Cytochrome P450 Oxidoreductase Deficiency: Identification and Characterization of Biallelic Mutations and Genotype-Phenotype Correlations in 35 Japanese Patients

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Context: Cytochrome P450 oxidoreductase (POR) deficiency is a rare autosomal recessive disorder characterized by skeletal dysplasia, adrenal dysfunction, disorders of sex development (DSD), and maternal virilization during pregnancy. Although multiple studies have been performed for this condition, several matters remain to be clarified, including the presence of manifesting heterozygosity and the underlying factors for clinical variability.

Objective: The objective of the study was to examine such unresolved matters by detailed molecular studies and genotype-phenotype correlations.

Patients: Thirty-five Japanese patients with POR deficiency participated in the study.

Results: Mutation analysis revealed homozygosity for R457H in cases 1–14 (group A), compound heterozygosity for R457H and one apparently null mutation in cases 15–28 (group B), and other combinations of mutations in cases 29–35 (group C). In particular, FISH and RT-PCR sequencing analyses revealed an intragenic microdeletion in one apparent R457H homozygote, transcription failure of apparently normal alleles in three R457H heterozygotes, and nonsense mediated mRNA decay in two frameshift mutation-positive cases examined. Genotype-phenotype correlations indicated that skeletal features were definitely more severe, and adrenal dysfunction, 46,XY DSD, and pubertal failure were somewhat more severe in group B than group A, whereas 46,XX DSD and maternal virilization during pregnancy were similar between two groups. Notable findings also included the contrast between infrequent occurrence of 46,XY DSD and invariable occurrence of 46,XX DSD and pubertal growth pattern in group A mimicking that of aromatase deficiency.

Conclusions: The results argue against the heterozygote manifestation and suggest that the residual POR activity reflected by the R457H dosage constitutes the underlying factor for clinical variability in some features but not other features, probably due to the simplicity and complexity of POR-dependent metabolic pathways relevant to each phenotype. **(*J Clin Endocrinol Metab* 94: 1723–1731, 2009)**

Cytochrome P450 oxidoreductase (POR) deficiency (PORD) is a rare autosomal recessive disorder caused by mutations in the gene encoding an electron donor for all microsomal P450 enzymes and several non-P450 enzymes (1–4). Salient clinical features of PORD include skeletal dysplasia referred to as Antley-Bixler syndrome (ABS), adrenal dysfunction, 46,XY and 46,XX disorders of sex development (DSD), and maternal virilization during pregnancy (3, 4). Such features are primarily ascribed to impaired activities of POR-dependent CYP51A1 (lanosterol 14α-demethylase) and SQLE

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Abbreviations: ABS, Antley-Bixler syndrome; CHX, cycloheximide; DSD, disorders of sex development; E_2, estradiol; FISH, fluorescent *in situ* hybridization; hCG, human chorionic gonadotropin; M, metabolite; NMD, nonsense-mediated mRNA decay; PCO, polycystic ovary; POR, cytochrome P450 oxidoreductase; PORD, POR deficiency; 17-OHP, 17α-hydroxyprogesterone; T, testosterone.

(squalene monooxigenase) involved in cholesterologenesis and CYP17A1 (17α-hydroxylase and 17,20 lyase), CYP21A2 (21-hydroxylase), and CYP19A1 (aromatase) involved in steroidogenesis (3, 4).

PORD has been identified in multiple patients (4). Mutations are diverse, including missense, nonsense, frameshift, and splice site mutations (4). Notably, however, A287P is the most common mutation in Caucasian patients, and R457H is the most prevalent founder mutation in Japanese patients (1–8). In addition, there is no patient with two apparently null mutations, suggesting that absence of a residual POR activity is incompatible with life (4–6). Clinical features are also variable, with a wide range of expressivity and penetrance. Indeed, ABS-compatible skeletal features and DSD are severely manifested by some patients and apparently absent in other patients (4–6). In addition, adrenal crisis remains relatively rare (4, 6), and maternal virilization is not a consistent feature (5, 6, 9).

To date, however, several critical matters remain to be clarified. First, although about 12% of patients have one apparently normal *POR* allele (4), it is uncertain whether such patients represent manifesting heterozygotes or have hidden aberrations in nonexamined region(s) (4, 10). Second, the underlying factors for the clinical diversity remain to be determined, although variable supporting activities of different POR mutants for target enzymes would have a certain role (5, 11, 12). Third, pubertal development and longitudinal growth have poorly been investigated.

To examine these matters, we analyzed the *POR* gene in affected patients and performed genotype-phenotype correlations in terms of the dosage effect of the R457H mutant.

Patients and Methods

Patients

This study consisted of 35 Japanese patients aged 0.1–23.8 yr (16 patients with 46,XY and 19 patients with 46,XX), including previously reported 23 cases (6, 8, 9) (Table 1). Of the 35 patients, 25 were sporadic cases and the remaining 10 were familial cases from families A–D. Twenty-three sporadic cases and four probands (cases 10, 15, 30, and 35) were ascertained by skeletal features and/or DSD, two sporadic cases (cases 1 and 5) by newborn mass screening for 21-hydroxylase deficiency, and the remaining six cases by familial studies.

Molecular analysis

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. The primers used in this study are shown in supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org. After taking written informed consent, peripheral blood samples were obtained from all the patients and the parents of 19 sporadic cases and two familial cases (families A and C). Subsequently, genomic DNA samples were subjected to direct sequencing for the *POR* exons 1–16, together with their flanking splice sites. To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and the two alleles were sequenced separately.

When lymphoblastoid cell lines were available, fluorescent *in situ* hybridization (FISH) analysis was performed with two long PCR products spanning exons 4–7 (probe 1) and exons 8–12 (probe 2). The two probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin. A spectrum green-labeled probe for *D7Z1* (CEP7) (Abbott, Abbott Park, IL) was used as an internal control. For a case with a probable microdeletion, RT-PCR was performed with a variety of primers, to determine the deletion size. Furthermore, to examine the occurrence of transcription failure in cases with apparent heterozygosity and that of the nonsense-mediated mRNA decay (NMD) in cases with premature truncation mutations, the lymphoblastoid cell lines available were incubated for 8 h with and without an NMD inhibitor cycloheximide (CHX; 100 μg/ml; Sigma, St. Louis, MO), and direct sequencing was performed for RT-PCR products (13, 14).

In addition to disease-causing mutations, we also examined the presence or absence of a common A503V variant that has been shown to have a mildly decreased supporting activity at least for CYP17A1 (~60%) (15), to investigate whether the A503V variant can function as a modifier of the clinical phenotype. To examine whether the A503V variant resides on the same allele carrying R457H, PCR products encompassing both the 457th and 503rd codons were subcloned and subjected to direct sequencing.

Clinical assessment

Skeletal features were assessed by bone survey. Adrenal function was evaluated by basal and ACTH-stimulated blood hormone values [250 $\mu g/m^2$ (maximum 250 μg) bolus iv; blood sampling at 0 and 60 min] and by urine steroid profiles determined by the gas chromatography/mass spectrometry using first morning urine samples in cases aged older than 6 months (16) (several urine steroid metabolites cannot be measured precisely during the first 6 months of age due to interference of unknown steroids derived from the fetal adrenocortex). DSD was clinically evaluated, as was pubertal development in boys aged older than 14.3 yr (mean +2 SD age for pubic stage 2) and in girls aged older than 12.8 yr (mean +2 SD age for breast stage 2) (17). When possible, basal blood pituitary-gonadal hormone values were also obtained as well as human chorionic gonadotropin (hCG)-stimulated testosterone (T) values (3000 IU/m^2 per dose im for 3 consecutive days; blood sampling on d 1 and 4). In addition, clinical records were surveyed for the data of 17-hydroxyprogesterone (17-OHP) values at the newborn mass screening, adrenal crisis, maternal virilization during pregnancy, polycystic ovary (PCO) in female cases, and body measurement.

Penile length, clitoral size, Tanner stage, testis size, age of menarche, and statural growth were assessed by age- and sex-matched Japanese reference data (17–20), as were hormone values (21–23). Because urine steroid metabolites (Ms) expressed in a logarithm scale grossly followed the normal distribution and showed marked change with age in control

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TABLE 1. Summary of molecular analyses

Patients				*POR* mutations	
Case	Karyotype	Age (yr)	Inheritance	Nucleotide changes[a]	Aminoacid changes
Group A: homozygotes for R457H					
1	46,XY	5.0	Sporadic	1370G>A/1370G>A	R457H/R457H
2	46,XY	23.8	Familial-A	1370G>A/1370G>A	R457H/R457H
3	46,XY	22.6	Familial-A	1370G>A/1370G>A	R457H/R457H
4	46,XY	6.7	Sporadic	1370G>A/1370G>A	R457H/R457H
5	46,XY	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
6	46,XX	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
7	46,XX	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
8	46,XX	2.0	Sporadic	1370G>A/1370G>A	R457H/R457H
9	46,XX	14.1	Sporadic	1370G>A/1370G>A	R457H/R457H
10	46,XX	15.0	Familial-A (P)	1370G>A/1370G>A	R457H/R457H
11	46,XX	3.0	Sporadic	1370G>A/1370G>A	R457H/R457H
12	46,XX	0.2	Sporadic	1370G>A/1370G>A	R457H/R457H
13	46,XX	0.1	Sporadic	1370G>A/1370G>A	R457H/R457H
14	46,XX	18.0	Sporadic	1370G>A/1370G>A	R457H/R457H
Group B: compound heterozygotes for R457H and an apparently null mutation					
15	46,XY	16.8	Familial-B (P)	1370G>A/601C>T	R457H/Q201X
16	46,XY	15.7	Familial-B	1370G>A/601C>T	R457H/Q201X
17	46,XY	14.8	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
18	46,XY	17.5	Sporadic	1370G>A/(15A>G)	R457H/Non-transcribed (G5G)[b]
19	46,XY	2.1	Sporadic	1370G>A/143delG	R457H/R48fsX63
20	46,XY	0.2	Sporadic	1370G>A/1665delG	R457H/Q555fsX612
21	46,XY	13.1	Sporadic	1370G>A/(−)[c]	R457H/DeltaExons 2–13[d]
22	46,XX	9.0	Sporadic	1370G>A/IVS7+1G>A	R457H/IVS7+1G>A
23	46,XX	14.8	Sporadic	1370G>A/1698-1699insC	R457H/Y567fsX574
24	46,XX	13.2	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
25	46,XX	12.9	Familial-B	1370G>A/601C>T	R457H/Q201X
26	46,XX	6.6	Sporadic	1370G>A/(−)[c]	R457H/Non-transcribed[b]
27	46,XX	4.2	Sporadic	1370G>A/(−)[c]	R457H/Non-transcribed[b]
28	46,XX	17.0	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
Group C: other compound heterozygotes					
29	46,XY	0.4	Sporadic	1370G>A/1386-1387insATCGCC	R457H/A462-S463insIA
30	46,XY	23.5	Familial-C (P)	1370G>A/1835-1858del[e]	R457H/L612-W620delinsR
31	46,XY	18.0	Familial-C	1370G>A/1835-1858del[e]	R457H/L612-W620delinsR
32	46,XY	17.9	Familial-D	1733A>G/1329-1330insC	Y578C/I444fsX449
33	46,XX	0.8	Sporadic	1370G>A/1738G>C	R457H/E580Q
34	46,XX	0.7	Sporadic	1370G>A/1042-1044delGTC	R457H/348delV
35	46,XX	0.5	Familial-D (P)	1733A>G/1329-1330insC	Y578C/I444fsX449

The genomic position corresponding to each mutation based on NC_000007.12 sequence at the National Center for Biotechnology Information database (Bethesda, MD) is as follows: R457H, 75452433G>A; Q201X, 75448386C>T; I444fsX449, 75452391-2insC; G5G, 75421261A>G; R48fsX63, 75421389delG; Q555fsX612, 75453099delG; IVS7 + 1G>A, 75448861G>A; Y567fsX574, 75453205-6insC; A462-S463insIA, 75452349-50insATCGCC; L612-W620delinsR, 75453432-55delTAAAGCAAGACCGAGAGCACCTGT; Y578C, 75453237A>G; E580Q, 75453245G>C; and 348delV, 75451086-88delGTC. Cases 1–3, 6–10, 15–18, 22–26, 29–33, and 35 have been reported previously (6, 8, 9), and the remaining 12 cases were first examined in this study. P, Proband.

[a] The A of the ATG encoding the initiator methionine residue of the predicted translation product is denoted position +1.

[b] The allele with G5G and the apparently normal alleles are not trasncribed into mRNA.

[c] The (−) symbol indicates the absence of a recognizable mutation on the exonic sequences.

[d] An intragenic microdeletion involving exons 2–13.

[e] 1835-1858delTAAAGCAAGACCGAGAGCACCTGT.

subjects of both sexes (854 males and 909 females), the M data of the patients were expressed as the SD score to allow for the comparison among patients of different sexes and ages.

Statistical analysis

Statistical significance of the frequency of clinical features was analyzed by the Fisher's exact probability test, and that of the median of nonpaired and paired variables was examined by the Mann-Whitney's *U* test and the Wilcoxon signed-rank test, respectively. $P < 0.05$ was considered significant.

Results

POR mutations

The results are summarized in Table 1. Direct sequencing revealed 12 types of mutations and one silent substitution (G5G) (Fig. 1A), with R457H being identified in 40 of the 58 alleles (~70%) in 25 sporadic cases and four probands of families A–D. Of the 12 mutations, R48fsX63, Q555fsX612, and 348delV were first identified in this study. These mutations were absent in 100 control subjects.

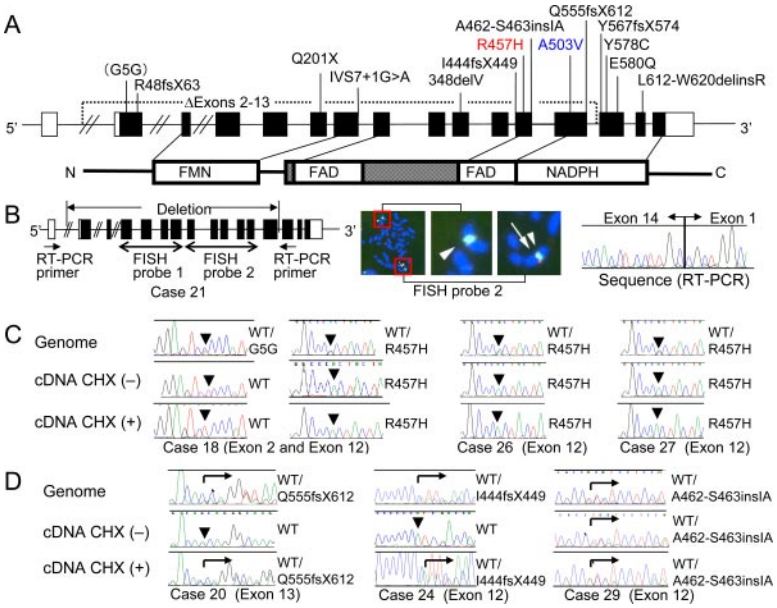

FIG. 1. Mutation analysis of *POR*. A, Schematic representation of the *POR* gene and the positions of identified mutations. The Japanese founder mutation R457H is shown in *red*, other disease-causing mutations in *black*, and the common A503V variant in *blue*. *Upper diagram*, The genomic structure comprising 16 exons. The *black* and *white boxes* denote the coding and the untranslated regions, respectively. *Lower diagram*, The protein structure consisting of the cofactor binding domains (FMN: flavin mononucleotide; FAD: flavin-adenine dinucleotide; and NADPH: nicotinamide-adenine dinucleotide phosphate, reduced) and the connecting domain (*stippled area*). B, FISH and RT-PCR sequencing analyses in case 21. *Left diagram*, The positions of the two FISH probes and those of the primers for RT-PCR. *Middle diagram*, FISH findings showing two signals for *DZ1* (*arrowheads*) and a single signal for *POR* (*arrow*) delineated by the FISH probe 2. *Right diagram*, RT-PCR sequencing indicating the fusion between exons 1 and 14 (the deletion of exons 2–13). C, Transcription failure in cases 18, 26, and 27. Although heterozygosity for R457H is delineated for the genomic DNA, RT-PCR sequencing indicates absent expression of the wild-type (WT) alleles in the three cases. Similarly, although heterozygosity for G5G is shown for the genomic DNA of case 18, RT-PCR sequencing reveals no expression of the G5G allele. Such lack of transcripts is not recovered by CHX. D, Nonsense-mediated mRNA decay in cases 20 and 24 but not case 29. Although heterozygosity for the mutations is shown for the genomic DNA, RT-PCR sequencing delineates the WT alleles only before CHX treatment and the heterozygosity after CHX treatment in cases 20 and 24. The NMD is not observed in case 29.

Fifteen cases were apparently homozygous for R457H, and hemizygosity was excluded in 14 of the 15 cases by parental analysis indicating heterozygosity for R457H in both parents (cases 1–3, 6–11, and 13) and by FISH analysis with two FISH probes (cases 4, 5, 12, and 14). Notably, however, FISH analysis delineated a heterozygous microdeletion in case 21, and RT-PCR sequencing analysis revealed loss of exons 2–13 in this case (Fig. 1B). The mother was heterozygous for R457H, and the father was heterozygous for the intragenic microdeletion.

Three cases were apparently heterozygous for R457H (cases 18, 26, and 27), although case 18 also had G5G. However, RT-PCR sequencing analysis using lymphoblastoid cell lines showed nearly complete absence of mRNA derived from the apparently normal alleles in the three cases (Fig. 1C). The mRNA remained undetected after CHX treatment, indicating transcription failure.

Of the 11 other types of mutations, the nonsense and four frameshift mutations (Q201X, R48fsX63, I444fsX449, Q555fsX612, and Y567fsX574) leading to premature termination and the conserved splice donor site mutation (IVS7+1G>A) appeared to be null mutations, whereas the remaining five mutations (Y578C, E580Q, 348delV, A462-S463insIA, and L612-W620delinsR) were unknown for residual activities. Indeed, RT-PCR sequencing analysis performed before and after CHX treatment in three cases with available lymphoblastoid cell lines demonstrated that the alleles carrying Q555fsX612 and I444fsX449 underwent NMD, whereas the allele harboring A462-S463insIA escaped NMD (Fig. 1D).

The common A503V variant was absent from cases of group A and was identified in four cases of group B (cases 22, 23, 26, and 27) and four cases of group C (cases 29–31, and 34). The eight cases with A503V were all compound heterozygotes with R457H and another mutation, and direct sequencing for subcloned PCR products encompassing both 457th and 503rd codons revealed lack of coexistence of R457H and A503V. Thus, it was indicated that the A503V variant was absent from all of the 47 alleles carrying R457H and was present on alleles carrying IVS7+1G>A, Y567fsX574, A462-S463insIA, L612-W620delinsR, and 348delV and on the two nontranscribed alleles.

Classification of the patients

On the basis of the above results, the 35 cases were classified into three groups: group A, homozygotes for R457H (cases 1–14); group B, compound heterozygotes for R457H and one apparently null mutation (cases 15–28); and group C, other types of compound heterozygotes (cases 29–35) (Table 1). The residual POR activity was predicted to be higher in group A than group B, although it was unknown for group C. In addition, group B was subclassified into A503V-positive cases (cases 22, 23, 26, and 27) and negative cases (cases 15–21, 24, 25, and 28).

Clinical features

The prevalence of each clinical feature in groups A–C is summarized in Table 2, together with its comparison between groups A and B. The sex ratio was similar between groups A and B, as was the median age.

ABS-compatible skeletal features were definitely more prevalent in group B than group A (Table 2 and supplementary Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). In particular, severe brachycephaly, elbow joint synostosis, and choanal stenosis were exclusively identified in group B.

Adrenal steroidogenic dysfunction was biochemically identified in all cases, with some difference between groups A and B. Blood ACTH was normal or elevated at the baseline, 17-OHP was normal or elevated at the baseline and above the normal range after ACTH stimulation, and cortisol was normal at the baseline but barely responded to ACTH stimulation (Fig. 2A). Significant difference between groups A and B was identified for basal 17-OHP value ($P = 0.044$) and basal and ACTH-stimulated cortisol values ($P = 0.018$ and $P = 0.022$). Urine Ms of progesterone and 17-OHP were elevated, whereas those of an-

TABLE 2. The prevalence of each clinical feature in groups A–C and its comparison between groups A and B

	Group A (n = 14)	Group B (n = 14)	Group C (n = 7)	Groups A *vs.* B (*P* value)
Sex (male:female)	5:9	7:7	4:3	0.35
Age (median, range, yr)	4.0 (0.1–23.8)	13.1 (0.2–17.5)	0.8 (0.4–23.5)	0.19
Skeletal features				
Any skeletal feature	7/14	14/14	7/7	0.0029
Brachycephaly (overt)	0/14	14/14	6/7[a]	0.000000025
Elbow joint synostosis[b]	0/14	7/14	4/7	0.0029
Arachnodactyly (overt)	5/14	14/14	7/7	0.048
Choanal stenosis	0/14	5/14	1/7	0.020
Joint contracture	7/14	14/14	7/7	0.0029
Adrenal dysfunction				
Adrenal crisis	0/14	4/14	1/7[c]	0.049
Detection by mass screening[d]	5/8	3/8	2/4	0.31
46,XY DSD				
Any genital feature at birth	1/5[e]	3/7[f]	3/4	0.42
Hypospadias	0/5	2/7	1/4	0.32
Cryptorchidism	0/5	3/7	2/4	0.16
Micropenis	1/5	2/7	3/4	0.64
46,XX DSD				
Any genital feature at birth	9/9[e]	7/7[f]	3/3	1.0
Clitoromegaly	8/9	5/7	3/3	0.40
Labial fusion	8/9	5/7	2/3	0.40
Common urogenital sinus	2/9	2/7	0/3	0.61
Maternal virilization	8/14	5/14	4/7	0.22
Pubertal failure, 46,XY				
Delayed (>2 SD) or no pubertal sign	0/2[g]	3/4[h]	2/3	0.20
Small testis (<2 SD)	0/2	2/4	1/3	0.40
Primary hypogonadism[i]	0/2	2/2	3/3	0.17
Pubertal failure, 46,XX				
Delayed (>2 SD) or no pubertal sign	3/3[g]	4/4[h]		1.0
Delayed (>2 SD) or no menses	0/2[j]	2/2		0.17
Primary hypogonadism[i]	3/3	3/3		1.0
Polycystic ovary	4/9	3/6	1/3	0.62

The denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature; thus, the differences between the denominators and numerators denote the number of patients evaluated to be negative for that feature.

[a] Severe craniosynostosis is absent in case 33 with two missense mutations.

[b] Humeroradial, humeroulnar, or radioulnar synostosis.

[c] Adrenal crisis has been manifested by case 35 with Y578C and I444fsX449.

[d] The measurement of 17-OHP in the mass screening for 21-hydroxylase deficiency has been performed since 1988 in Japan.

[e,f] DSD is more frequent in 46,XX cases than 46,XY cases in groups A ($P = 0.0050$) and B ($P = 0.035$).

[g,h] The *P* values between 46,XY and 46,XX cases are 0.19 for group A and 0.50 for group B.

[i] Elevated gonadotropins (LH and/or FSH) and/or decreased T or E_2, as compared with age- and sex-matched reference data.

[j] Only a few vaginal spottings.

drostenedione, 11-deoxycortisol, cortisol, and aldosterone grossly remained within the normal range (Fig. 2B). The M ratio indicating 17α-hydroxylase activity remained almost normal, consistent with the elevation of both substrates and products, whereas the M ratios indicating 17,20 lyase and 21-hydroxylase activities were grossly decreased. Significant difference between groups A and B was identified for Ms of progesterone ($P = 0.044$), those of 17-OHP ($P = 0.022$), those of aldosterone ($P = 0.0084$), and M ratio indicating 17,20 lyase activity ($P = 0.011$). Adrenal crisis was observed only in group B with a significant difference between groups A and B, whereas the detection frequency of elevated 17-OHP in mass screening was similar between groups A and B (Table 2).

DSD was more prevalent in 46,XX cases than 46,XY cases in both groups A and B (Table 2, footnote, and supplementary Fig. 2). 46,XY DSD in group A was micropenis in one case, and that in group B included more severe phenotypes. By contrast, 46,XX DSD was invariably identified in both groups A and B. Maternal virilization during pregnancy was often found in groups A and B with a similar prevalence. Serum T of case 20, aged 0.2 yr in group B, was 6.5 and 7.6 nmol/liter (1.9 and 2.2 ng/ml) before and after hCG stimulation, respectively.

Pubertal development was apparently normal in two 46,XY cases of group A and one of four 46,XY cases in group B and was invariably affected in 46,XX cases in both groups A and B (Table 2). In family A of group A, cases 2 and 3 exhibited full pubertal development with testis volume of 20 ml, whereas case 10 had obvious pubertal failure with Tanner B2 stage. T value of case 18, aged 17.5 yr in group B, was low at the baseline (0.7 nmol/liter,

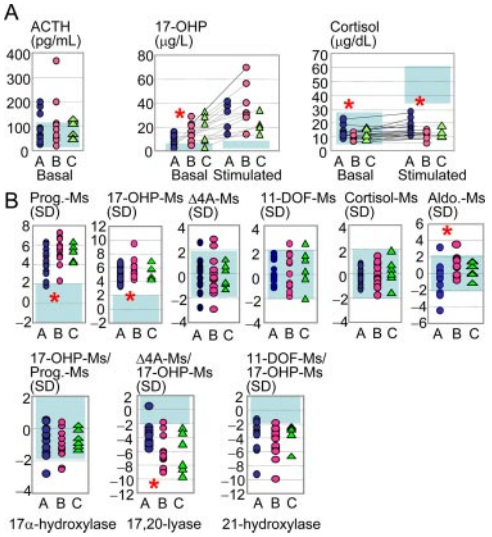

FIG. 2. Adrenal steroidogenic dysfunctions in groups A–C. *Light blue areas* represent the normal ranges. *Red asterisks* indicate the presence of significant differences between groups A and B. A, Basal and ACTH-stimulated blood hormone values. B, Basal urine steroid M values. Prog, Progesterone; Δ4A, androstenedione; 11DOF, 11-deoxycortisol; Aldo, aldosterone.

0.2 ng/ml) and poorly responded to hCG stimulation (1.0 nmol/liter, 0.3 ng/ml). PCO was observed in infantile or pubertal cases with a similar frequency between groups A and B, and cases 22 and 24 had ovarian torsion. Notably, bilateral ovarian cysts of case 10 markedly reduced in size after treatment with estradiol (E_2) (supplementary Fig. 3).

Long-term growth patterns were obtained in eight cases (Fig. 3). Whereas childhood heights tended to be high in both groups A and B, pubertal growth was different between the two groups. Cases in group A lacked obvious pubertal growth spurt but continued to grow for a long term, attaining tall adult heights, whereas those in group B showed rather compromised pubertal growth with worsening of scoliosis (supplementary Fig. 1).

There was no phenotypic difference between A503V-positive and -negative cases of group B (supplementary Table 2). In addition, the phenotypes in group C were grossly similar to those in group B (Table 2). In particular, craniosynostosis was identified in all cases except for case 33 with R457H and E580Q, and adrenal crisis was manifested by case 35 with Y578C and I444fsX449.

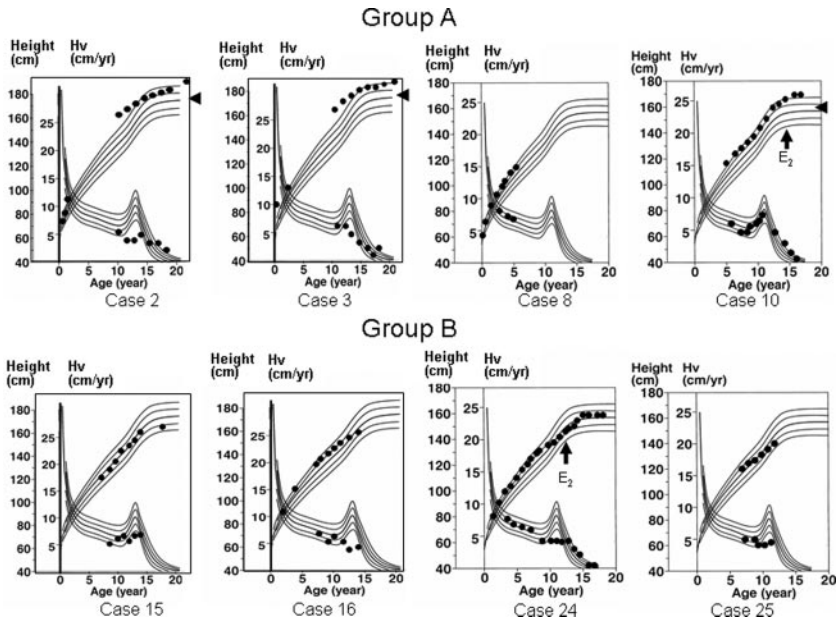

FIG. 3. Growth charts of eight cases plotted on the sex-matched longitudinal growth curves for the normal Japanese children (+2 SD, +1 SD, the mean, −1 SD, and −2 SD). The *triangles* in cases 2, 3, and 10 represent the target heights. Cases 10 and 24 are placed on E_2 replacement therapy. Hv, Height velocity.

Discussion

Molecular studies

Detailed molecular studies were performed in this study, providing two notable findings. First, all 35 cases were found to be homozygotes or compound heterozygotes for *POR* mutations including intragenic microdeletion and transcription failure. Because the microdeletion was found in case 21 with apparent R457H homozygosity, such a microdeletion might be hidden in the previously reported patients with apparent homozygosity (1, 5). Similarly, because transcription failure was invariably identified in cases 18, 26, and 27 with apparent heterozygosity, it may also underlie in the previously reported patients with apparent heterozygosity (4, 5, 10). In this regard, it is likely that the three cases carry a mutation in a hitherto unidentified *cis*-regulatory sequence(s) for the transcription of *POR*, as has been reported for several genes (24).

Second, RT-PCR sequence analysis indicated the occurrence of NMD in the two frameshift mutations (I444fsX449 and Q555fsX612). In this context, all the premature termination codons caused by the nonsense and the four frameshift mutations satisfy the positional conditions for the occurrence of NMD that functions as an mRNA surveillance mechanism to prevent the formation of aberrant proteins (13, 14). Thus, it is likely that the remaining three mutations (Q201X, R48fsX63, and Y567fsX574) are also null mutations subject to NMD *in vivo*.

Genotype-phenotype correlations

Genotype-phenotype correlations also provide several informative findings. Skeletal features were clearly different between groups A and B. Because cholesterol production in skeletal tissues is carried out in a simple one way manner (Fig. 4), this would explain why the skeletal phenotype is obviously dependent on the R457H dosage, reflecting the residual activity. It is likely that the threshold level for the development of severe skeletal phenotypes resides between a single copy and two copies of the R457H residual activity.

Adrenal steroidogenic dysfunction was grossly similar between groups A and B, although it was somewhat milder in group A than group B. Such a relatively minor role of R457H dosage in adrenal steroidogenesis

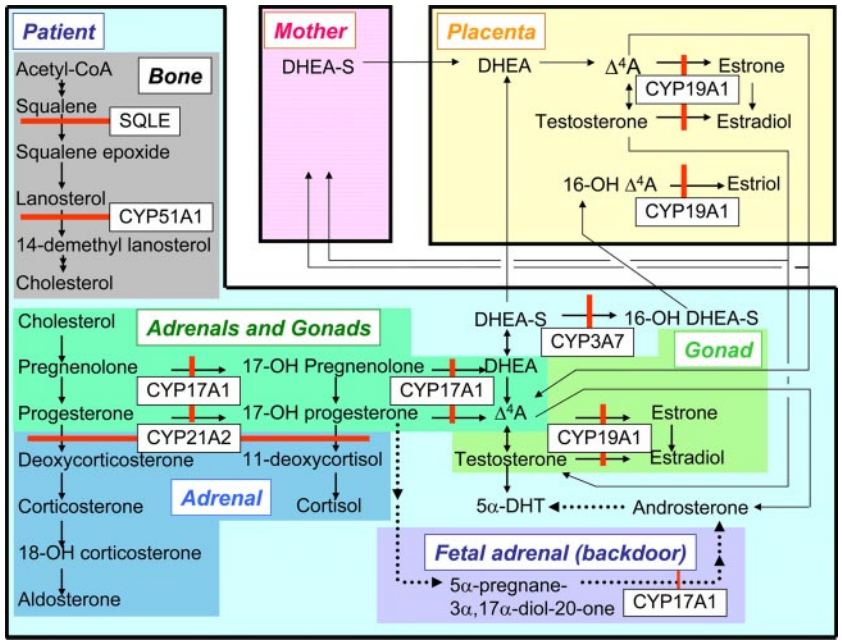

FIG. 4. Simplified schematic representation indicating impaired cholesterologenesis and steroidogenesis in PORD. DHEA, Dehydroepiandrosterone; DHEA-S, DHEA sulfate; Δ^4A, androstenedione; DHT, dihydrotestosterone. SQLE, CYP51A1, CYP17A1, CYP21A2, CYP19A1, and CYP3A7 are POR-dependent enzymes. The important Ms only are shown, and the reaction steps in which some Ms are omitted are indicated by two tandem *arrows*. Note that the amount of estriol synthesized in the placenta far exceeds the total amount of estrone and E_2 (~10 times) (34).

may primarily be due to the complexity of steroidogenesis in PORD (Fig. 4). For example, both production and degradation of 17-OHP are carried out by POR-dependent enzymes, and such enzymatic reactions would depend on the R457H dosage and the differential supporting activity of the R457H protein for target enzymes as well as the amount of substrates and products. Furthermore, the basal cortisol values imply that the baseline steroidogenic capacity can grossly be sustained, even in group B. Indeed, whereas basal blood 17-OHP values were significantly higher in group B than group A, some of them remained within the normal range, and several cases of both groups were not detected in neonatal mass screening. Nevertheless, the R457H dosage would have important clinical relevance, because the ACTH-stimulated blood cortisol was drastically reduced especially in group B, and adrenal crisis was observed only in group B. Furthermore, because 17,20 lyase activity alone was significantly different between groups A and B (Fig. 2B), this would provide further support for the previous finding that 17,20 lyase activity is the most sensitive index of defective POR activity (5, 15).

46,XY DSD was not so remarkable, whereas 46,XX DSD was invariably identified. This suggests a mildly reduced androgen production in genetic males and a definitely excessive androgen production in genetic females. In this context, there are three androgen sources during the fetal life in PORD, *i.e.* the fetal testis, backdoor pathway, and placenta (3, 4, 9, 25, 26) (Fig. 4). For fetal testicular T production specific to 46,XY cases, placental hCG-stimulated T production around the critical period for sex development would be more compromised in group B than group A because testicular T production is performed in a simple one-way manner, as in cholesterologenesis. Furthermore, because T responses to hCG stimulation were reduced, at least in the two examined cases of group B, this implies the compromised maximum T production capacity. By contrast, the backdoor- and placenta-derived androgen productions common to both 46,XY and 46,XX cases may be similar between groups A and B: 1) whereas 17-OHP as the source metabolite for the backdoor pathway is higher in group B than group A, the supporting activity for fetal adrenal CYP17A1 involved in the backdoor pathway would be lower in group B than group A; and 2) whereas fetal adrenal derived dehydroepiandrosterone as the source metabolite for placental androgens would be lower in group B than group A (4, 9, 25), the residual supporting activity for placental CYP19A1 would be lower in group B than group A. Thus, the total amount of androgens would be relatively well preserved in 46,XY cases with a mild difference in the fetal testis-derived T between groups A and B and invariably and similarly increased in 46,XX cases of both groups A and B. Furthermore, this notion explains why maternal virilization during pregnancy was similar between groups A and B because it is primarily due to androgens of the placental origin rather than the fetal gonadal or the backdoor origin (3, 4, 25).

Assessment of pubertal development was possible in a limited number of patients. However, pubertal development appeared to differ between groups A and B and between 46,XY and 46,XX cases. In this regard, T and E_2 biosynthesis during puberty is also performed in a simple one-way manner, and T production is mediated by CYP17A1 and E_2 production is mediated by both CYP17A1 and CYP19A1 (Fig. 4). Thus, gonadal steroid production would depend on the R457H dosage, with T production being less compromised than E_2 production. In addition, our observation suggests the frequent occurrence of PCO in infancy and puberty when gonadotropins are physiologically elevated (27) and the beneficial effect of estrogen replacement therapy in the amelioration of PCO.

Evaluation of growth pattern also remained fragmentary. However, two implications are possible. First, the intrinsic skeletal abnormalities may be relevant to the growth pattern. Indeed, relative tall stature in childhood may be compatible with the elongation of long bones as indicated by arachnodactyly and dolichostenomelia, and worsening of scoliosis during puberty in group B would also be consistent with the low POR activity (supplementary Fig. 1). Second, the spontaneous pubertal growth pattern of cases 2 and 3 without scoliosis is considered to represent a mild form of that of male patients with aromatase deficiency (28, 29). Such a qualitatively similar but quantitatively different pubertal growth pattern would be explained by assuming a drastically attenuated but not abolished *in vivo* supporting function of the R457H protein for aromatase.

Lastly, clinical features were similar between A503V-positive and -negative cases in group B. However, this would not argue

against a possible phenotypic effect of mildly hypomorphic A503V, because A503V of the four cases in group B was present on the alleles carrying apparently null mutations. Thus, it remains unknown whether A503V can modify phenotypic features in PORD, although the previous study argues against a modifying effect of A503V on clinical phenotypes in 21-hydroxylase deficiency (30). Furthermore, because A503V was absent from all of 47 alleles carrying R457H, this would provide further support for the previous notion that R457H is a founder mutation accompanied by a specific haplotype (6, 7). Thus, whereas A503V was identified in only eight of the 70 alleles (11.4%) in this study, this frequency is obviously biased by the high prevalence of R457H in Japanese patients. Rather, the frequency of A503V in R457H-negative alleles suggests that the prevalence of A503V is considerably high in the Japanese population, as reported in other populations (from 19.1% in African American to 36.7% in Chinese American) (15).

Remarks and conclusion

It should be pointed out that the results are totally based on the studies of Japanese patients. In this regard, A287P is common in Caucasian patients (4, 5), and clinical studies in 10 A287P-positive patients including three homozygotes (five with 46,XY and five with 46,XX) have suggested phenotypic similarities and differences between R457H-positive patients and A287P-positive patients: 1) skeletal phenotype is usually obvious and appears to be grossly dependent on the A287P dosage; 2) 46,XY DSD is variable and is apparently independent of the A287P dosage; 3) 46,XX DSD is also variable and absent in one A287P homozygote and one of four compound heterozygotes with A287P; and 4) maternal virilization during pregnancy is not described (1, 2, 5, 31, 32). Thus, skeletal phenotype would be explained by assuming that both R457H and A287P have drastically lost supporting activities for CYP51A1 and/or SQLE involved in cholesterologenesis, although functional studies have not been performed. Furthermore, clinical features relevant to steroidogenic dysfunction would be grossly consistent with the previous *in vitro* functional data. It has been reported that R457H yields only 1–3% supporting activities for 17α-hydroxylase and aromatase, and virtually no activity for 17,20 lyase, whereas A287P provides supporting activities of about 40% for 17α-hydroxylase, about 20% for 17,20 lyase, about 70% for 21-hydroxylase, and about 100% for aromatase (1, 5, 11, 33). Thus, the relative activities of frontdoor and backdoor pathways would be different largely between R457H-positive and A287P-positive patients, and placental T production would remain minor, if any, in A287P-positive patients. Collectively, the Japanese data would not apply simply to other populations.

In conclusion, the present study in Japanese patients argues against the heterozygote manifestation and suggests that the residual POR activity reflected by the R457H dosage constitutes the underlying factor for the clinical variability in some features but not other features, probably because of the simplicity and the complexity of the POR-dependent metabolic pathways relevant to each phenotype. Further studies including genotype-phenotype analyses in various ethnic groups will permit a better clarification of the molecular and clinical characteristics of PORD.

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