J Hum Genet (1999) 44:312–317

ORIGINAL ARTICLE

Aki Asanuma · Toshihiro Ohura · Eishin Ogawa
Sachiko Sato · Yutaka Igarashi · Yoichi Matsubara
Kazuie Iinuma

Molecular analysis of Japanese patients with steroid 21-hydroxylase deficiency

Received: March 29, 1999 / Accepted: May 11, 1999

Abstract We have designed a rapid and convenient strategy to determine nine of the most common mutations in the 21-hydroxylase gene (*CYP21*). The frequency of the mutations was investigated in 34 Japanese patients affected with congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency. We characterized 82% of the CAH chromosomes. The most frequent mutations were a C/A to G substitution in intron 2 in the salt-wasting form of the disease and an *I172N* in the simple virilizing form. Three *de novo* mutations were found. Two homozygous mutations (*S268T* and *N493S*) were detected by direct sequencing of all exons of *CYP21* in two siblings, who had a normal genotype at all positions screened. We successfully applied these methods for prenatal diagnosis in one family. These procedures proved to be sensitive and rapid for the detection of the most common known mutations in the *CYP21* gene and may be useful for genetic screening.

Key words Genetic screening · Genetic disease · 21-Hydroxylase deficiency · PCR

Introduction

Congenital adrenal hyperplasia (CAH; MIM *201910) is one of the most common forms of inborn errors of metabolism. Steroid 21-hydroxylase (21-OH) deficiency is a cortisol biosynthesis autosomal recessive disorder that accounts for 90%–95% of CAH cases. A wide spectrum of clinical variants exists, from severe or classical forms, which are evident at birth, to mild, late-onset nonclassical forms. Severe forms include two groups of patients: those with a complete lack of 21-OH function (salt-wasting, SW), and those with partial impairment of 21-OH (simple virilizing, SV) (White et al. 1987).

The affected enzyme, 21-OH, is encoded by an active gene, *CYP21*, located on the short arm of chromosome 6, with an adjacent inactive pseudogene (*CYP21P*). Many mutations responsible for the disease have been described (Rodrigues et al. 1987; Higashi et al. 1988b, 1991; Wu and Chung 1991). Most of these mutations are results of gene conversion events between the functional *CYP21* gene and the pseudogene (*CYP21P*) acting as a reservoir of mutations (Higashi et al. 1988a). Approximately 90% of the cases are caused by nine specific point mutations and deletions in the *CYP21* gene: *P30L*, 655C/A> G in intron 2 (*i2g*), *del 8-bp* in exon 3, *I172N*, triple substitution in exon 6 (*E6 cluster*), *V281L*, *1761insT*, *Q318X*, and *R356W*. We designed rapid and convenient PCR-based methods to screen for these known mutations in the *CYP21* gene and characterized 34 Japanese patients with 21-OH deficiency.

A. Asanuma · T. Ohura (✉) · E. Ogawa · S. Sato · K. Iinuma
Department of Pediatrics, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
Tel. +81-22-717-7286; Fax +81-22-717-7290
e-mail: tohura@ped.med.tohoku.ac.jp

Y. Igarashi
Igarashi Childrn's Clinic, Sendai, Japan

Y. Matsubara
Department of Medical Genetics Tohoku University School of Medicine, Sendai, Japan

Materials and methods

Patients

Thirty-four Japanese CAH patients, including three siblings, were studied. There was no consanguinity between the parents of these patients. In 18 families (20 patients), DNA samples from the parents also were analyzed. Twenty-six patients suffered from the salt-wasting (SW) form of the disease and 8 from the simple virilizing (SV) form. No patient was classified as having the nonclassical form. The patients were diagnosed on the basis of an elevated plasma 17-hydroxyprogesterone. CAH with SW was characterized by the onset of hyperkalemia, hyponatremia, dehydration, and shock requiring treatment with both mineralocorticoids and glucocorticoids. The SV form was identified by the presence of ambiguous genitalia in females.

After informed consent was obtained from the patients and/or their parents, genomic DNA was prepared from their peripheral blood leukocytes.

PCR amplification and digestion of PCR products

Polymerase chain reaction (PCR) amplification reactions were carried out in 50-μl reaction mixtures containing 50 mM KCl, 10 mM TRIS-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (W/V) gelatin, 200 μM of each dNTP, 1 μM of each nucleotide primer, 1 μg of genomic DNA, and 2.5 units *Taq* DNA polymerase (TaKaRa, Kyoto, Japan). The PCR products were digested with appropriate restriction enzymes (New England Biolabs, Beverly, MA, USA) according to the manufacturer's protocol.

Detection of *CYP21* gene deletions

Deletion of the *CYP21* gene was detected by means of PCR with primers 1 and 5 (Table 1) and subsequent digestion with *Taq*I restriction enzyme (Ogawa et al. 1993). Although the PCR products (210 bp) could be a mixture of both *CYP21* and *CYP21P* sequences, a *Taq*I restriction site is present only in *CYP21P* (Fig. 1). Therefore, in normal subjects, equal-intensity 210- and 187-bp bands could be detected. If one of the *CYP21* genes was deleted, the intensity of the 210-bp band decreased relative to that of the 187-bp band. If both the *CYP21* genes were deleted, only the 187-bp band could be detected.

Amplification of the *CYP21* gene

Genomic DNA was amplified in two segments by PCR with primers 1 and 2 (fragment 1) and 3 and 4 (fragment 2), which selectively amplify the *CYP21* gene (Table 1). Primers 2 and 3 were specific for sequences in the nonpseudogene DNA (Tajima et al. 1993b). Fragment 1 represented a 952-bp segment extending from exon 1 to exon 3. Fragment 2 was 2070-bp and extended from exon 3 to beyond exon 10. Thirty cycles of amplification were used, each consisting of denaturation for 30 s at 94°C, annealing for 30 s at 60°C (primer 1/2) or 64°C (primer 3/4), and extension for 30 s (primer 1/2) or 120 s (primer 3/4) at 72°C. If the sample had the *CYP21* deletion or an 8-bp deletion in exon 3 (*del 8-bp*), no PCR products were generated with these primers.

Detection of seven point mutations

For the second round of PCR, 1 μl of the PCR product from each first-stage amplification was used with the appropriate primers listed in Table 1. Thirty cycles of amplification were used, each consisting of a denaturation step for 30 s at 94°C, an annealing step for 30 s at 58° or 60°C, and an extension step for 30 s at 72°C. Using fragment 1 as a template, we performed a second round of PCR to detect the *P30L* and *i2g* mutations. Fragment 2 was used as a template to detect the *I172N*, *E6 cluster*, *V281L*, *Q318X*, and *R356W* mutations. The volume of the reaction mixture for PCR was 50 μl and 5 μl of each PCR product was incubated for at least 2 h with 5–10 units of the appropriate restriction enzyme. Electrophoresis was performed using a 3%–6% agarose gel and visualized by ethidium bromide staining.

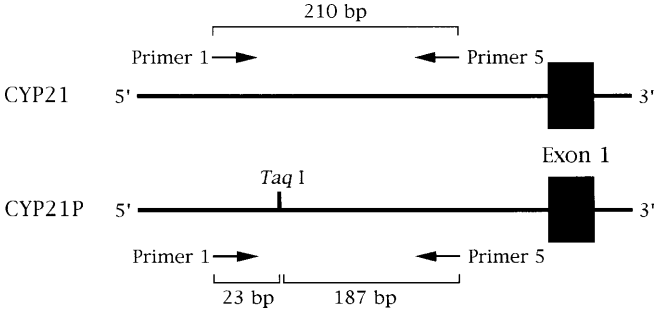

Fig. 1. Locations of primer 1 and 5 in CYP21 and CYP21P. A *Taq*I restriction site is present only in CYP21P

Table 1. PCR primers used for amplification of *CYP21* gene

Primer 1	−231–−211	5′-TGC ATT TCC CTT CCT TGC TTC-3′
Primer 2	+701–+721	5′-GCA GGG AGT AGT CTC CCA AGG-3′
Primer 3	+701–+721	5′-CCT TGG GAG ACT ACT CCC TGC-3′
Primer 4	+2749–+2770	5′-AGG GGT TCG TAC GGG AGC AAT A-3′
Primer 5	−42–−22	5′-CTG AGG TGC CAC TTA TAG CTC-3′
Primer 6	+67–+88	5′-AAG CTC CGG AGC CTC CAC CTC ***G***-3′
Primer 7	+195–+214	5′-AGA TCA GCC TCT CAC CTT GC-3′
Primer 8	+524–+547	5′-TGG GGC ATC CCC AAT CCA GGT CCC-3′
Primer 9	+656–+677	5′-ACC AGC TTG TCT GCA GGA GGA ***T***-3′
Primer 10	+1000–+1020	5′-TCT CCG AAG GTG AGG TAA CA ***T***-3′
Primer 11b	+1375–+1395	5′-AGC TGC ATC TCC ACG ATG TGA-3′
Primer 11a	+1375–+1397	5′-TCA GCT GCT TCT CCT CGT TGT GG-3′
Primer 12	+1373–+1396	5′-GAT CAC ATC GTG GAG ATG CAG CTG-3′
Primer 13	+2129–+2153	5′-TGG GCC GTG TGG TGC GGT GGG GCA A-3′
Primer 14	+1975–+1993	5′-CCA GAT TCA GCA GCG ACT G-3′
Primer 15	+2113–+2136	5′-TGG GGC AAG GCT AAG GGC ACA AC ***C***-3′

Underlined primers are antisense primers
Italic letters indicate modified nucleotides

V281L and *Q318X* mutations

Because these mutations abolish restriction enzyme recognition sites, we performed nested PCR using primers 12 and 13, followed by *Apa*LI or *Pst*I digestion. When the *V281L* mutation is present, the *Apa*LI restriction site is lost, and when the *Q318X* mutation is present, the *Pst*I site is lost. Digestion of the PCR products with *Apa*LI yielded 375-, 311-, and 95-bp fragments if the *V281L* mutation was absent, and 686- and 95-bp fragments if the mutation was present. Digestion with *Pst*I yielded 299-, 204-, 158-, and 120-bp fragments if the *Q318X* mutation was absent, and 457-, 204-, and 120-bp fragments if the mutation was present.

P30L, *i2g*, *I172N*, and *R356W* mutations

We developed a rapid, modified PCR assay using mismatch primers to detect these mutations.

P30L mutation

Using primers 6 and 7, a 148-bp fragment containing the 89C > T mutation site was amplified. The mismatched G in primer 6 introduced an *Acc*II restriction site in the normal sequence. Digestion of the PCR product with *Acc*II yielded 126- and 22-bp fragments if the mutation was absent and a 148-bp fragment if the mutation was present.

i2g mutation

A modified PCR was performed for detection of the 655C/A > G mutation using primers 8 and 9. Primer 9 was designed to introduce a *Sau*3AI restriction site in the mutant PCR products. Digestion of the PCR product yielded a 156-bp fragments if the mutation was absent and 133- and 23-bp fragments if the mutation was present.

I172N mutation

Using primers 3 and 10, we performed nested PCR to detect the 999T > A mutation. Primer 10 was designed to introduce an *Nde*I site into the normal products. Digestion of the PCR products with *Nde*I yielded a 320-bp fragment if the mutation was absent and 297- and 23-bp fragments if the mutation was present.

R356W mutation

A modified PCR amplification was performed to detect the *R356W* mutation using primers 14 and 15. Primer 15 was designed to introduce an *Eco*52I restriction site in the normal PCR products. Digestion of the PCR product yielded a 162-bp fragment if the mutation was present and a 136-bp fragment if the mutation was absent.

E6 cluster mutation

E6 cluster mutations were analyzed by means of allele-specific PCR. Two PCRs were performed, one reaction using primers 3 and 11b detected the normal allele, and the other with primers 3 and 11a detected the mutant allele. If the sample was normal and homozygous, then a product would be generated only from the reaction containing the normal primer. Conversely, if the sample was homozygous mutant, a product would be generated only from the reaction containing the mutant primer. If, however, the sample was heterozygous, products would be generated from both the normal and mutant reactions (Wilson et al. 1995).

1761insT mutation

When none of the seven mutations or deletions could account for one of the diseased alleles, we sequenced exon 7 of the *CYP21* gene from the patient to determine whether there was an insertion of T at nucleotide 1761 (*1761insT*).

Direct sequencing

Patients 29 and 30 (siblings) were screened for the most common known mutations, but none of these mutations were detected. We further analyzed these patients by direct sequencing of all the exons of the *CYP21* gene. The primers used to amplify each exon of *CYP21* are listed in Table 2. For the amplification of exon 3, the first stage of PCR was performed using primers 8 and 11b (specific for the active gene). All nested PCR products were directly sequenced on a Pharmacia LKB A.L.F. DNA Sequencer (Uppsala, Sweden) using a Thermo-Sequenase cycle sequencing kit (Amersham, Buckingham Shire, UK).

Prenatal diagnosis

Prenatal diagnosis was requested from the family of patient 15, who was homozygous for the *i2g* mutation. After obtaining informed consent, whole blood was collected from her parents and sister. Chorionic villus sampling was performed at 12 weeks gestation. DNA was extracted by a standard procedure and analyzed by a modified PCR as described in Materials and methods.

Results

The distribution of the mutations detected in *CYP21* is shown in Table 3. Among the 68 chromosomes, 56 (82%) were characterized by screening for the seven most common point mutations and deletions. Both chromosomes were affected by one of these mutations in 25 (73%) patients, 6 (18%) patients had only one copy of one of these mutations, and 3 (9%) patients harbored none of the tested mutations. The most frequent mutations were *i2g* (26%),

Table 2. PCR primers used for direct sequencing

Exon 1		
Primer rev-1	−45–−26	5′-CTT GAG CTA TAA GTG GCA CC-3′
Primer KS-2	+244–+261	5′-ACC CTC TCC GTC ACC TCC-3′
Exon 2		
Primer rev-3	+257–+274	5′-AGG GTC CTC TCT CCG CTG-5′
Primer KS-4	+452–+470	5′-TAA GAC CAG CCT GGG CAA C-3′
Exon 3		
Primer rev-5	+605–+624	5′-GAA GGT CAG GCC CTC AGC TG-3′
Primer KS-6	+853–+873	5′-TAC TGT GAG AGG CGA GGC TGA-3′
Exon 4–5		
Primer rev-7	+890–+907	5′-TGC ACA GCG GCC TGC TGA-5′
Primer KS-8	+1239–+1257	5′-CCT ACA ACC CAG GGG TGT C-3′
Exon 6		
Primer rev-9	+1270–+1288	5′-GTG GAG GGA GAG GCT CCT T-3′
Primer KS-10	+1471–+1490	5′-AGA ACC CGC CTC ATA GCA AT-3′
Exon 7		
Primer rev-11	+1520–+1540	5′-CAC TCT CTA CTC CTC TCC CCA-3′
Primer KS-12	+1808–+1825	5′-TGG CCA GGT TGC TGG GAA-3′
Exon 8		
Primer rev-13	+1923–+1940	5′-TGG AGG CTG GGC AGC TGT-3′
Primer KS-14	+2211–+2229	5′-TGG AGT TAG AGG CTG GCC A-3′
Exon 9		
Primer rev-15	+2177–+2196	5′-GAT GAG TGA GGA AAG CCC GA-3′
Primer KS-16	+2374–+2391	5′-ACC AGC CTC CAC CAC ATT-3′
Exon 10		
Primer rev-17	+2701–+2721	5′-TGA ACG CCT CCC CAC CCA-3′
Primer KS-18	+2749–+2770	5′-AGG GGT TCG TAC GGG AGC AAT A-3′

Antisense primers are underlined

Table 3. Distribution of deletions, large gene conversions, and nine common mutations in 34 Japanese CAH patients

	SW	SV	Total
P30L	0	1	1
i2g	15	3	18
del 8-bp	0	0	0
I172N	2	6	8
E6 cluster	1	0	1
V281L	0	0	0
1761insT	0	0	0
Q318X	6	0	6
R356W	9	3	12
C.A.[a]	2	0	2
Deletion	7	1	8
Not detected	10	2	12
Total	52	16	68

CAH, congenital adrenal hyperplasia
[a] Complex allele: I172N + R356W and V281L + 1761insT + Q318X

R356W (18%), a deletion of the *CYP21* gene (12%), *I172N* (12%), and *Q318X* (9%). The *P30L*, *E6 cluster*, and *1761insT* mutations were rare, and the *del 8-bp* in exon 3 was not detected in this study. The distribution of the mutation frequencies in Japanese populations does not differ significantly from those previously reported (Speiser et al. 1992), except for the frequency of the deletion of the *CYP21* gene. The frequency of a deletion of the *CYP21* gene in Japan is lower than in Western countries (20%–30%) (Wilson et al. 1995; Speiser et al. 1992; Wedell et al. 1994). Two families in this series had one allele with more than one mutation. Patient 19 had the *I172N* and *R356W* mutations on his maternal allele while patient 21 carried the *V281L*, *1761insT*, and *Q318X* mutations on her paternal allele. These complex alleles probably resulted from large gene conversions or multiple mutation events.

Analysis of the segregation of point mutations and deletions in the 18 CAH pedigrees (20 patients) showed three *de novo* mutations (8% allelic frequency; an *i2g* mutation in patient 17, a *P30L* in patient 31, and an *R356W* in patient 33). The *de novo* mutation rate for this disease is reportedly low (<1%) (Wedell et al. 1994; Barbat et al. 1995; Speiser et al. 1994), although it has been reported to be higher in Japanese populations (~20%) (Tajima et al. 1993a,b). Our result supports the conclusion that the incidence of *de novo* mutations among Japanese is high.

Direct sequencing of *CYP21* in patients 29 and 30 revealed homozygous *S268T* and *N493S* mutations (Fig. 2). Their parents were heterozygous for these two mutations. The *S268T* mutation has been reported to be a normal polymorphism (Rodrigues et al. 1987; Wu and Chung 1991), although the functional effect of the *N493S* mutation has never been analyzed. Barbat et al. (1995) described the *N493S* mutation as a disease-causing mutation, but Wedell and Luthman (1993) considered this change to be normal polymorphism. Ordonez-Sánchez et al. (1998) found a very high frequency of the *N493S* mutation in a Mexican population, and the proportion of homozygosity for the *N493S* substitution was higher for patients. Rodrigues et al. (1987) also reported a patient in whom a hemizygous *N493S* mutation was combined with the *S268T* mutation. It is possible that the *N493S* substitution may result in decreased enzymatic activity only when combined with the effect of the *S268T* mutation, and that these two changes appear in linkage disequilibrium. A synergistic effect of partially inacti-

vating mutations has already been documented for the *CYP21* gene (Nikoshkov et al. 1997).

The DNA analysis of a fetus from family 15 revealed that only one of the chromosome 6 alleles carried the *i2g* mutation (Fig. 3), predicting that the fetus would be unaffected. After delivery, this prediction was confirmed by postnatal DNA analysis and hormonal studies.

Discussion

We have designed a coordinated strategy to detect the nine most common 21-hydroxylase mutations. Depending upon the mutation to be detected, we applied one of two simple strategies: digestion of PCR-amplified gene fragments with appropriate restriction enzymes or the use of modified PCR methods employing mismatch primers followed by restriction analysis. In contrast to previous methods, such as dot blot analysis, single-strand conformation polymorphism (using radioactive probes), or allele-specific PCR (which amplifies normal and mutant alleles in different tubes) (Speiser et al. 1992; Ogawa et al. 1993; Tajima et al. 1993b; Wilson et al. 1995), this strategy could characterize six common *CYP21* gene mutations (*P30L*, *i2g*, *I172N*, *V281L*, *Q318X*, and *R356W*) by using ethidium bromide-stained agarose gel and six common restriction enzymes. Because we could not develop a modified PCR approach for the *E6 cluster* mutation, we applied allele-specific PCR to detect this mutation. Using these rapid and convenient PCR-based methods, we successfully screened 34 CAH patients. Our results indicate that most (76%; 52/68) of the CAH mutations may be detected by screening for five mutations (*i2g*, *I172N*, *Q318X*, *R356W*, and deletion of *CYP21*).

The most frequent mutations found in the SW form of the disease were *i2g* (29% of the SW chromosomes), *R356W* (17%), *deletion* (13%), and *Q318X* (11%). In the SV form, *I172N* was very frequent (37% of the SV chromosomes). Phenotype–genotype correlations may be drawn by considering the phenotype of patients homozygous or hemizygous for these mutations (Table 4). Individuals homozygous or hemizygous for *i2g*, the *E6 cluster*, *Q318X*, and *R356W* had an SW phenotype, suggesting that these mutations were responsible for the SW phenotype. Conversely, those who were homozygous or hemizygous for the *I172N* mutation had an SV phenotype. However, the phenotypes of the patients who were compound heterozygotes could not be predicted from their genotypes with complete certainty. For instance, patients whose genotypes were *i2g*/*R356W* could have either the SV or SW phenotype (see Table 4).

Patient 9, who was clinically diagnosed as having SW, had an affected younger brother (patient 10) who had not developed salt-losing symptoms. Similarly, patient 7, who did not have SW, had a younger sister (not shown in Table 4) who died of hypovolemic shock. Indeed, the mutation *i2g*, which leads to aberrant splicing, was found in both clinical forms. This may be explained by differences in the rate of correct splicing in the adrenals of the patients (White and New 1992; White et al. 1994). It is also possible that the phenotype may depend on individual variations, a term that must include not only other genetic factors but also nongenetic factors such as environmental stress.

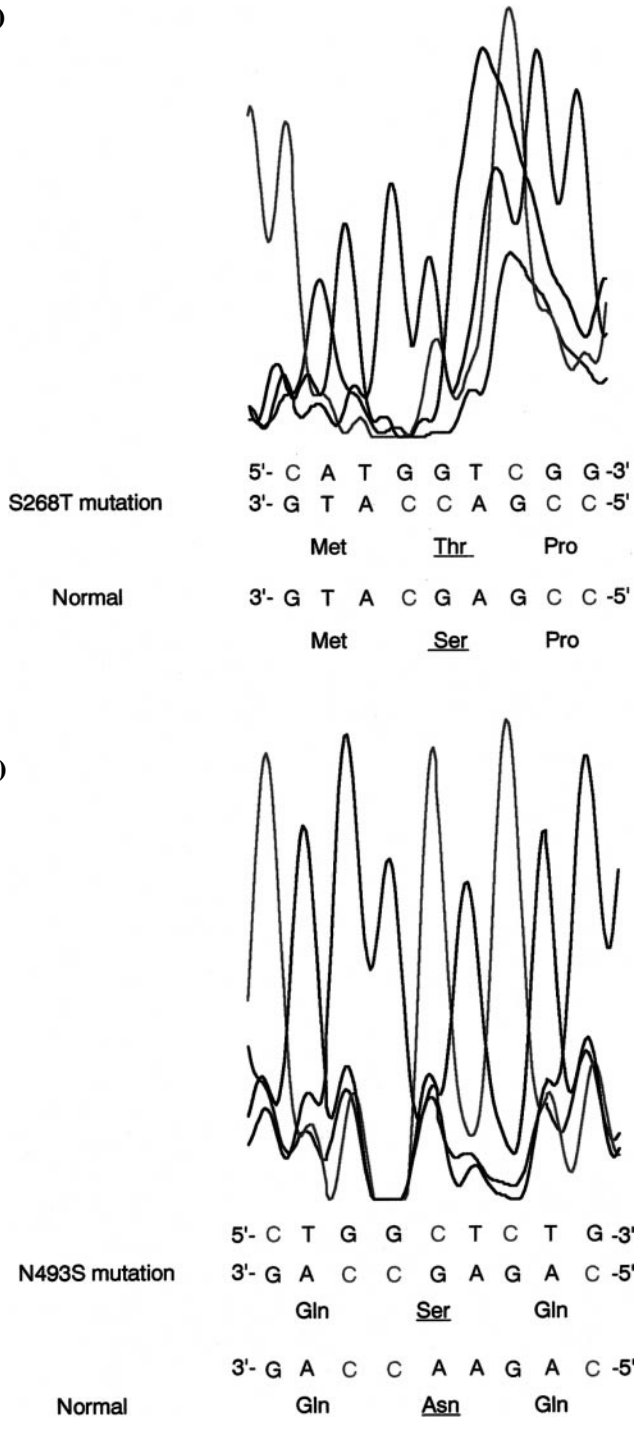

Fig. 2a, b. Direct sequence analysis of genomic DNA from patient 29 (antisense strand). Homozygous S268T (**a**) and N493S (**b**) mutations were detected

Since the introduction of neonatal screening for CAH, most children are diagnosed before salt-wasting symptoms develop (Pang et al. 1988). We have found that genotyping is a very useful tool for predicting disease severity in CAH

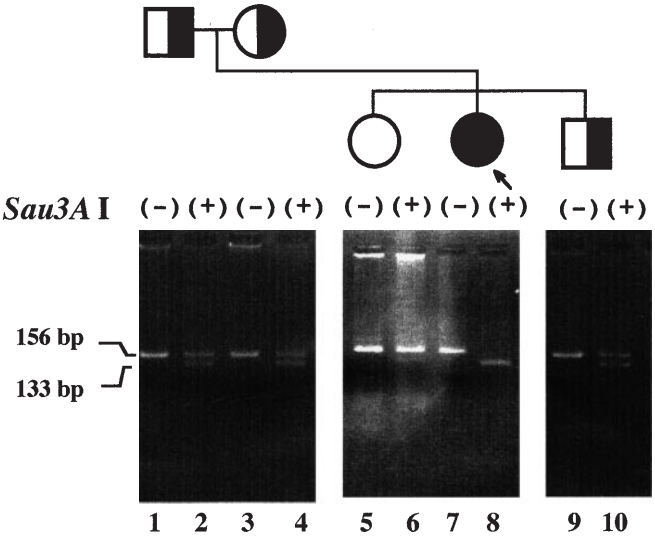

Fig. 3. DNA analysis was performed by PCR as described in Materials and methods. Patient 15 (*lanes 7,8*) is a homozygote for the *i2g* mutation. The patient's father (*lanes 1,2*) and mother (*lanes 3,4*) both are heterozygotes for this mutation; her sister (*lanes 5,6*) is normal. A fetus (*lanes 9,10*) is a heterozygote for this mutation

patients. However, caution is needed when analyzing the *CYP21* genes to avoid misinterpretation of the genotype data because the *CYP21* gene in patients with CAH may have undergone considerable variations in the gene arrangement as well as in the number of point mutations. With proper vigilance, however, a rapid and accurate diagnosis of CAH can be made by typing the *CYP21* mutations.

In conclusion, PCR-based screening methods are useful in genotyping the Japanese 21-OH-deficient population. Our results showed a close, but not complete, correlation between the molecular defect and the clinical expression of this disease. Therefore, the methods described here are suitable for genetic screening, including the prenatal diagnosis of disease.

Table 4. Genotype–phenotype correlation

Genotype	Phenotype	Patient no.
i2g/i2g	SW	5, 6, 8, 15
i2g/del	SW	28
E6 cluster/del	SW	27
Q318X/Q318X	SW	2, 12
R356W/R356W	SW	11
R356W/del	SW	1
I172N/I172N	SV	16
I172N/del	SV	25
i2g/R356W	SW	9, 23, 32
i2g/R356W	SV	7, 10

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