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Identification of novel and rare *CYP21A2* variants in Chinese patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Jing Xu, Pin Li*

Department of Endocrinology, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200062, China

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ABSTRACT

Objective: 21-hydroxylase deficiency (21-OHD) is the most common cause of congenital adrenal hyperplasia due to *CYP21A2* gene mutation. The aim of study is to expand *CYP21A2* mutational spectrum in the Chinese population and to provide novel genetic information in terms of ethnic diversity.

Design and methods: 95 Chinese suspected 21-OHD patients with phenotypes varying from salt-wasting (SW) to nonclassic symptoms were recruited. The clinical characteristics were retrospectively analyzed. Sanger sequencing and multiplex ligation-dependent probe amplification were used to detect point mutations and large gene deletions, respectively.

Results: 20 different mutant alleles were detected in 35 patients with 21-OHD. The most common variant was c.293-13A/C > G (30.0%), followed by p.I173N (20.0%), large gene conversions (14.3%), large gene deletions (11.4%), and p.R484Pfs*58 (4.3%). Remarkably, we identified a novel F450L variant, *in silico* predicted to be associated with the salt-wasting form. Two variants including p.R409C and p.R427H, previously considered as conserved in specific ethnicities due to a founder effect, were detected in our cohort. Further, a rare p.H63L + p.V70L variant, hitherto only observed in the Chinese population, *in trans* with different variants corresponding to the salt-wasting form resulted in diverse phenotypes.

Conclusions: One novel and four rare variants of *CYP21A2* gene corresponding to severe phenotypes were identified in our cohort. Two variants including p.R409C and p.R427H have wider ethnic distributions. Therefore, the sequence of *CYP21A2* gene must be analyzed carefully in case rare or novel deleterious variants exist. Our findings improve the understanding of *CYP21A2* mutational spectrum in 21-OHD patients and contribute to the precise diagnosis and prenatal counseling.

1. Introduction

Congenital adrenal hyperplasia (CAH, OMIM#201910), first described in 1865 [1,2], is a group of autosomal recessive diseases resulting from variants in genes encoding enzymes responsible for adrenal steroidogenesis. More than 95% of CAH cases are caused by 21-hydroxylase deficiency (21-OHD) due to the *CYP21A2* gene mutation [3,4], characterized by inadequate cortisol and aldosterone synthesis and androgen excess. Depending on the clinical severity, 21-OHD is classified into three subtypes: classic salt-wasting (SW), classic simple virilizing (SV), and nonclassic (NC). The classic types affect 1 in 15,000 live births [5–7]. The prevalence of nonclassic type varies widely between different ethnicities as 3.7% in Ashkenazi Jews, 1.9% in Hispanics, 1.6% in Yugoslavs, 0.3% in Italians, and 0.1% in other Caucasians [8].

The SV form is characterized by virilization of the external genitalia in newborn girls and by precocious pseudopuberty in both sexes. The SW form presents with a severe life-threatening adrenal crisis (vomiting, diarrhea, dehydration, hyponatremia, and hyperkalemia) in the neonatal period in addition to the features of the SV form. Approximately 70% of classic CAH patients have the SW form [9]. NCCAH, a mild form, retains up to 50% of normal enzyme activity and has normal external genitalia at birth. NC patients may manifest at any age with signs of androgen excess, such as premature pubarche, hirsutism, primary amenorrhea, acne, and subfertility, or may be asymptomatic [10].

The gene for 21-hydroxylase, *CYP21A2*, is located on chromosome 6p21.3, about 30 kb away from its highly homologous pseudogene *CYP21A1P*. Approximately, 70% of *CYP21A2* disease-causing variants are *CYP21A1P*-derived variants due to gene conversions, the transfer of inactive pseudogene into the active *CYP21A2* gene [11]. 25%–30% are due to large gene deletions [12,13]. Although the genotype generally

* Corresponding author.
E-mail address: lipin21@126.com (P. Li).

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correlated well with the phenotype, divergence in phenotypes has been observed in studies worldwide. Certain variants can cause different CAH phenotypes [14]. In addition, due to improvements in technology, an increasing number of rare variants have been identified that may contribute to the development of 21-OHD. All these above bring difficulties in the diagnosis and phenotype prediction.

In the present study, we reported the findings of molecular genetic analysis in 35 patients with 21-OHD in order to provide more information for a precise diagnosis. Among them, one novel p.F450L variant was identified along with several rare variants.

2. Materials and methods

2.1. Subjects

A total of 95 unrelated Chinese patients with suspected 21-OHD were recruited from our endocrinology clinic between October 2013 to June 2018. Among them, 35 patients (10 males and 25 females) were diagnosed according to clinical manifestations and basal 17-hydroxyprogesterone (17-OHP) levels above 30 nmol/L [4] and were also confirmed by the molecular genetic analysis. Three patients were found to carry one severe variant (R357W or I2G) with a basal 17-OHP level above 303 nmol/L. In addition, three patients with a basal 17-OHP level below 30 nmol/L were found to carry a heterozygous variant (I2G, promoter mutations, or V238G). No variant was detected in other 54 patients with variable 21-OHD phenotypes. More experiments such as whole-exome sequencing are needed to find out the cause of their phenotypes especially for three patients with a basal 17-OHP level over 303 nmol/L. Those patients without a molecular diagnosis were excluded for further analysis.

Both parents of each proband were also sequenced to confirm the allelic configuration of variants and to determine which variants were *de novo*. All the patients were ethnic Chinese and none of their parents were consanguineous. Written informed consent for genetic testing was obtained from all participants and the study was approved by the Ethics Committee of Shanghai Children's Hospital.

2.2. Direct sequencing of CYP21A2 gene

Genomic DNA was extracted from the peripheral blood using a QIAamp DNA Blood Mini Kit (Qiagen, Germany). The *CYP21A2* gene was specifically amplified in two overlapping fragments with primers 1 and 2, listed in Table 1. Each 50 μL PCR mixture contained 5 μL 10× PCR buffer, 4 μL of 2.5 mM dNTP mixture, 2 μL of each primer (10 μM), 1.25 U *TaKaRa Taq* HS DNA polymerase (Takara, Japan), and 30 ng DNA template. The PCR amplification condition was as follows: a denaturation step at 95 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 30s, annealing at 62 °C for 45 s, and extension at 72 °C for 4 min, with a final extension step at 72 °C for 10 min. The PCR products were purified and then sequenced using standard methods. *CYP21A2* variants were named according to Human Gene Nomenclature Guidelines (http://varnomen.hgvs.org/, accession number: NM_000500.7).

2.3. MLPA analysis

For suspected CAH patients in whom biallelic variants of *CYP21A2* were not detected in Sanger sequencing, MLPA was performed according to the manufacturer's protocol using a commercial SALSA MLPA probemix P050-C1 CAH kit (MRC Holland, Netherlands). The amplification products were detected using an ABI 3500DX Genetic Analyzer (Applied Biosystems, Japan). The raw data were analyzed using Coffalyser software (MRC Holland, Netherlands).

Table 1
Primers of *CYP21A2* gene.

No.	Sequence (5′-3′)	Exon	Fragment size (bp)
1	F: GTGGAACCAGAAAGCTGACTCTGG R: GCATCTCCACGATGTGA	1–5	1673
2	F: CCTGTCCTTGGGAGACTACT R: TCTCGCACCCCAGTATGACT	4–10	2212

2.4. Classification of patients based on genotype

The patients with 21-OHD were classified into five groups: Null, A, B, C, and D according to the method described previously [11,15] with some modifications. Since CAH is an autosomal recessive disorder, the phenotype in compound heterozygotes was determined by the mildest variant retaining the most enzyme activity.

Group Null included patients with biallelic variants resulting in a complete loss of enzyme activity (gene deletion, 8-bp deletion in exon 3, E6 cluster, p.Q319*, p.R357W, p. L308Ffs*6, p.R409C, p.R427H, and p.R484Pfs*58).

Group A included patients with homozygous c.293-13A/C > G (I2G) variants or heterozygous I2G variant *in trans* with one variant of group Null.

Group B included patients with homozygous p.I173N variants or heterozygous p.I173N variant *in trans* with one variant of group Null or group A.

Group C included patients with homozygous mild variants (*e.g.* p.V282L and p.P31L) or heterozygous mild variant *in trans* with one variant of groups Null, A, or B.

Group D included patients carrying rare variants with unknown *in vitro* effects on enzyme activity.

All the female patients with different degrees of external genital virilization were also graded according to the Prader score [16].

2.5. In silico analysis

The effect of the novel variant was predicted using three tools: Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT, and Provean (http://provean.jcvi.org/index.php). Multiple sequence alignments were performed by DNAMAN version 6.0 (Lynnon Biosoft, USA) to analyze the conservation of amino acid residues across different species. PyMOL version 2.2 (http://pymol.org/2/) was used to analyze the possible effect of the corresponding amino acid substitution on the structure of 21-hydroxylase, on the basis of a three-dimensional model of human CYP21A2–17-OH-progesterone complex (PDB ID code: 5VBU) [17].

3. Results

3.1. Clinical findings of patients with 21-OHD

Among the 35 patients, thirteen (4 males) presented the SW form. Nine female patients with a diagnosis age ranging from 12 days to 1.2 years exhibited both the signs of adrenal crisis and atypical external genitalia. Three male patients presented with salt-wasting in their neonatal period. One male presented with signs of adrenal crisis and precocious puberty at the age of 5.7 years. However, only one patient (Patient 11) was diagnosed from neonatal screening. Fifteen females with a diagnosis age ranging from 1 to 14.8 years displayed the SV form. Six males presented precocious puberty aged from 0.6 to 10 years. As for females, a total of nine patients showed ambiguous external genitalia at birth, wherein three of them (2 SW and 1 SV) were reared as males. In addition, one female patient (Patient 35) was diagnosed as the nonclassic form due to the absence of menses without secondary sexual characteristics at the age of 15.8 years. Detailed information on the genotype and phenotype is provided in Table 2.

Table 2
Phenotype and genotype of 35 patients with 21-OHD.

Patient	Karyotype	Sex of rearing	Age	Allele 1	Allele 2	Phenotype	Group	Expected phenotype
1	46, XX	M	2 m	R357W	R484Pfs*58	SW	NULL	SW
2	46, XY	M	5y8m	Del	R484Pfs*58	SW	NULL	SW
3	46, XY	M	2 m	Del	I2G	SW	A	SW/SV
4	46, XY	M	1 m	Del	I173N	SW	B	SV
5	46, XX	F	1y2m	Q319*	I173N	SW	B	SV
6	46, XX	F	5 m	Del	I2G	SW	A	SW/SV
7	46, XY	M	23 d	8 bp deletion	I2G	SW	A	SW/SV
8	46, XX	F	2 m	R484Pfs*58	H63L;V70L	SW	D	NA
9	46, XX	M	14 d	I2G	I2G	SW	A	SW/SV
10	46, XX	F	12 d	Del	I2G	SW	A	SW/SV
11	46, XX	F	24 d	R427H	I173N	SW	B	SV
12	46, XX	F	14 d	I173N	F450L	SW	D	NA
13	46, XX	F	2 m	P31L;I2G;8 bp deletion	P31L;I2G;8 bp deletion	SW	NULL	SW
14	46, XY	M	1 y	I2G	I2G	SV	A	SW/SV
15	46, XX	M	3y8m	I2G	F405L	SV	D	NA
16	46, XY	M	10y1m	Del	R357W	SV	NULL	SW
17	46, XX	F	6 y	Del	I173N	SV	B	SV
18	46, XX	F	12 y	I2G	c. − 126C > T;c. − 113G > A;c. − 110T > C; c. − 103A > G;P31L	SV	B	SV
19	46, XX	F	5 y	I2G	I2G	SV	A	SW/SV
20	46, XY	M	7 m	I2G	I173N	SV	B	SV
21	46, XX	F	1y1m	I173N	R409C	SV	B	SV
22	46, XX	F	6y6m	L308Ffs*6	I2G	SV	A	SW/SV
23	46, XX	F	3y10m	Del	I173N	SV	B	SV
24	46, XY	M	4 y	L308Ffs*6	I2G	SV	A	SW/SV
25	46, XX	F	5y6m	I2G	I173N	SV	B	SV
26	46, XX	F	5y2m	I2G	I173N	SV	B	SV
27	46, XX	F	11y3m	I2G	I173N	SV	B	SV
28	46, XY	M	4y6m	I2G	I173N	SV	B	SV
29	46, XY	M	4 y	I2G	I173N	SV	B	SV
30	46, XX	F	1y9m	Q319*	8 bp deletion	SV	NULL	SW
31	46, XX	F	14y10m	c. − 126C > T;c. − 113G > A;c. − 110T > C; c. − 103A > G;H63L;8 bp deletion	c. − 126C > T;c. − 113G > A;c. − 110T > C	SV	C	NC
32	46, XX	F	4 y	I2G	c. − 126C > T;c. − 113G > A;c. − 110T > C; c. − 103A > G;P31L	SV	B	SV
33	46, XX	F	1 y	c. − 126C > T;c. − 113G > A;c. − 110T > C; c. − 103A > G;P31L;I2G;8 bp deletion	I173N	SV	B	SV
34	46, XX	F	11 y	c. − 126C > T;c. − 113G > A;c. − 110T > C; c. − 103A > G;H63L;I2G;8 bp deletion	E6 cluster	SV	NULL	SW
35	46, XX	F	15y9m	R357W	H63L;V70L	NC	D	NA

Del, large gene deletions; NA, not applicable.

3.2. Mutation spectrum of CYP21A2 gene in patients with 21-OHD

20 different mutant alleles were identified. Fig. 1 shows the distribution of point mutations identified in our work. These variants were nearly scattered throughout the entire coding region except for exon 5. And the rare variants were mainly distributed at both ends of the enzyme.

The most common variant was I2G (30.0%), followed by I173N (20.0%), large gene conversions (14.3%), large gene deletions (11.4%), R357W (4.3%), R484Pfs*58 (4.3%), and 8-bp deletion on exon 3 (2.9%). Five types of rare point mutations located on exons 9–10 accounted for 10% of alleles including four missense and one insertion-deletion variants (Table 3). Genetic findings in the parents were consistent with an autosomal recessive pattern of inheritance. No *de novo* variants were found. One novel variant, c.1348T > C (p.F450L), was detected in one patient (Fig. 2A).

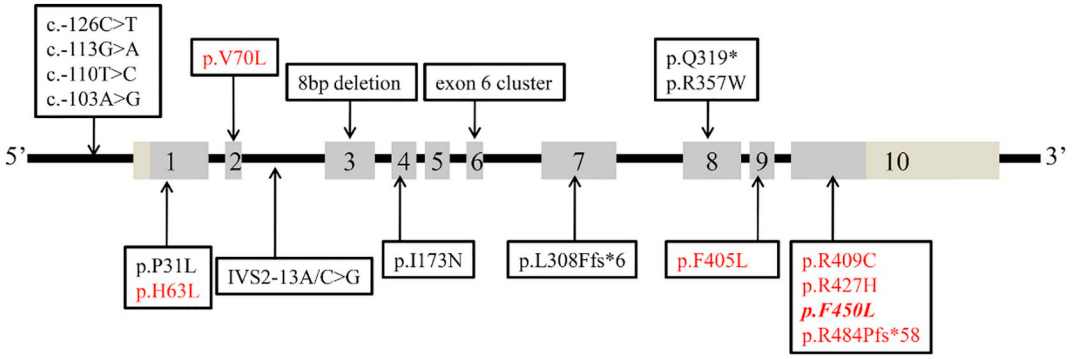

Fig. 1. Point mutations of the *CYP21A2* gene identified in the present study. Gene conversions and rare variants are respectively colored in black and red. The novel variant is highlighted in bold and italic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Allelic frequency of *CYP21A2* variants in 21-OHD patients.

DNA level	Protein level	Location on *CYP21A2*	Mutation type	Mutated allele N	Mutated allele %
Micro-conversion*					
c.293-13A/C > G	I2G	Intron 2	Splice	21	30.0
c.332_339delGAGACTAC	p.G111Vfs*21	Exon 3	Deletion	2	2.9
c.518T > A	p.I173N	Exon 4	Missense	14	20.0
c.[710T > A;713T > A;719T > A]	E6 cluster	Exon 6	Missense	1	1.4
c.923dupT	p.L308Ffs*6	Exon 7	Insertion	2	2.9
c.955C > T	p.Q319*	Exon 8	Nonsense	2	2.9
c.1069C > T	p.R357W	Exon 8	Missense	3	4.3
Rare variant					
c.1213T > C	p.F405L	Exon 9	Missense	1	1.4
c.1225C > T	p.R409C	Exon 10	Missense	1	1.4
c.1280G > A	p.R427H	Exon 10	Missense	1	1.4
c.1348T > C	p.F450L	Exon 10	Missense	1	1.4
c.1451_1452delGGinsC	p.R484Pfs*58	Exon 10	InDel	3	4.3
Large gene deletion				8	11.4
Large gene conversion*					
c.[−126C > T;−113G > A;−110T > C]		5'UTR		1	1.4
c.[−126C > T;−113G > A;−110T > C;−103A > G;92C > T]	p.P31L	5'UTR-Exon1		2	2.9
c.[−126C > T;−113G > A;−110T > C;−103A > G;92C > T;293−13A/C > G; 332_339delGAGACTAC]	p.[P31L;I2G;8bp deletion]	5'UTR-Exon3		1	1.4
c.[−126C > T;−113G > A;−110T > C;−103A > G;188A > T; 332_339delGAGACTAC]	p.[H63L;8bp deletion]	5'UTR-Exon1,3		1	1.4
c.[−126C > T;−113G > A;−110T > C;−103A > G;188A > T;293−13A/C > G; 332_339delGAGACTAC]	p.[H63L;I2G;8bp deletion]	5'UTR-Exon3		1	1.4
c.[188A > T;208G > T]	p.[H63L;V70L]	Exon1-2		2	2.9
c.[92C > T; 293−13A/C > G; 332_339delGAGACTAC]	p.[P31L;I2G;8bp deletion]	Exon1-3		2	2.9

* For variants without MLPA probes, gene conversion was implied by the combination of variants detected in *CYP21A2* and the fact that those variant nucleotides are present in the pseudogene.

3.3. Genotype-phenotype correlation

The overall genotype-phenotype correlation was 77.4% (24/31). Detailed information is provided in Table 2. In group Null (predicted to be the SW form), three out of six patients showed the SW form, while the others presented the SV form carrying Del/R357W, Q319*/8-bp deletion, or large gene conversion/E6 cluster compound heterozygotes. In group A (predicted to be the SW/SV form), five out of nine patients presented the SW form, while the remaining showed the SV form including two patients with I2G/I2G and two patients with I2G/L308Ffs*6. In group B (predicted to be the SV form), three out of fifteen patients presented with the SW form with I173N/ Del, I173N/Q319*, and I173N/R427H, respectively. In group C (predicted to be the NC form), one patient carrying the promoter mutations (c.-126C > T, c.-113G > A, c.-110T > C) *in trans* with a large gene conversion exhibited the SV form. In group D (carrying variants with unknown effects on enzyme activity), two patients presented the SW form, one presented the SV form, and one presented the NC form.

3.4. In silico analysis of the novel p.F450L variant

Multiple amino acids alignments of CYP21A2 across the species revealed that the F450 residue was highly conserved among its orthologues (Fig. 2B). The three-dimensional model structure of human CYP21A2–17-OH-progesterone complex showed that the F450 residue was far from the active site and surrounded by hydrophobic residues (Fig. 2C).

4. Discussion

In the present study, we identified 20 different mutant alleles in 35 patients with 21-OHD. Similar to previous studies in the Chinese population [11,18], the most common variants of the *CYP21A2* gene were I2G, large gene deletions/conversions, and I173N variants. The V282L variant which has a relatively high frequency in other populations was not detected in our cohort. Zhang et al. reported that the V282L variant accounted for 25% of alleles in the NC patients [19]. Therefore, the absence of V282L variant may be attributed to the low proportion of NC patients in our study. Moreover, seven rare point mutations were located at both ends of the enzyme including H63L, V70L, F405L, R409C, R427H, F450L, and R484Pfs*58 variants.

In 2016, the V70L variant was first described in a Chinese cohort [11]. In the present study, it was found in two unrelated Chinese girls (Patient 8, 35). To date, it has not been identified in other populations, which may result from an ancient founder effect. Remarkably, all the V70L variants were located on the same allele with the H63L variant in these patients, while the severe variants of Q319* + R357W, R357W, or R484Pfs*58 were on the other allele, leading to the SV, NC, or SW forms, respectively. We designed different primers for the amplification and sequencing of the gene. MLPA was also carried out. However, no other variants were found. According to *in vitro* studies, the isolated H63L variant reduced enzyme activity to a similar extent as that of the V282L variant, whereas a synergistic effect occurred when it was associated with another variant on the same allele. For instance, its association with the P454S variant conserved the enzyme activity at an intermediate level between isolated H63L and I173N variants [20]. Nevertheless, H63L + V70L variants *in trans* with different variants correlated with the SW form resulted in different phenotypes. As a result, further functional experiments are needed in order to confirm the combined effect of the H63L and V70L variants.

The F405L variant was previously described in a female patient who exhibited the NC form with the V282L/F405L genotype [21]. In the present work, this variant was observed in a girl (Patient 15) at a diagnosis age of 3.7 years due to the ambiguous genitalia with Prader

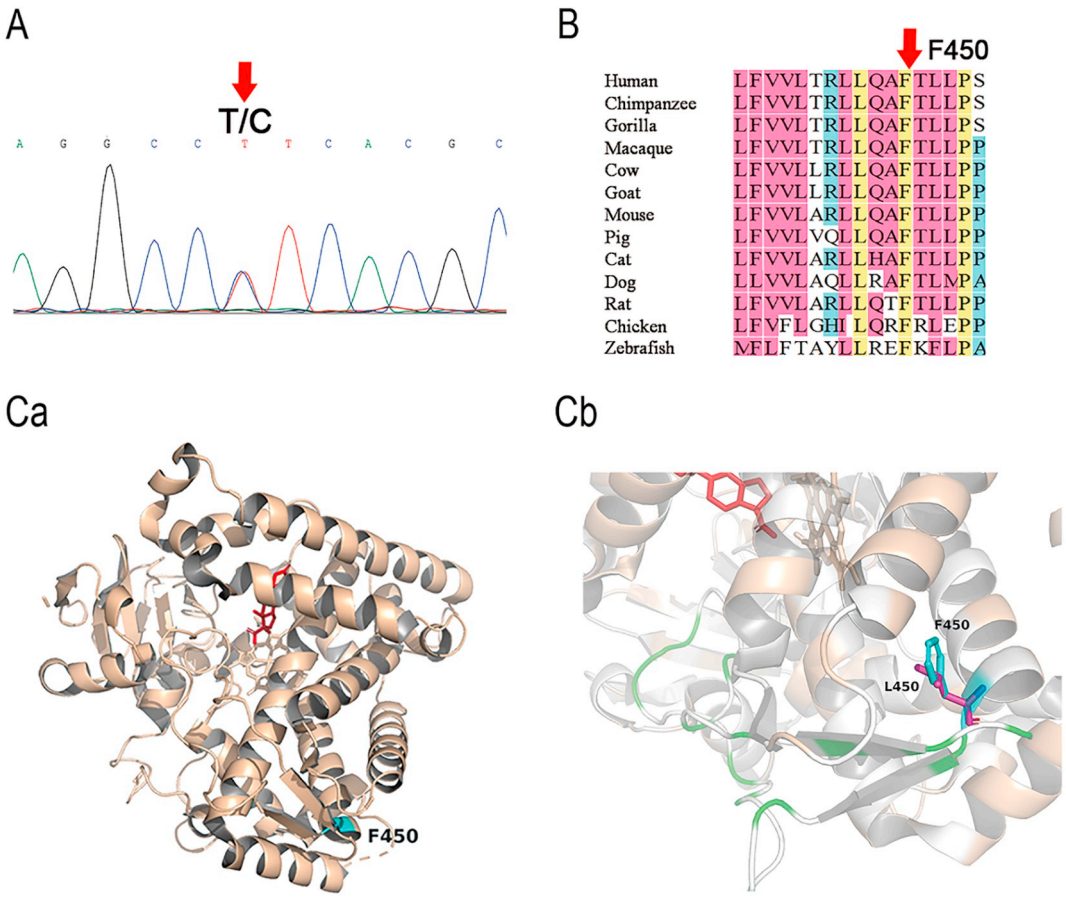

Fig. 2. A, DNA sequence of the *CYP21A2* gene in patient 12 showing a T to C transition at nucleotide 1348 in heterozygous form, resulting in the phenylananine to Leucin mutation at codon 450. Arrow indicates the location of variant. B, Alignment of amino acid sequences of CYP21A2 orthologues. The region of CYP21A2 protein with Phe450 of different organisms is shown. Arrow indicates the location of variant. C, The structure of CYP21A2–17-OH-progesterone complex with the F450L variant. Ca, overall view of the complex colored in *wheat*. 17-OH-progesterone and the F450 residue are colored in *red* and *cyan*, respectively. Cb, close-up view of the mutated site with hydrophobic amino acids colored in *gray*. Mutated residue is highlighted with carbon atoms colored in *cyan* (wild type) and *magenta* (mutant). The C-terminal end (residues 450–484, except for hydrophobic residues) is shown in *green*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

score of 4. The I2G variant retaining less than 1% of normal enzyme activity was found on the other allele. The V282L variant corresponds to the residual 50% enzyme activity, and the variants that reduce enzyme activity close to 2% cause the SV form. Since the phenotype is determined by the variant retaining the most enzyme activity, we hypothesized that the F405L variant could lead to a residual activity close to 2%, thus the genotypes of V282L/F405L and I2G/F405L correspond to the NC and SV phenotypes, respectively. Furthermore, the basal 17-OHP level in our patient was 730 nmol/L. A random concentration of 17-OHP above 303 nmol/L was commonly observed in the classic form [4], which was compatible with our hypothesis.

The R409C variant had only been described previously in Brazilian patients, and was considered to result in complete impairment of enzyme activity [22,23]. Microsatellite analysis suggested a gene founder effect in Brazilian population. Surprisingly, this variant was also observed in a Chinese girl with SV form in our cohort (Patient 21), which revealed that the R409C variant was not restricted to Brazilians and may present in other populations. The patient in our study exhibited the SV phenotype with a Prader score of 3 corresponding to the R409C/I173N genotype. Computed tomography scans also demonstrated bilateral adrenal hyperplasia.

The R427H variant was previously described in Austrian, Spanish, and Brazilian populations [23,24]. Like the R409C variant, a founder effect was also observed in these populations. However, the R427H variant was detected in one Chinese girl in our cohort (Patient 11), suggesting a wider ethnic distribution of this variant. In addition, previous observations implied that the R427H variant may produce a moderate-to-severe effect on enzyme activity correlated to the SV form. *In vitro* expression experiments showed that the R427H variant retained extremely low enzyme activity [24]. The patient in our cohort exhibited the SW manifestation with the I173N/R427H genotype. Although the I173N variant predominantly gives rise to the SV form, it can also result in SW form somehow, which may contribute to the genotype-phenotype discordance in this patient. Otherwise, it may be due to the presence of other defects.

We also identified a novel variant in a SW patient (Patient 12) at a diagnosis age of 14 days. The F450L variant on exon 10 was not found in ExAC, gnomAD, 1000Genomes, and HGMD databases. It was predicted to be damaging, probably damaging, and deleterious by SIFT, Ployphen-2, and Provean, respectively. Alignments of amino acid sequences across the species revealed that the F450 residue was highly conserved among CYP21A2 orthologues, indicating its importance in normal enzymatic function. *In silico* modeling of the mutated CYP21A2–17-OH-progesterone complex showed that the F450 residue was located in the loop that preceded the β-sheet at the C-terminus of the protein. Although the residue was far from the active site and surrounded by hydrophobic residues, the relatively smaller size of the mutant leucine residue could result in conformational instability due to the increased flexibility of the C-terminal end. Functional studies would be necessary to determine the ultimate influence of the novel variant.

According to ACMG guidelines, the F450L variant is categorized to be "likely pathogenic variant" because it belongs to PM2 (absent from controls in population databases), PM3 (for recessive disorders, detected *in trans* with a pathogenic variant), PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product), and PP4 (patient's phenotype is highly specific for a disease with a single genetic etiology) in ACMG [25].

In general, the genotype correlated well with the phenotype, but discordance existed in some cases, especially for the SV patients [26]. As for the I173N variant, phenotype diversity has been reported ranging from 59%–85% in many populations [15]. The overall genotype-phenotype correlation in our cohort was 77.4%. The SV form occurred in 76.9% of patients who harbored heterozygous I173N variant *in trans* with severe variants. However, the limitation of our study is the small sample size. The investigation on genotype-phenotype correlation in larger populations would be more eligible for meaningful results.

In summary, our findings have improved the understanding of *CYP21A2* mutational spectrum in 21-OHD patients, providing novel genetic insights helpful for genetic counseling and prenatal screening. First, we identified a novel variant, F450L, *in silico* predicted to be associated with the SW form. Second, two rare variants including R409C and R427H previously considered to be conserved in specific ethnicities due to a founder effect were also identified in the Chinese population. Third, a H63L + V70L variant that was only detected in Chinese patients *in trans* with different SW variants was found to lead to diverse phenotypes. Consequently, the sequence of *CYP21A2* gene must be analyzed carefully in case rare or novel deleterious variants exist. When diagnosis and treatments are concerned, phenotype prediction must be made with caution.

Conflict of interest

The authors declare that there is no conflict of interest.

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