 1 μg of pRK-GH1, a human growth hormone expression plasmid, which was used as an internal control of transfection efficiency. The cells were suspended in Dulbecco modified Eagle medium (Invitrogen) containing 10% fetal calf serum and transferred to 35 × 10–mm FALCON tissue culture dishes (Nippon Becton Dickinson, Tokyo, Japan). Twenty-four hours after transfection, 5 μg/mL of 17α-hydroxyprogesterone (Sigma Chemical, St Louis, MO) was added to the media. After another 24 hours of incubation, the media and cells were collected. The amount of 11-deoxycortisol in the media was determined by high-performance liquid chromatography as described previously [9], and that of human growth hormone was determined by an immunoradiometric assay kit (Daiichi Radioisotope Laboratories, Tokyo, Japan). The experiments, each performed in quadruplicate, were carried out 4 times; and data are shown as mean ± SEM.

2.4. Northern blot and reverse transcription PCR analyses

Total RNA was extracted from the transfected cells with an ISOGEN kit (Nippon Gene, Tokyo, Japan). Twenty micrograms of total RNA was electrophoresed on a 1% agarose gel and transferred to the Hybond-N+ membrane. The membrane was subjected to hybridization with a digoxigenin-labeled *CYP21A2* complementary DNA (cDNA) probe, which was prepared using a set of primers (5′-GAACTACCCGGACCTGTC-3′, corresponding to nucleotides 306-323, and 5′-CTGCATCTCCACGATGT-GATCCCTC-3′, corresponding to the reverse complement of nucleotides 696-720 of *CYP21A2* cDNA [5]) and a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The hybridized probes were detected according to the manufacturer's instruction (Roche Diagnostics).

First-strand cDNA was synthesized in a 20-μL reaction mixture containing 1 μg of total RNA, 0.5 μg of oligo $(dT)_{12\text{-}18}$, 0.5 mmol/L each of dNTPs, 10 mmol/L DTT, 80 units of ribonuclease inhibitor, 200 units of SuperScript II

Reverse Transcriptase, and 1× First-Strand Buffer (Invitrogen, Carlsbad, CA). The resultant cDNA was subjected to PCR amplification with a forward primer, 5′-TCGGTGGGAGGGTACCTGAA-3′, corresponding to nucleotides −122 to −103 and a reverse primer, 5′-TAAGCCTCAATCCTCTGCAGCGGA-3′, corresponding to the reverse complement of nucleotides 1923 to 1946 of *CYP21A2* cDNA [5]. The PCR products were fractionated, isolated, and directly sequenced using the Thermo Sequenase kit.

2.5. Western blot analysis

Aliquots of the whole cell lysates were applied to Western blot analysis using a polyclonal antibody raised against a recombinant human 21-hydroxylase protein in a rabbit, which was kindly provided by Dr Bon-chu Chung (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) [10].

3. Results

3.1. Southern blot and sequencing analyses

Southern blot analysis of genomic DNA from the patient and his parents revealed neither large conversions nor deletions of *CYP21A2* gene.

Direct sequencing analysis of the patient's *CYP21A2* gene revealed a homozygous C to A conversion at −9 position of intron 9, which was designated *IVS9-9C>A* (Fig. 1A). No

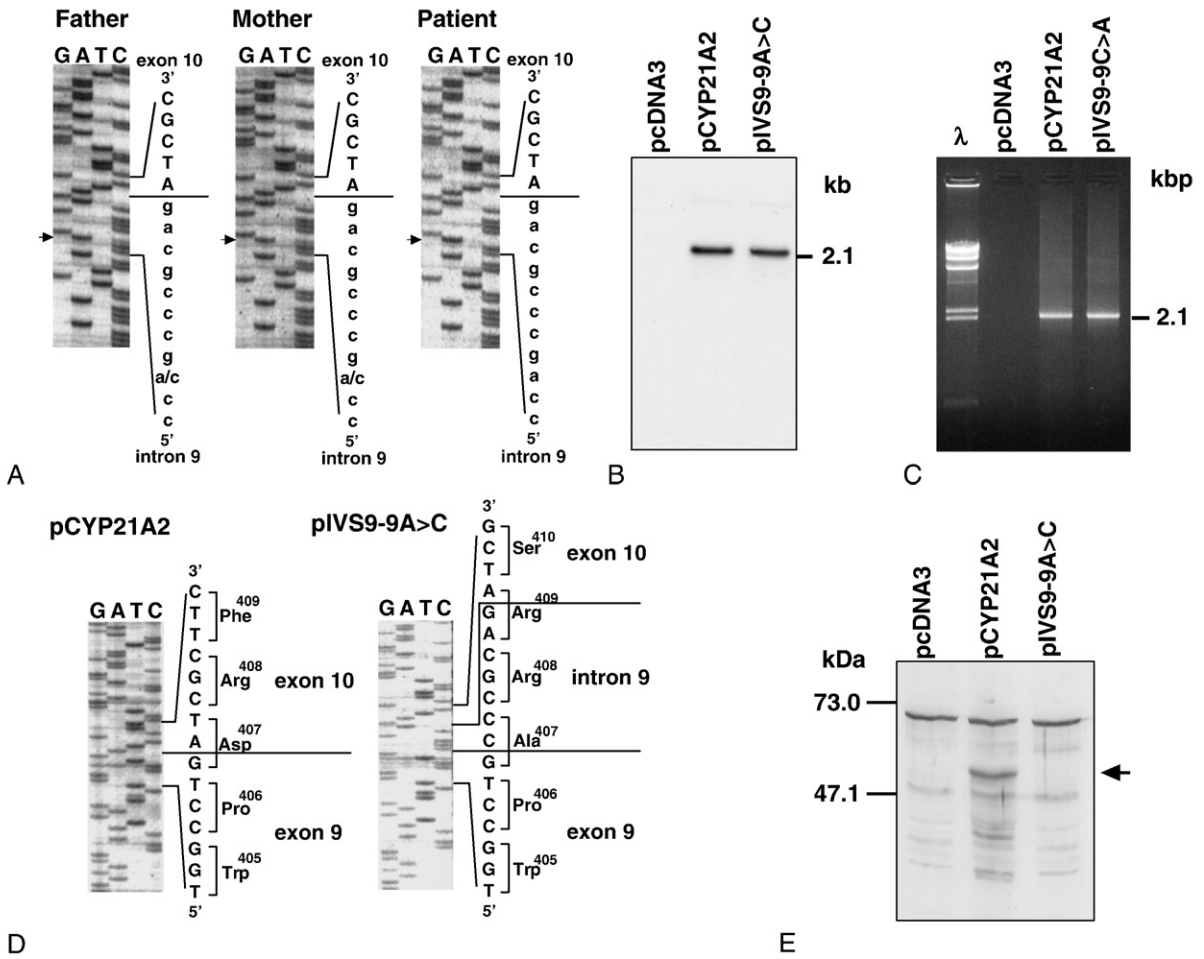

Fig. 1. Molecular analysis of defects in the patient. A, Sequencing analysis of the *CYP21A2* gene demonstrates that the patient is homozygous for an IVS9-9C>A mutation and that the parents are heterozygous for the same mutation as indicated by the arrows. B, Northern blot analysis reveals that both the wild-type and IVS9-9C>A mutant *CYP21A2* genes (designated *pCYP21A2* and *pIVS9-9C>A*, respectively) give rise to approximately 2.1-kilobase transcripts of *CYP21A2*, whereas the empty pcDNA3 plasmid (pcDNA3) yields no *CYP21A2* transcripts. C, Reverse transcription PCR analysis of *CYP21A2* mRNA indicates that both the wild-type and IVS9-9C>A mutant *CYP21A2* genes yield approximately 2.1–kilo base pair products, whereas the empty pcDNA3 plasmid generates no products. λ indicates *Hin*dIII digested λ DNA, which was used as size markers. D, Direct sequencing analysis of the RT-PCR products demonstrates that the IVS9-9C>A mutation creates an aberrant splice acceptor site at −7 position of intron 9 resulting in an insertion of the last 7 nucleotides of intron 9 in mutant *CYP21A2* mRNA. E, Western blot analysis reveals that the wild-type *CYP21A2* gene produces a 52-kd 21-hydroxylase protein as denoted by the arrow, whereas the IVS9-9C>A mutant *CYP21A2* gene and the empty pcDNA3 plasmid yield no immunoreactive protein.

other mutations were found in the patient's *CYP21A2* gene. The parents were heterozygous for the IVS9-9C>A mutation (Fig. 1A). The IVS9-9C>A mutation was not detected in the *CYP21A2* gene of 50 healthy subjects. The IVS9-9C>A mutation was not detected in the *CYP21A1P* pseudogene of the patient, parents, and 50 healthy subjects, either.

3.2. In vitro expression study of CYP21A2

To determine the functional consequences of the IVS9-9C>A mutation, we transiently expressed the *CYP21A2* gene with or without the mutation in COS-1 cells. The cells transfected with the expression plasmid containing the wild-type *CYP21A2* (pCYP21A2) efficiently converted 17α-hydroxyprogesterone to 11-deoxycortisol, whereas the cells transfected with the empty pcDNA3 plasmid or the plasmid containing the mutant *CYP21A2* (pIVS9-9C>A) did not at all (Table 1).

To characterize the transcripts of the wild-type and mutant *CYP21A2* genes, we performed Northern blot and reverse transcription (RT) PCR analyses. Northern blot analysis detected equivalent amounts of *CYP21A2* mRNA of about 2.1 kilobases in the COS-1 cells transfected with pCYP21A2 and in those with pIVS9-9C>A, but not in the cells transfected with the empty plasmid (Fig. 1B). The RT-PCR amplification of *CYP21A2* mRNA from the cells transfected with pCYP21A2 or pIVS9-9C>A yielded a product of about 2.1 kilo–base pairs, whereas the amplification from the cells transfected with the empty plasmid gave no products (Fig. 1C). Direct sequencing of the products demonstrated that all the introns were correctly spliced out in the wild-type gene transcripts, whereas the last 7 nucleotides of intron 9 were retained in the IVS9-9C>A mutant gene transcripts (Fig. 1D).

To determine immunoreactive 21-hydroxylase, we performed Western blot analysis, which detected a 21-hydroxylase protein of 52 kd in the COS-1 cells transfected with pCYP21A2; but no immunoreactive 21-hydroxylase was detected in the cells transfected with pIVS9-9C>A or the empty plasmid (Fig. 1E).

4. Discussion

We have described the IVS9-9C>A mutation in the *CYP21A2* gene in a patient with the salt-wasting form of 21-OHD. The IVS9-9C>A mutation has not been previously reported [1,2,6,7]; thus, this mutation appears to be a novel mutation. It is very likely that the IVS9-9C>A mutation has occurred independently from the *CYP21A1P* pseudogene because none of the investigated *CYP21A1P* alleles carry this mutation.

The novel IVS9-9C>A mutation apparently does not alter the coding sequences or the consensus splice site sequences [11], so the effect of the mutation on the 21-hydroxylase activity needs to be determined. The transient expression of the wild-type and IVS9-9C>A mutant *CYP21A2* genes demonstrates that the IVS9-9C>A mutation creates an aberrant splice acceptor site at −7 position of intron 9 and totally inactivates the authentic splice acceptor site of intron 9. The resultant mRNA is expected to cause a shift in the open reading frame after codon 407 with termination at codon 524 instead of codon 495 in the wild type. It is presumed that the mutant 21-hydroxylase has no enzymatic activity because it is devoid of the functionally critical heme-binding C428 residue [12]. This presumption is confirmed by the functional determination, which indicates that the IVS9-9C>A mutation totally abolishes 21-hydroxylase activity as estimated by the amount of 11-deoxycorisol converted from 17α-hydroxyprogesterone. Failure to detect mutant 21-hydroxylase protein by Western blot analysis suggests that the amino acid alteration in the C-terminus renders the mutant protein very labile. Thus, we conclude that the IVS9-9C>A mutation is a novel splicing mutation leading to total loss of enzymatic activity and immunoreactive protein. Clinical presentations of the patient as the severe salt-wasting form of 21-OHD are in good agreement with these results of the expression studies.

To date, 7 intronic mutations in the *CYP21A2* gene have been reported, that is, IVS2-13A/C>G [13], IVS7+1G>C [14], IVS1-2A>G [15], IVS7+2T>G [16], IVS2+1G>A [17], IVS2-2A>G [18], and IVS2+5G>A mutations [19]. The IVS2-13A/C>G mutation, derived from the *CYP21A1P* pseudogene, comprises around 25% of all classic 21-OHD alleles [1,2] and is demonstrated to cause abnormal splicing of *CYP21A2* transcripts and almost total loss of enzymatic activity [13,20]. The remaining 6 mutations, not apparently originating from the *CYP21A1P* pseudogene, alter the consensus splice site sequences [11] and thus putatively interfere with proper splicing of *CYP21A2* mRNA. However, only one of these mutations, IVS2+1G>A, is proven to affect splicing from mRNA studies [21]; and the effects of these mutations on 21-hydroxylase activity have never been determined. It is reported that the disease severity correlates well with the residual enzymatic activity of mutant *CYP21A2* in patients with 21-OHD, and prenatal treatment is effective to reduce virilization of external genitalia in female patients with the classic form [1,2]. Therefore, functional characterization of mutant *CYP21A2* as we have done in the present study is requisite to predict the disease phenotype and offer appropriate genetic and prenatal counseling.

Table 1
21-Hydroxylase activity of wild-type and mutant *CYP21A2* expressed in COS-1 cells

Plasmid	11-deoxycortisol (ng/dish)
pcDNA3	<1.0
pCYP21A2	79.2 ± 5.6
pIVS9-9C>A	<1.0

Data are shown as mean ± SEM from 4 independent experiments. pcDNA3 indicates the empty pcDMA3 plasmid; pCYP21A2, the expression plasmid containing the wild-type *CYP21A2* gene; pIVS9-9C>A, the expression plasmid containing the IVS9-9C>A mutant *CYP21A2* gene.

In conclusion, we have demonstrated a novel splicing mutation in an intron of *CYPA21A2* that gives rise to classic salt-wasting 21-OHD.

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