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Three novel *CYP17A1* gene mutations (A82D, R125X, and C442R) found in combined 17α-hydroxylase/17,20-lyase deficiency

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ABSTRACT

The aim of this study was to analyze the structure and functional consequences of 3 novel mutations (A82D, R125X, and C442R) of the *CYP17A1* gene found in 2 patients with combined 17α-hydroxylase/17,20-lyase deficiency (17OHD). Two Chinese 46, XY female patients were diagnosed with 17OHD based on clinical findings and biochemical results. The *CYP17A1* gene was analyzed by polymerase chain reaction direct sequencing. An in vitro expression system was performed in HEK293 cells to analyze the mutant P450c17 activity compared with the wild type. Analysis of the *CYP17A1* gene sequences in patient 1 showed compound heterozygous mutations A82D (g.417 C > A) in exon 1 and Y329*fs* (g.4869 T > A, 4871del) in exon 6; DNA sequencing analysis in patient 2 revealed compound heterozygous mutations R125X (g.2045 C > T) in exon 2 and C442R (g.6457 T > C) in exon 8. The mutations A82D, R125X, and C442R have not been reported previously. The functional study demonstrated that the A82D, R125X, and C442R mutations almost completely eliminate enzymatic activity. These results, which indicate that Ala 82 and Cys 442 are crucial for both 17-hydroxylase and 17,20-lyase activities, help define the structure-function relationship of the *CYP17A1* gene. The novel mutations A82D, R125X, and C442R further clarify the patients' clinical manifestations of combined 17OHD.

1. Introduction

Congenital adrenal hyperplasia resulting from combined 17α-hydroxylase/17, 20-lyase deficiency (17OHD) is a rare autosomal recessive disease with an approximate incidence of 1:50 000 to 1:100 000 in newborns [1]. 17α-Hydroxylase is the key enzyme required for the synthesis of cortisol; it catalyzes the 17α-hydroxylation reaction of progesterone and pregnenolone into 17α-hydroxyprogesterone and 17α-hydroxypregnenolone, respectively. Impaired 17α-hydroxylase activity leads to decreased cortisol synthesis and compensatory hypersecretion of adrenocorticotropic hormone (ACTH). The excess ACTH stimulates hyperplasia of bilateral adrenal glands and overproduction of mineralocorticoids such as 11-

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deoxycorticosterone, which causes hypertension, hypokalemia, and suppressed plasma renin and aldosterone activity in 17OHD patients [2,3]. 17,20-Lyase is essential to the production of sex steroids; it converts 17α-hydroxyprogesterone and 17α-hydroxypregnenolone into androstenedione and dehydroepiandrosterone (DHEA), respectively, the precursors of sex hormone. A defect in 17,20-lyase results in the insufficient sex hormone production, causing 17OHD patients to have an absence of pubertal development and disorders of sex development [2-4]. All patients present with the female phenotype, but have varied genotypes: 46, XX individuals (genotypic female) show primary amenorrhea and lack of secondary sex characteristics; 46, XY individuals (genotypic male) always present with infantile female or ambiguous external genitalia and intraabdominal or inguinal testes, but no uterus, oviduct, or upper part of the vagina due to normal Müllerian duct inhibition.

Both 17α-hydroxylase and 17,20-lyase are encoded by one human *CYP17A1* gene, which resides in 10q24.3 and is expressed in several steroidogenic tissues, including the adrenal cortex, ovaries, and testes [5,6]. To date, approximately 90 mutations, including missense mutations, nonsense mutations, insertions, deletions, and splice site defects, have been reported in 17OHD patients (www.hgmd.cf.ac.uk/ac/gene.php?gene=CYP17A1). Because the severity of clinical manifestations in 17OHD is determined by the residual mutant P450c17 enzyme activity, the mutations affecting the heme binding site or steroid substrate binding site often demonstrate combined 17α-hydroxylase/17,20-lyase deficiency, whereas the mutations affecting the redox-partner binding site only lead to isolated 17,20-lyase deficiency [7,8]. Therefore, the mutations in the *CYP17A1* gene, especially point mutations, directly reflect the functions of the given amino acid in the P450c17 enzyme.

Although the imperfect activity of P450c17 caused by *CYP17A1* gene mutations cannot be restored, the symptoms such as hypertension and hypokalemia can be reversible with the treatment of exogenous glucocorticoid, such as prednisone, by inhibiting the hypersecreted ACTH. 46, XX patients are treated with the addition of conjugated estrogens to retain secondary sex characteristics; 46, XY patients are further advised to undergo a cryptorchidectomy in case of malignant tumors of the testes, owing to the irreversible malformation of external and internal genitalia, as well as psychological factors [9-11].

Here, we describe 2 combined 17OHD 46, XY female patients with 3 novel mutations (A82D, R125X, and C442R), which provide further insights to *CYP17A1* gene functions.

2. Materials and methods

2.1. *Case report*

Table 1 summarizes the clinical, biochemical, and hormonal findings in the 2 patients.

2.1.1. *Patient 1*

The patient was a 22-year-old Chinese woman who complained of primary amenorrhea and tall stature. First, she was

Table 1 – Summary of clinical characteristic, hormonal, and molecular genetic findings

	Patient 1		Patient 2		
Mutation g.DNA level	g.417 C>A;	g.4869 T>A, 4871del	g.2045 C>T;	g. 6457 T>C	
Mutation protein level	A82D	Y329fs	R125X	C442R	
Chromosome karyotype	46, XY		46, XY		
Age at diagnosis (y)	22		30		
Height (cm)	178		180		
Weight (kg)	48		50		
Blood pressure (mm Hg)	180/120		160/110		
Tanner stages	B1, P1		B1, P1		
Other	Bone age delay (7 y)		Right femur neck fracture and severe osteoporosis		
	Basal	Postdema	Basal	Postdema	Reference value
K+ (mmol/L)	3.15		1.4		3.5~5.5
PRA supine (ng/mL)	0.04		0.01		0.5~0.79
Aldosterone supine (ng/mL)	0.02		0.04		0.059~0.174
E2 (pmol/L)	<20		32		<54.4~127
Testosterone (nmol/mL)	1.21		0.91		8.34~32.11
LH (IU/L)	20.54	<0.69	24.3	<0.69	1.24~8.62
FSH (IU/L)	70.99		41.1		1.27~16.26
Progesterone (nmol/L)	15.5	<0.64	26.6	<0.64	0.5~2.8
DHEAS (μmol/L)	<0.41	<0.41	<0.41	<0.41	1.12~15.41
Androstenedione (nmol/L)	<1.05	<1.05	<1.05	<1.05	3.5~7.5
ACTH (pg/mL)	279	7.73	316	8.07	0~46
Cortisol (nmol/L)	70.6	28.3	62.4	<27.6	138-690

Postdema refers to the value after 2 days with large-dose dexamethasone administration. PRA indicates plasma renin activity; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

given standard hormone replacement therapy because of pelvic ultrasound detecting a primordial uterus and absent ovaries. She was then sent to the internal medicine clinic for further diagnosis. The detailed physical examinations showed that she was 178 cm tall and weighted 48 kg. She grew significantly taller in the past 3 years (from 167 to 178 cm). Her blood pressure was 180/120 mm Hg. The patient had infantile female external genitalia and a rudimentary vagina of 3 to 4 cm in length with a normal external vagina opening. Neither breast development nor pubic hair was found (Tanner stage 1). Her bone age was 7 years old according to her left hand radiograph, which suggested significantly delayed bone growth. Further ultrasound detection showed widespread hyperplasia in bilateral adrenal glands and intraabdominal testes. The karyotype was 46, XY. Serum potassium was 3.15 mmol/L. Plasma steroid analysis showed high levels of progesterone, human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH), and ACTH and low levels of estradiol (E2), testosterone, plasma renin activity, aldosterone, dehydroepiandrosterone sulfate (DHEAS), androstenedione, and cortisol. No cortisol response after large-dose dexamethasone stimulation was detected (Table 1). The patient was diagnosed with combined 17OHD and administrated prednisone 10 mg daily at 8:00 AM. One month later, her serum potassium was 3.45 mmol/L and her blood pressure declined to the reference range (145/90 mm Hg).

2.1.2. *Patient 2*

The patient was a 30-year-old Chinese woman who came to our hospital complaining of sudden fatigue, thirst, and polyuria after surgery for a right femur neck fracture. Her serum potassium at admission was 1.4 mmol/L, which was first attributed to insufficient fluid infusion after the operation. A detailed medical history revealed that the patient presented at 15 years old owing to a complaint of primary amenorrhea and the absence of pubertal development; a pelvic ultrasound obtained at that time confirmed the patient as having neither uterus nor ovaries. She was also diagnosed with hypertension, without headache, by routine physical examination when she was 20 years old. The physical examination showed that she was 180 cm in height and 50 kg in weight; blood pressure was 160/110 mm Hg. She had infantile female external genitalia with a slightly enlarged clitoris. Pubic hair and breast development were completely absent (Tanner stage 1). Abdominal computed tomography scan showed bilateral adrenal hyperplasia. Quantitative computed tomography of the lumbar spine (L2-4) revealed a markedly decreased bone mineral density less than –3.7 SD (0.682 g/cm^3 calcium hydroxyapatite, 61% of normal young adults), which suggested osteoporosis. The patient's karyotype was 46, XY. A series of hormones were measured, and the results were consistent with combined 17OHD. The patient was treated with prednisone twice daily: 10 mg at 8:00 AM and 5 mg at 3:00 PM. After 1 week of treatment, her serum potassium and blood pressure returned to reference ranges. No ethinyl estradiol was administrated because she rejected laparotomy to remove her suspected intraabdominal testes. Treatment with calcium and cholecalciferol was also initiated for osteoporosis and prevention of refracture.

2.2. *Hormone measurements*

Serum ACTH, cortisol, luteinizing hormone, follicle-stimulating hormone, progesterone, E2, testosterone, DHEAS, and androstenedione were measured by a commercial chemiluminescence kit in the Immulite 2000 system (Siemens, Los Angeles, USA). Serum aldosterone, and plasma renin activity were measured by a commercial radioimmunoassay kit. The results were compared with normal values established in our laboratory.

2.3. *Polymerase chain reaction and DNA sequencing*

After informed consent was obtained from the patients and their parents, their genomic DNA was extracted from peripheral blood leukocytes using classic phenol/chloroform methods. All 8 coding exons of the *CYP17A1* gene were amplified by polymerase chain reaction (PCR) using primers designed by software Primer Premier 5.0 (PREMER Biosoft International, Palo Alto, CA), with intronic regions to span all exons and the intron/exon boundaries. The PCR amplification was performed in 50-μL reaction volumes, containing 100 ng genomic DNA, 1 μmol/L of each dNTP, 1.5 μmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), and 1 U Tag DNA polymerase (Takara, Dalian, China). After initial denaturation at 94°C for 5 minutes, the reactions were followed by amplification for 30 cycles with denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes after the last cycle. The PCR products were directly sequenced and analyzed by the ABI PRISM 3730 DNA sequencer (Applied Biosystem by life Technologies, Calsbad, California, USA). The T-A clone sequencing was subsequently completed when direct sequencing showed doubled signals.

2.4. *In vitro functional studies*

The wild-type complementary DNA (cDNA) of the *CYP17A1* gene was inserted into pcDNA3.1 (+) (Invitrogen by Life Technologies Company, Shanghai, China) between the *Hin*dIII and *Bam*HI restriction enzyme sites. Site-directed mutagenesis was performed with 2 primers designed for the nucleotide change, *pfu* DNA polymerase (Takara, Dalian, China), and wild-type cDNA of the *CYP17A1* gene. The primers were designed as followed: A82D-forward, 5′-ccaccaccagctggacaaggaggtg-cttatt-3′; A82D-reverse, 5′-aataagcacctccttgtccagctggtggtgg-3′; Y329*fs*-forward, 5′-ggtgaagaagaagctcaagaggagattgaccag-3′; Y329*fs*-reverse,5′-ctggtcaatctcctcttgagcttcttcttcacc-3′; R125-X-forward, 5′-acactggcagctgcattgaaggctggcgatgg-3′; R125X-reverse, 5′-ccatcgccagccttcaatg-cagctgccagtgt-3′; C442R-forward, 5′-caggacctcgctcccgtataggtgagatcct-3′; and C442R-reverse, 5′-aggatctcacctatacgggagcgaggtcctg-3′. The PCR product was digested with *Dpn*I restriction enzyme at 37°C for 1 hour and then transformed into *Escherichia coli* DH5α. The positive clones were picked out, and the full-length mutant cDNA was sequenced to confirm the site-directed mutations.

HEK-293 cells were seeded in 60-mm plates and incubated with 5 mL Dulbecco modified Eagle medium (Hyclone, Logan, USA) containing 10% fetal calf serum. Cells were transfected

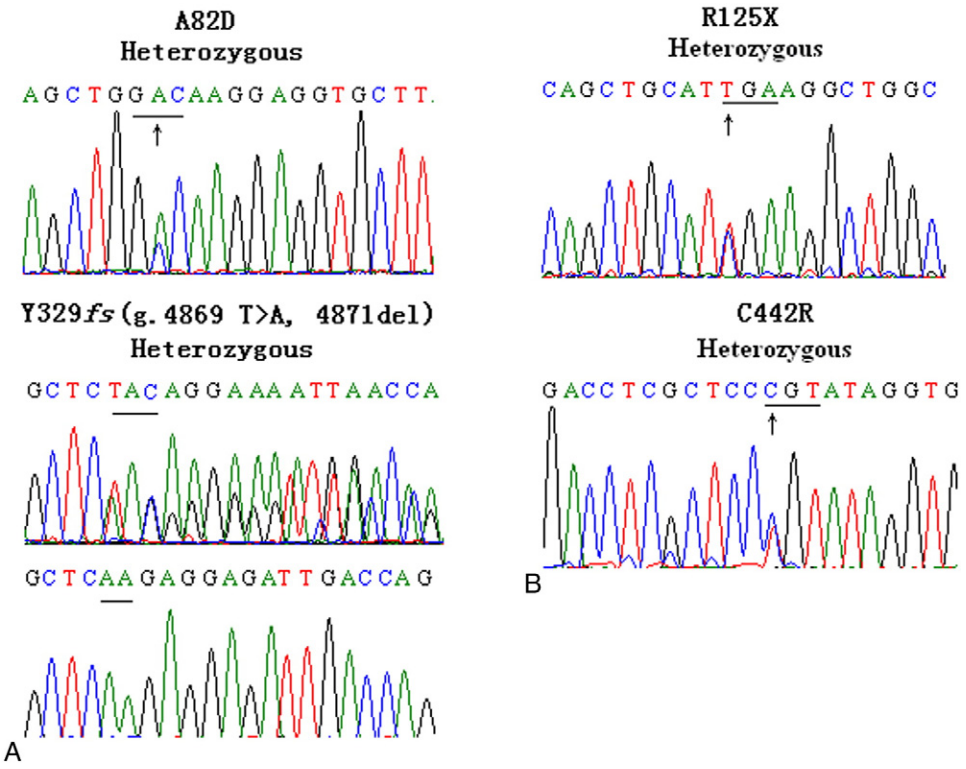

Fig. 1 – CYP17A1 gene mutations found in patient 1 (A) and patient 2 (B). A, Direct sequencing results showed the heterozygous mutations A82D in exon 1 and Y329fs (g.4869 T>A, 4871del) in exon 6 of patient 1 with T-A clone sequencing due to double signals by PCR direct sequencing. B, Direct sequencing results showed the heterozygous mutations R125X in exon 2 and C442R in exon 8 of patient 2.

with 8.0 μg total plasmid DNA, including pcDNA3.1-C17-wt, pcDNA3.1-C17-A82D, pcDNA3.1-C17-R125X, pcDNA3.1-C17-C442R, pcDNA3.1-C17-Y329*fs*, using 20 μL Lipofectamine 2000 (Invitrogen by Life Technologies Company, Shanghai, China) for each reaction. To assess the activity of 17α-hydroxylase and 17,20-lyase, 4 μg/mL of progesterone was added to the media 24 hours after transfection. After another 36 hours of incubation, the media were collected and prepared for high-performance liquid chromatography (AB SCIEX, Foster, California, USA) to detect the amount of 17α-hydroxyprogesterone and androstenedione. All the experiments were conducted at least 4 times.

3. Results

3.1. *Serum steroid hormone concentration results*

Results are shown in Table 1.

3.2. *Mutation analysis*

Direct sequencing analysis of the *CYP17A1* gene in patient 1 revealed a compound heterozygous state in exon 1 and exon 6 (Fig. 1A). The novel missense mutation in exon 1 (g.417 C > A) results in a substitution from alanine to aspartic acid at amino acid position 82 (A82D). The mutation in exon 6 (g.4869 T > A, 4871del; Y329*fs*) was reported previously [12]. Segregation analysis showed that A82D was a maternal inheritance and that Y329*fs* (g.4869 T > A, 4871del) was a paternal inheritance.

Mutation analysis of patient 2 showed a compound heterozygous state with 2 novel mutations: a nonsense mutation (g.2045 C > T) in exon 2, leading to a premature stop codon in position 125 (R125X), and a missense mutation (g.6457 T > C) in exon 8, which resulted in an amino acid substitution of arginine for cysteine in position 442 (C442R) (Fig. 1B). Neither mutation has been reported previously. Analysis of the patient's parental DNA demonstrated that the novel R125X mutation was inherited from her father, who is a heterozygous carrier. The other novel mutation C442R was inherited from her mother, who is also a heterozygous carrier.

Table 2 – 17-Hydroxylase/17,20-lyase activity of wild-type and mutant P450c17 expressed in HEK293 cells

Plasmid	17α-Hydroxyprogesterone (ng/dish)	Androstenedione (ng/dish)
pcDNA3.1-C17	5778 ± 167	136 ± 24
pcDNA3.1	<1	<1
A82D mutant	202 ± 36	6.3 ± 1.6
Y329fs mutant	<1	<1
R125X mutant	<1	<1
C442R mutant	<1	<1
A82D/Y329fs	69 ± 14	2.3 ± 1.1
R125X/C442R	<1	<1

3.3. *In vitro functional analysis*

To evaluate the enzyme activity of P450c17 with 3 novel mutations, A82D, R125X, and C442R, we transiently expressed the wild-type, A82D, R125X, and C442R mutant P450c17 in HEK293 cells. The cells expressing the wild-type *CYP17A1* gene showed high efficiency in converting progesterone to 17α-hydroxyprogesterone and converting 17α-hydroxyprogesterone to androstenedione. The functional analysis of the A82D mutation showed approximately 3.5% activity compared with the wild-type P450c17. Both the expressed R125X mutant P450c17 and the expressed C442R mutant P450c17 displayed almost complete inactivity of 17α-hydroxylase and 17,20-lyase (nearly 0% of the wild-type P450c17) (Table 2).

When cells expressed both A82D and Y329*fs* mutant P450c17, only 1.2% of enzyme activity remained; and when both R125X and C442R mutant plasmids were transfected into cells, almost all enzyme activity was lost compared with the wild-type P450c17 (Table 2).

4. Discussion

We report 2 Chinese patients with combined 17OHD caused by novel mutations in the *CYP17A1* gene. The mutations A82D, R125X, and C442R have not been reported previously. In vitro functional experiments of these mutations provided strong evidence for the pathological effect of the identified mutations.

The missense A82D mutation substitutes the conserved neutral alanine residue to an acidic aspartic acid residue. Functional expression analysis revealed that this mutation only retained 3.5% of activity of both 17α-hydroxylase and 17,20-lyase, which reconfirms the importance of the A82 residue to the structure and the function of P450c17. The mutation Y329*fs* has been repeatedly identified in Chinese patients with combined 17OHD [12-14], which suggests that this mutation has been derived from a single founder gene with high frequency in the Chinese population. The nonsense mutation R125X, which causes a loss of approximately 75% of the P450c17 enzyme, including of a part of substrate-binding pocket, the entire heme-binding site, and the entire redox-partner site [7], completely ablates the enzymatic functions. The missense mutation C442R disrupts the most conserved cysteine in the heme-binding site of the P450c17 enzymes, the ligand for the heme iron [7]. The functional expression study demonstrated that the C442R mutation led to complete inactivity of the enzyme. The compound heterozygous mutations A82D/Y329*fs* and R125X/C442R are the causes of combined 17OHD in our 2 patients, respectively.

Both patients manifested significant hypertension, and patient 2 also displayed severe hypokalemia. These symptoms were relieved after regular glucocorticoid therapy. *CYP17A1* enzyme activity needs to retain roughly one fourth of its catalytic capability to prevent the onset of mineralocorticoid-dependent hypertension [15]. In fact, approximately 10% to 15% of 17OHD patients present with normotension or normokalemia despite the mutation that leads to complete elimination of 17α-hydroxylase/17,20-lyase activity [16]. Interestingly, the age of onset for patients with mild hypertension or hypokalemia is relatively younger than that of majority combined 17OHD patients with severe hypertension and hypokalemia [11,12,17,18]. Some patients display a different severity of hypertension with the same mutation of the *CPY17A1* gene, even though the mutation functional study demonstrated enzyme activities were almost eliminated [19-21]. The degree of accumulation of mineralocorticoid precursors, such as deoxycorticosterone, owing to the long-term activity of compensation pathway might contribute to the degree of hypertension and hypokalemia, in addition to the *CYP17A1* genotype.

Both 46, XY patients exhibit the female phenotype with intraabdominal testes, but no Müllerian structures. In vitro functional analysis shows that the 3 novel mutations severely impair the dual function of P450c17, both 17α-hydroxylase and 17,20-lyase, required to synthesize sex hormones, which accounts for the extremely low concentrations of serum testosterone, DHEAS, and androstenedione in the 46, XY female phenotype. However, normal Müllerian inhibitor factor in the 46, XY genotype led to Müllerian duct inhibition; and the normal *SRY* gene results in testes development.

Both patients presented with tall stature, although their parents were all with normal stature. Tall stature in 17OHD patients has been reported in several cases and always accompanies delayed bone age and/or osteoporosis [17,22]. The underlying mechanism of this finding is still unclear. Interestingly, in our patient 1, her stature changed significantly in recent years. This clue suggests that tall stature in 17OHD patients might be the result of the decompensated mineralocorticoid precursors and decreased sex hormones, which might interfere with calcium metabolism and bone development. The decreased sex hormone inhibits epiphyseal arrest. However, the normal calcium metabolism is mainly regulated by vitamin D3, which is cholecalciferol, classified as a kind of secosteroid, and exerts its physiologic function only with active metabolite $1,25(OH)_2D_3$. The 1α-hydroxylase is also a P450 enzyme and expressed in the renal proximal tubule that catalyzes the hydroxylation of 25-hydroxyvitamin D3 into 1, $25(OH)_2D_3$ [23]. It is speculated that the accumulated mineralocorticoid precursors might interfere with 1α-hydroxylase activity in 17OHD patients, which hinders calcium homeostasis. The exact mechanism needs further study.

The mutation of the *CYP17A1* gene and its remnant function provide the direct molecular basis of the clinical manifestations of gene defects. The time span without glucocorticoid treatment is also an influential factor on the severity of disease. In summary, the 3 novel mutations found in our 2 Chinese patients, and their phenotypic manifestations, are important to define *CYP17A1* gene function and to provide valuable information for patient management and genetic counseling.

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