NOTE

Nonclassic Steroid 21-Hydroxylase Deficiency due to a Homozygous V281L Mutation in CYP21A2 Detected by the Neonatal Mass-Screening Program in Japan

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Abstract. Since 1989, neonatal mass screening for congenital adrenal hyperplasia (CAH) has been carried out in Japan. The mass screening has detected not only the patients with the classic form of steroid 21-hydroxylase deficiency (21-OHD), but also those with the nonclassic (NC) form of 21-OHD, and the molecular basis in these patients has been elucidated. However, the homozygous V281L mutation in CYP21A2, the common mutation in the NC form in Caucasians, has not been described in Japanese patients, implying at least two possibilities; 1) the V281L mutation itself might be very rare in Japanese, and 2) nonclassic 21-OHD patients bearing the V281L mutation might be barely detectable by the mass-screening program, hence overlooked in Japan. In the present study, we describe a Brazilian girl with the NC form of 21-OHD, who was pointed out to have mildly elevated 17α-hydroxyprogesterone in blood by the mass screening in Japan. Genetic analysis revealed that the patient was homozygous for the V281L mutation, and that the parents were heterozygous for the V281L mutation. Thus, the NC patients due to the homozygous V281L mutation can be detectable by the mass-screening program for CAH in Japan, and further accumulation and analysis of the NC patients should elucidate the frequency of the V281L allele in Japan.

Key words: Steroid 21-hydroxylase deficiency, P450c21, CYP21A2, Gene, Mutation

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program for CAH based on the measurement of 17α-hydroxyprogesterone (17-OHP) in blood samples collected on filter paper has been carried out since 1989. Since then, the benefits of the program have been recognized [11]. The mass screening has detected the NC form of 21-OHD as well as the classic form, and the molecular basis of the NC form has been elucidated [12–14]. However, the homozygous V281L mutation has not been described in 21-OHD patients in Japan [12–19].

In the present study, we report a Brazilian patient with the NC form of 21-OHD due to a homozygous V281L mutation, who was detected by the mass screening in Japan.

**Materials and Methods**

**Patient**

The patient was a daughter of healthy Brazilian parents. The patient was born after an uneventful 38-week and 4-day gestation. Her birth weight was 3400 g. The patient was pointed out to have mildly elevated 17-OHP (8.8 ng/ml) (cut-off value, 5 ng/ml) in the blood sample taken at day 5 for the neonatal mass screening in Japan. The blood 17OHP level increased to >15 ng/ml in the second sample, and thus she was referred to our hospital at the age of 2 months. When she was first seen, she weighed 5760 g, and did not show any symptoms of CAH such as ambiguous genitalia, generalized skin pigmentation, and failure to thrive. Blood sugar was normal (86 mg/dl). Serum Na, K and PRA were normal (142 mEq/l, 5.5 mEq/l, 5.7 ng/ml/h, respectively). Plasma ACTH was also normal (22.6 pg/ml), but serum 17-OHP was slightly elevated (9.2 ng/ml). Since the patient continued to have slightly high serum 17-OHP (7.9 ng/ml at 3 months of age), serum adrenal steroids were measured before and after ACTH stimulation at 3 months of age (Table 1). Serum cortisol and aldosterone were normal basally, and they increased normally in response to ACTH stimulation [20, 21]. Basal and ACTH-stimulated 17-OHP levels were high [20], and fell within the range of the NC patients in the nomogram reported by New et al. [22]. Based on these clinical and endocrinological findings, the patient was diagnosed as having the NC form of 21-OHD. On the last examination at 1 year and 3 months of age, she showed normal growth and development, and had no adrenal crisis without medication.

*Southern blot and sequencing analysis of the CYP21A2 gene*

The genetic study was reviewed and approved by the Institutional Review Board at the National Center for Child Health and Development, and the written informed consent for the genetic analysis was obtained from the parents.

The genomic DNA of the patient and her parents was isolated from whole blood by proteinase K digestion and phenol/chloroform extraction. Three micrograms of genomic DNA were digested with either Taq I (New England Biolabs, Inc., Beverly, MA, USA) or Bgl II (New England Biolabs, Inc., Beverly, MA), then electrophoresed on a 1% agarose gel and transferred to a Hybond-N+ membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The membranes were subjected to hybridization with the digoxigenin-labeled probes of CYP21A2, which were prepared using three sets of primers (5'-TCGGTGGGAGGGTACCTGAA-3', corresponding to nucleotides –122 to –103 and 5'-GGGCCAGAGCGAGATCA-3', corresponding to nucleotides 209 to 225; 5'-GATCACATCGTGGAGATGCAGCTG-3', corresponding to nucleotides 1373 to 1396 and 5'-ATGTGCCAGTGCCCTTCCAGGAG-3', corresponding to

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**Table 1.** Basal and ACTH-stimulated serum adrenal steroids in the patient at 3 months of age

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Normal Females</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Stimulated</td>
</tr>
<tr>
<td>17-OHP (ng/ml)</td>
<td>13</td>
<td>95</td>
</tr>
<tr>
<td>Cortisol (µg/dl)</td>
<td>11.5</td>
<td>32.6</td>
</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>150</td>
<td>950</td>
</tr>
</tbody>
</table>

* Reference values in normal females aged <1 year are based on the previous data [20, 21].
the reverse complement of nucleotides 1668 to 1690; and 5'-CCAGTGGTACCAAGCTCACTC-3', corresponding to nucleotides 3002 to 3021 and 5'-GCT CATCTCTGCGCTTGCTT-3', corresponding to the reverse complement of nucleotides 3424 to 3443 [6]) and a PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). The hybridized DNA fragments were detected according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

The entire CYP21A2 gene was specifically amplified using a forward primer, 5'-TCGGTGGGAGGACCTGAA-3' (underlined are the nucleotides not shared with CYP21A1), corresponding to nucleotides –122 to –103 and a reverse primer, 5'-TAAGCCTCAATCCCTTCGACCGGA-3' (underlined are the nucleotides not shared with CYP21A1), corresponding to the reverse complement of nucleotides 3144 to 3167 [6]. Polymerase chain reactions (PCR) were performed in a 50-µl mixture containing 0.2 µg of genomic DNA, 200 µM each of dNTPs, and 500 nM of the forward and reverse primers, 2.5 U Ex Taq DNA polymerase, and 1 x Ex Taq Buffer (Takara Shuzo Co., Ltd., Otsu, Japan). 30 cycles of denaturation at 94°C for 1 min and at 98°C for 20 sec, and extension at 68°C for 15 min were followed by an additional extension at 72°C for 10 min. The amplified PCR products were fractionated and isolated on a 1% agarose gel (Bio-Rad Laboratories, Richmond, CA, USA), and were directly sequenced with appropriate primers using a Thermo Sequenase kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

**Results**

Southern blot analysis of genomic DNA from the patient and her parents revealed neither large conversions nor large deletions of CYP21A2 gene (data not shown).

Direct sequencing analysis of the entire CYP21A2 gene in the patient revealed a homozygous missense mutation V281L, which changed codon 281 (GTG) encoding Val to TTG encoding Leu (Fig. 1). No other mutations were found in the patient. The parents were heterozygous for the V281L mutation (Fig. 1).

**Discussion**

In the present study, we described a Brazilian patient with the NC form of 21-OHD who was detected by the neonatal mass screening in Japan. The patient was a girl without any symptoms of CAH such as ambiguous genitalia, generalized skin pigmentation, and failure to thrive. Blood sugar, serum electrolytes, and endocrinological data were normal except for basal and ACTH-stimulated 17-OHP levels. The patient grew normally without medication thus far. These findings were completely compatible with the NC form of 21-OHD. We performed the southern blot analysis and the sequencing analysis of the entire CYP21A2 gene, and identified only the homozygous
V281L mutation. Thus, we concluded that the homozygous V281L mutation is the cause of the NC form of 21-OHD in our patient. It goes without saying that the genetic analysis is a valuable diagnostic tool to confirm the diagnosis in cases with slightly elevated 17-OHP levels.

To our knowledge, CYP21A2 gene mutations have been elucidated in 22 Japanese NC patients from 19 families, [12–14, 18, 19]. Among the total 39 independent alleles, the P30L and I172N mutations have been identified in 8 (20.5%) and 14 (35.9%) alleles, respectively. Of note, one NC patient detected by the newborn mass-screening program was a compound heterozygote with the V281L mutation in a paternal allele and the I172N mutation in a maternal allele [14]. So far this is the only Japanese patient bearing the V281L mutant allele, and the homozygous V281L mutation has not been described in Japan [12–19]. On the contrary, the V281L mutation was reported to be most frequent in the Caucasian NC patients [9], and accounted for 40% of the NC alleles in Brazil [10]. There are at least two ways to explain the paucity of the V281L mutation in the Japanese NC patients. The first is to hypothesize that the V281L mutation itself might be very rare in Japanese. The second is to hypothesize that the NC patients bearing the V281L mutation might be barely detectable by the mass-screening program, hence overlooked in Japan. Recently, Wilson et al. analyzed the CYP21A2 gene in a large number of 21-OHD patients, and demonstrated significant ethnic variations in the prevalence of the mutant CYP21A2 alleles [23]. Interestingly, they reported that the V281L mutation was not observed in the Chinese patients, whereas the V281L mutation is most frequent in the Ashkenazi Jews with the NC form, and they also speculated that the NC form of 21-OHD is rare or had not been clinically diagnosed in Chinese [23]. In any case, the discovery of our patient suggests that the Japanese mass-screening program is sensitive enough to detect some NC patients caused by the homozygous V281L mutation, and further accumulation and genetic analysis of the Japanese NC patients are necessary to elucidate the incidence of the NC form and the frequency of the V281L mutant allele in Japan.

In conclusion, we reported a Brazilian patient with the NC form of 21-OHD, who was detected by the Japanese neonatal mass-screening program, and we identified a homozygous V281L mutation in the CYP21A2 gene in the patient.

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References


