



Functional Identification of Compound Heterozygous Mutations in the *CYP17A1* Gene Resulting in Combined 17 α -Hydroxylase/17,20-Lyase Deficiency

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Background: We previously reported a patient with congenital adrenal hyperplasia (CAH) with compound heterozygous mutations in the cytochrome P450 17A1 (*CYP17A1*) gene. One allele had a p.His373Leu and the other a new p.Glu383fsX36 mutation. The aim of this study was to investigate the functional properties of a new allele present in a compound heterozygote of *CYP17A1*.

Methods: To understand how p.His373Leu and p.Glu383fsX36 affect P450c17 enzymatic activity, wild type and mutant *CYP17A1* cDNAs were cloned into flag-tagged pcDNA3 vector and introduced into human embryonic kidney cells 293T (HEK293T) cells. Protein expression levels of CYP17A1 were then analyzed. And the activities of 17 α -hydroxylase and 17,20-lyase of CYP17A1 were evaluated by measuring the conversion of progesterone to 17 α -hydroxyprogesterone and of 17 α -hydroxypregnenolone to dehydroepiandrosterone, respectively. In addition a computer model was used to create the three-dimensional structure of the mutant CYP17A1 enzymes.

Results: Production of the p.His373Leu mutant protein was significantly lower than that of the wild type protein, and the p.Glu383fsX36 protein was hardly produced. Similarly the enzymatic activity derived from the p.His373Leu mutant vector was significantly lower than that obtained from the wild type vector, and little activity was obtained from the p.Glu383fsX36 vector. Three-dimensional modeling of the enzyme showed that p.His373 was located in region important for heme-binding and proper folding. Neither the p.His373Leu nor the p.Glu383fsX36 mutant protein formed a heme-binding structure.

Conclusion: Enzyme activity measured in both mutants disappeared completely in both 17 α -hydroxylase and 17,20-lyase. This result accounts for the clinical manifestations of the patient with the compound heterozygous *CYP17A1* mutations.

Keywords: Adrenal hyperplasia, congenital; 17-Alpha-hydroxylase; 17,20-Lyase; Steroid 17-alpha-hydroxylase

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by mutations of the cytochrome P450

17A1 (*CYP17A1*) gene [1,2]. This gene encodes the enzyme cytochrome P450c17, which possesses both 17 α -hydroxylase and 17,20-lyase activities [3,4]. Mutations in the *CYP17A1* gene are associated with combined 17 α -hydroxylase/17,20-lyase defi-

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ciency (17OHD) and isolated 17,20-lyase deficiency (ILD) [5,6]. Most defects in *CYP17A1* impair both enzymatic activities and cause complete or partial combined 17OHD [6,7]. Abnormal enzymatic activity of *CYP17A1* can present as isolated 17 α -hydroxylase deficiency, ILD, or combined 17OHD [5,7].

We previously described a patient with compound heterozygous mutations in the *CYP17A1* gene. Genetic analysis revealed that one allele had a missense mutation c.1118A>T (p.His373Leu), which is a frequently observed mutation type, and the other allele had a novel 1-bp deletion, c.11148delA, causing a frameshift (p.Glu383fsX36) [8]. Although the patient had primary amenorrhea, sexual infantilism and minimal body hair, she had no hypokalemia (3.6 mEq/L) and hypertension (130 to 110/80 to 70 mm Hg).

The hallmark of 17OHD, first described in 1966 [6], is hypertension and hypokalemia, including sexual infantilism. The classic presentation of 17OHD in phenotypically female individuals is hypertension including primary amenorrhea and minimal body hair [6,9,10]. The clinical signs tend to be milder in cases with mutations retaining partial enzyme activity than where there is complete loss of enzyme activity [5,11], and the phenotypes of the latter individuals are highly variable [1,12]. The common mutation sites in *CYP17A1* differ among ethnic groups [8,13,14]. This phenomenon can be explained as Founder effect in autosomal recessive genetic disease [15]. The relative 17 α -hydroxylase/17,20-lyase activities of *CYP17A1* mutants have been shown to vary in *in vitro* assays [5,16,17], and, remarkably, the degree of hypertension can vary between individuals with the same *CYP17A1* genotype [8,14,18]. In this study, the effect of the mutations on 17 α -hydroxylase/17,20-lyase activities was assessed by *in vitro* studies on the *CYP17A1* enzymes expressed from vectors of the individual mutant alleles present in the compound heterozygote.

METHODS

Research case

The case has been published previously [8]. Briefly, a 21-year-old woman has experienced intermittent hypokalemia episodes since she was 2 years old. Puberty development was absent until 17 years old. The pubic hair and axillary hair were not visible, and a slight hyperpigmentation of the skin was found. Blood pressure was 130 to 110/80 to 70 mm Hg. Serum potassium level was in the low normal range (3.6 mEq/L). Plasma adrenocorticotropic hormone (ACTH) was significantly elevated. Plasma progesterone, follicle stimulating hormone and luteinizing hor-

mones were increased and serum estradiol and cortisol levels were significantly decreased. However, plasma renin activity and aldosterone levels were within normal ranges. Abdominal computed tomography showed bilateral adrenal hyperplasia. The study protocol was approved by the Institutional Review Board of the Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea (OC16TISI0099).

Subcloning of the *CYP17A1* gene

Reverse transcription polymerase chain reaction (RT-PCR) products of total RNA extracted from the proband were subcloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA), and the cloned inserts were analyzed by PCR and Big-Dye terminator cycle sequencing using an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Construction of *CYP17A1* expression vectors

To investigate separately the mutations c.1118A>T and c.1148delA, we first created an expression vector for the wild type (WT) gene. The WT *CYP17A1* gene was amplified from control complementary DNA (cDNA) using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland). A HindIII site was introduced into the upstream primer (5' CTATAGGGAGACCCAAGCTTCCTCCTTGTCGCCCTAGAG 3') of P450c17 and a KpnI site into the downstream primer (5' GTCCTTGTAATCGGTACCG-GTGCTACCCCTCAGCCTGGG 3'). The PCR fragments were ligated with T4 ligase (TaKaRa, Kyoto, Japan) between the HindIII and KpnI sites of pcDNA3 vector with a C-terminal flag tag (Fig. 1). Mutant (mutant type 1 [MT1]-flag/pcDNA3 and mutant type 2 [MT2]-flag/pcDNA3) expression vectors were constructed by mutating the WT *CYP17A1* expression vector (WT-flag/pcDNA3) with a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The expression vectors were confirmed by BigDye Terminator cycle sequencing with the ABI 3730XL DNA sequencer.

Transfection

Human embryonic kidney cells 293T (HEK293T) cells were harvested at nearly 80% confluence, suspended with trypsin/ethylenediaminetetraacetic acid (EDTA) solution and washed with phosphate-buffered saline (PBS). The cells were transfected using an implemented electroporation device system according to the manufacturer's instructions (Neon Transfection System, Invitrogen). For transfection, cells (1×10^6) were re-suspended in buffer containing 5 μ g plasmid DNA (WT-flag/pcDNA3, MT1-flag/pcDNA3, or MT2-flag/pcDNA3). Parameter

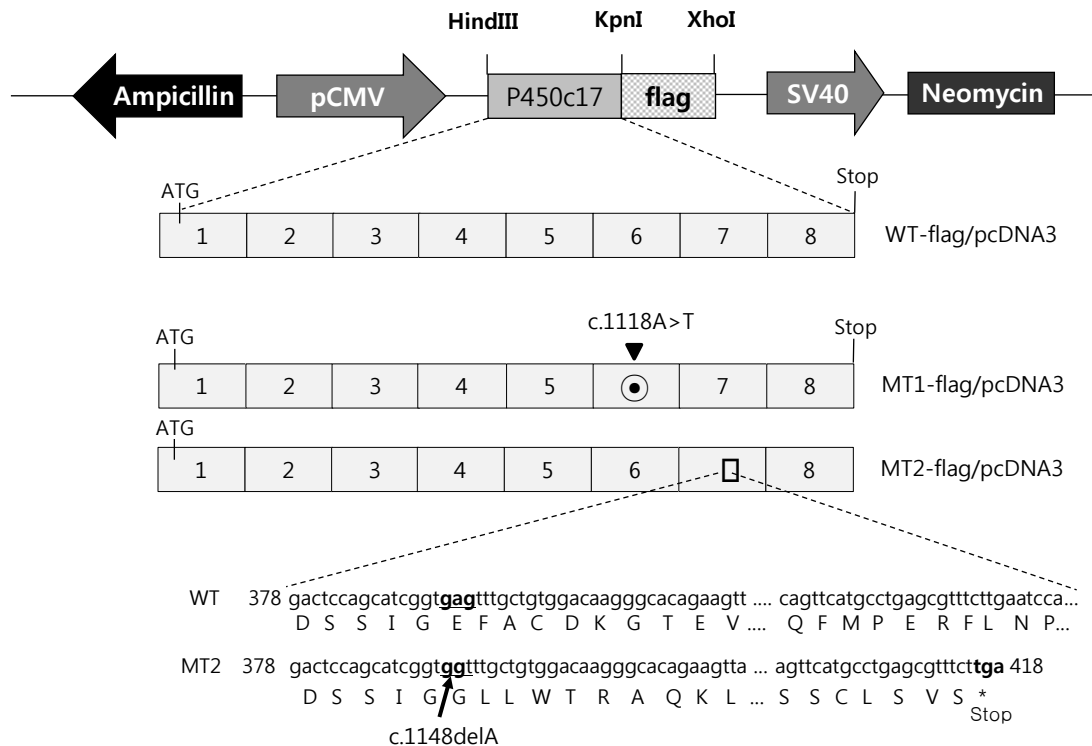


Fig. 1. Schematic representation of the constructs used to measure P450c17 enzyme activity produced in human embryonic kidney cells 293T (HEK293T) cells. The entire coding regions of c.1118A>T, c.1148delA, and wild type (WT) cytochrome P450 17A1 (*CYP17A1*) complementary DNAs (cDNAs) were subcloned into the HindIII and KpnI site of pcDNA3 vector with a C-terminal flag tag. The positions of restriction enzymes, start (ATG) and stop (tga) codons, and HindIII, KpnI, and XhoI sites, are shown. Exons are indicated with arabic numerals and the mutation sites (c.1118A>T and c.1148delA) are indicated. The 1 bp deletion (bp position 1148 in c.1148delA, indicated by the arrow) results in a shift of the reading frame and a termination codon after 36 amino acids. pCMV, promoter cytomegalovirus; SV40, simian virus 40; MT1, mutant type 1; MT2, mutant type 2.

set 1,100 pulse voltage, 40 pulse widths, and one pulse were used. After the electroporation, cells were seeded in 6-well plates by adding 3 mL of medium supplemented with 10% fetal bovine serum (FBS) without antibiotic supplements.

Western blotting construction of *CYP17A1* expression vectors

After 48 hours of transfection, the HEK293T cells were lysed with lysis buffer and centrifuged. The pellets were discarded, and protein concentrations were measured in the supernatants. Aliquots containing 20 μ g protein were fractionated on 10% a denaturing polyacrylamide gels. After transferring the proteins to membranes, the membranes were incubated with monoclonal mouse anti-flag M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) followed by goat anti-mouse immunoglobulin G-horse-radish peroxidase (IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The *CYP17A1* signal was detected with enhanced chemiluminescence (ECL) select (GE Healthcare Life

Sciences, Uppsala, Sweden). We used β -actin to assess protein loading.

Immunocytochemistry of flag-tagged proteins

Transfected cells were grown on glass coverslips, washed twice with PBS, fixed with 4% paraformaldehyde (Biosesang, Seongnam, Korea) for 20 minutes at room temperature and incubated in PBS containing 0.25% Triton X-100 (Calbiochem, San Diego, CA, USA). After blocking in normal donkey serum, they were incubated overnight at 4°C with a 1:500 dilution of monoclonal mouse anti-flag M2 antibody (Sigma-Aldrich). The bound antibody was stained with 1:200 dilution of rhodamine-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA, USA). And the nuclei were loaded onto a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector, Burlingame, CA, USA). Fluorescence images were obtained using a confocal laser microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

17 α -Hydroxylase and 17,20-lyase activity of transfected cells

HEK293T cells were transfected with each construct and cultured for 48 hours. Then various concentrations (0, 1.0, and 2.0 μ M) of progesterone (P) (Sigma-Aldrich) and 17 α -hydroxypregnenolone (17-PREG) (Sigma-Aldrich) were added to the cells, which were further cultured for 6 hours. The medium was removed and the concentrations of 17 α -hydroxyprogesterone (17-OHP) (DRG Instruments GmbH, Marburg, Germany) and dehydroepiandrosterone (DHEA) (DRG Instruments GmbH) were measured using an enzyme-linked immunosorbent assay (ELISA) kit.

Three-dimensional structure modeling

To better understand how the new p.Glu383fsX36 and previously reported p.His373Leu CYP17A1 variants affect P450c17 enzyme activity, we created a three-dimensional computer model

using SWISS-MODEL (<http://swissmodel.expasy.org>, Biozentrum University of Basel, Basel, Switzerland). We analyzed the computer models of CYP17A1 using the Swiss PDB viewer (<https://spdbv.vital-it.ch>, Swiss Institute of Bioinformatics, Lausanne, Switzerland) and UCSF Chimera version 1.11.2 (<https://www.cgl.ucsf.edu>, University of California, San Francisco, CA, USA).

RESULTS

Subcloning of the compound heterozygous CYP17A1

Sequence analysis showed that the patient had a compound heterozygous mutation, p.His373Leu derived from mother and a novel frameshift leading to p.Glu383fsX36 derived from father (Fig. 2A). Subcloning and sequencing of the RT-PCR products of total RNA extracted from the leukocytes of the proband re-

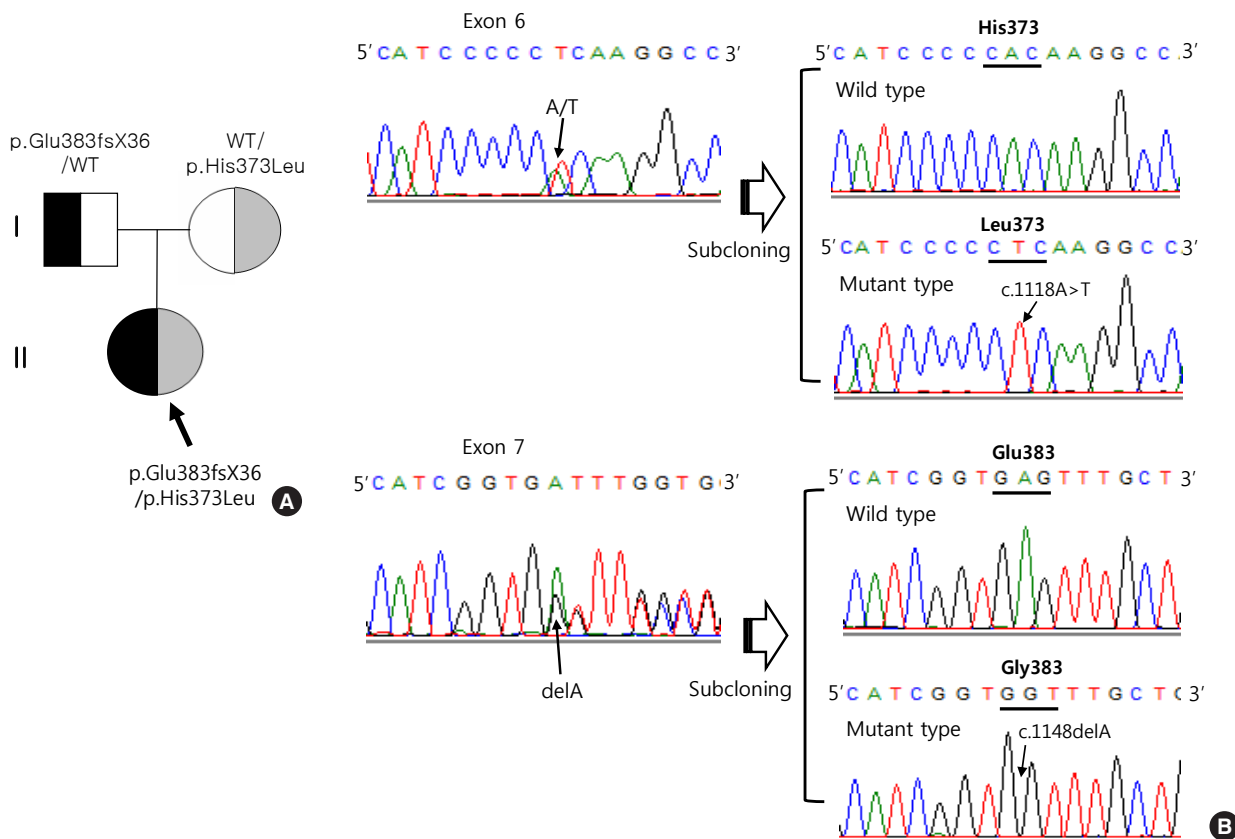


Fig. 2. The compound cytochrome P450 17A1 (*CYP17A1*) heterozygote. (A) Pedigree of 17 α -hydroxylase/17,20-lyase deficient family. Individuals are represented as follows: male (square); females (circles). The black symbol represents the c.1148delA allele and the gray symbol represents the c.1118A>T allele. The arrow indicates the compound heterozygous proband. The proband inherited mutations c.1118A>T and c.1148delA from her mother and father, respectively. (B) Subcloning of the compound heterozygous *CYP17A1* gene. Polymerase chain reaction products of RNA from the proband's leukocytes were subcloned and sequenced to confirm heterozygous mutations. The c.1148delA mutation was found to be in exon 7 and the c.1118A>T mutation in exon 6. WT, wild type; A/T, A and T.

Table 1. Comparison of p.His373 Mutations of *CYP17A1* Gene Reported in the Literature

Literature	Study case					
	[8]	[27]	[28]	[29]	[30]	[31]
Age, yr	21	23	3.5	14	23	6
Ethnic	Korean	Korean	Japanese	Caucasian	Chinese	Japanese
p.His373 mutation	p.His373Leu	p.His373Leu	p.His373Asn	p.His373Asp	p.His373Leu	p.His373Leu
Opposite allele mutation	p.Glu383fs	p.His373Leu	p.Phe53del	p.Arg96Trp	p.Asp487_Phe489del	p.Leu247del
Karyotype	46,XX	46,XX	46,XY	46,XX	46,XX	46,XY
Sexual infantilism	Yes	Yes	Yes	Yes	Yes	Yes
Hypertension	No	Yes	No	Yes	Yes	Yes
Hypokalemia	No	No	No	Yes	Yes	Yes

vealed that the c.1148delA mutation was located in exon 7 and the c.1118A>T mutation in exon 6 (Fig. 2B).

Comparison of p.His373 mutations of *CYP17A1* gene reported in the literature

The p.His373 mutations are common in East Asia and showed complete type of 17 α -hydroxylase/17,20-lyase deficiency without any pubertal development. The severity of phenotype was variable among cases, even though the same genetic mutation of the *CYP17A1* gene was found (Table 1). It seems that genotype-phenotype correlation is affected by not only mutation type of the *CYP17A1* gene but also karyotype. This is the fourth case of 17 α -hydroxylase/17,20-lyase deficiency in Korea.

Expression of the wild type and P450c17 mutations in HEK293T cells

In order to see whether the heterozygous mutations (p.His373Leu and p.Glu383fsX36) cause 17OHD, *CYP17A1* cDNA constructs carrying the WT and the two mutants separately (c.1118A>T, c.1148delA) were overexpressed in HEK293T cells. We analyzed P450c17 protein expression by Western blotting and immunofluorescence. Enzyme protein expression was significantly lower in cells harboring either mutant construct than in those harboring the WT, and the c.1148delA clone on its own produced no enzyme protein (Fig. 3A, B).

Enzyme activity

To confirm the results of the Western blotting studies, we measured the P450c17 enzyme activity produced by HEK293T cells harboring these various constructs. Activity was evaluated as conversion of progesterone to 17-OHP and conversion of 17-PREG to DHEA. After incubation with 1.0 and 2.0 μ M proges-

terone, the concentrations of 17-OHP generated by the WT construct were 305 and 744 nmol/L, respectively. By comparison the 17-OHP concentrations produced by the c.1118A>T construct were 12.1 and 21.9 nmol/L, respectively, and those of the c.1148delA construct were 6.1 and 8.8 nmol/L, respectively, while the 17,20-lyase activity of the c.1118A>T and c.1148delA mutants were almost zero (Fig. 3C).

Three-dimensional structure modeling

Three-dimensional structure modeling of the enzyme showed that the amino acid p.His373 residue is located within a region critical for heme-binding. The p.His373Leu mutation prevented the incorporation of heme and thus caused loss of catalytic activity. The absence of His at position 373 rendered the protein unable to form a hydrogen bond. And the c.1148delA mutant structure also does not contain a heme molecule and lacks enzyme activity in spite of the presence of His at position 373. Thus, it appears that the mutant enzymes are predicted to be largely non-functional (Fig. 4).

DISCUSSION

CAH is characterized by hypokalemic hypertension and sexual infantilism and is caused by mutations in *CYP17A1* [14,19]. The *CYP17A1* gene encodes an enzyme of the cytochrome P450 superfamily, P450c17 which catalyzes a 17 α -hydroxylase reaction to form 17-hydroxysteroid, and a 17,20-lyase reaction to cleave 21-carbon 17-hydroxysteroid into 19-carbon 17-keto androgen precursor [3]. Mutations in the *CYP17A1* gene can cause complete (or partial), combined (or isolated) 17 α -hydroxylase/17,20-lyase deficiencies [7,19]. The prevalence of CAH is the highest in Brazil [17,20]. ILD is extremely rare

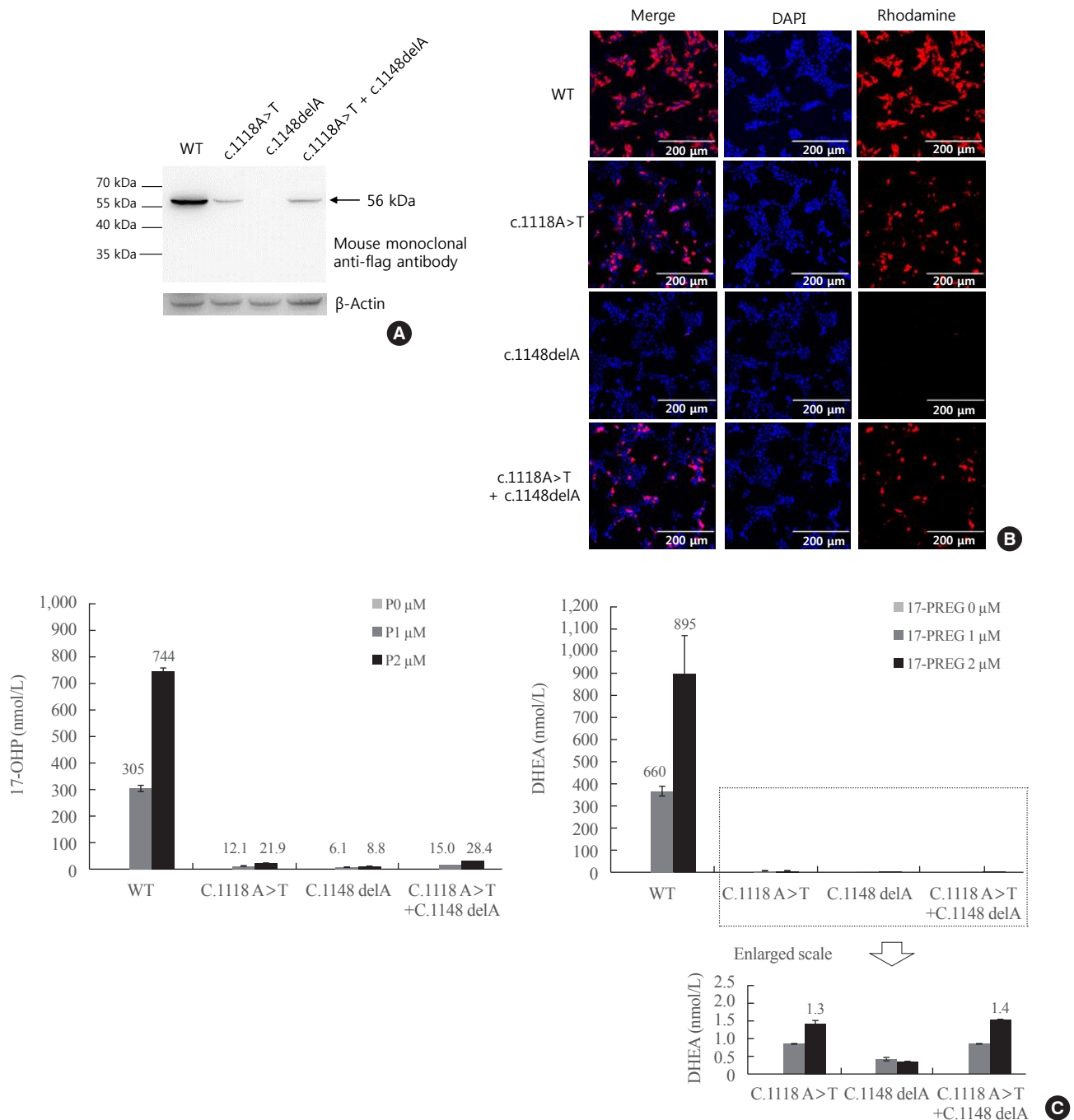


Fig. 3. Expression and enzyme activities of P450c17 protein produced by cytochrome P450 17A1 (*CYP17A1*) complementary DNA (cDNA) overexpressed in human embryonic kidney cells 293T (HEK293T) cells. (A, B) Analysis of P450c17 proteins by Western blotting (WB) and immunofluorescence, respectively. Expression of c.1118A>T and the doubly transfected clone (p.H373L and p.E383fsX36) in HEK293T cells is significantly lower than that of wild type (WT) and no expression of c.1148delA mutant is detected. (C) Enzyme activity. Production of 17 α -hydroxyprogesterone (17-OHP) in the presence of various concentrations of progesterone (P) (0, 1.0, and 2.0 μ M) and production of dehydroepiandrosterone (DHEA) in the presence of various concentration of 17 α -hydroxypregnenolone (17-PREG) (0, 1.0, and 2.0 μ M). HEK293T cells were transfected with WT, c.1118A>T, c.1148delA, and co-transfection. Compared to the enzymatic activity against WT (744 nmol/L), the 17 α -hydroxylase activity for the p.His373Leu mutant was reduced to 21.9 nmol/L and the 17,20-lyase activity for the p.Glu383fsX36 mutant was almost zero (1.3 nmol/L). Data are mean values of P450c17 activity assayed in triplicate. Data are expressed as mean \pm SD ($n=3$). DAPI, 4',6-diamidino-2-phenylindole.

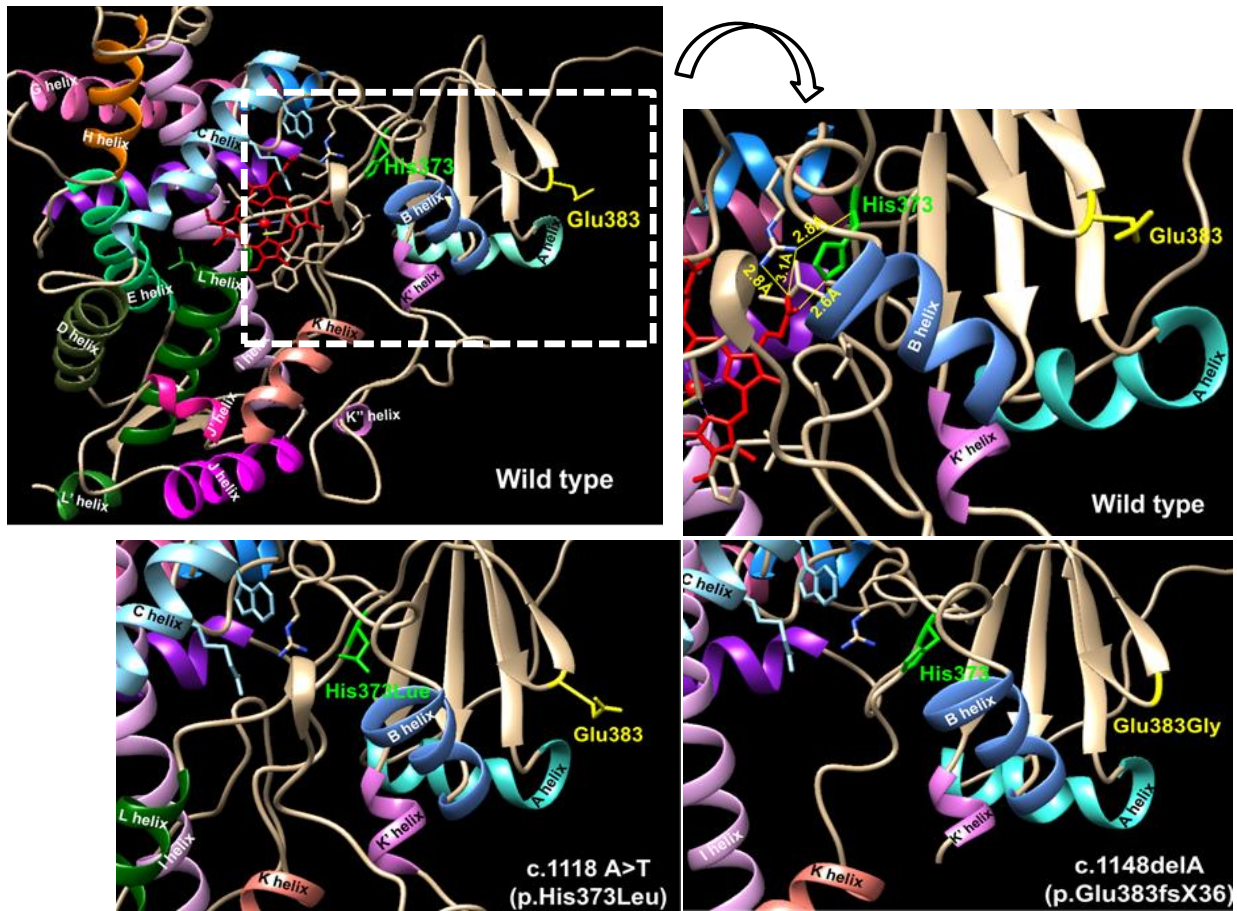


Fig. 4. Computer model of cytochrome P450 17A1 (CYP17A1) (PDB ID; 3SWZ). A, a ribbon diagram of the protein backbone, with heme (red) and highlighting p.His373, p.Glu383, p.His373Leu, p.Glu383Gly. Also shown are residues p.His373 and p.His373Leu in green and p.Glu383 and p.Glu383Gly in yellow. Substitution of His by Leu at amino acid position 373 results in loss of catalytic activity. Mutation of the His-deficient mutant at position 373 or substitution of Glu with Gly at position 383 prevents the incorporation of a heme group, rendering the mutant enzyme inactive. The imidazole side chain of His is a common ligand for the metal protein and is part of the catalytic site of the enzyme. His can participate in acidic catalysis by proton and non-proton states, but Leu does not bind to heme because there is no imidazole branch. Images were created in Swiss PDB viewer (Swiss Institute of Bioinformatics) and UCSF Chimera version 1.11.2 (University of California).

[21,22]. In ILD, hypertension and hypokalemia are not detected as clinical symptoms of 17OHD since most 17-hydroxylation activity is preserved. Biochemical evidence of partial 17-hydroxylation defects can be obtained without clinical symptoms of hypertension or hypokalemia [23].

Previously we identified a compound heterozygous mutation, p.His373Leu/p.Glu383fsX36, in the *CYP17A1* gene [8]. Subcloning and sequencing using TOPO-TA cloning of RT-PCR products of proband RNA revealed that the c.1148delA mutation in exon 7 and the c.1118A>T mutation in exon 6 of the contralateral allele (Fig. 2). This compound heterozygous mutations appear to cause 17 α -hydroxylase and 17,20-lyase deficiency. The patient had a complete absence of secondary sexual development with hypergonadotropic hypogonadism, but no

hypertension and low-renin hypokalemia. The classic presentation of 17OHD in phenotypic females includes hypertension and primary amenorrhea [6,9,10]. Hypertension is typically seen in early adulthood and is a serious condition [24-26] and the extents of hypertension and hypokalemia vary from case to case (Table 1) [8,27-31]. Shin et al. [32] examined the clinical characteristics of six 17OHD and found that all six patients had hypertension and hypokalemia. Clinical symptoms such as hypertension and hypokalemia in women are less severe than those in men with the same compound heterozygous mutations, suggesting that phenotype expression of the *CYP17A1* gene depends on the karyotype [8,14,33].

In our case, the molecular approach helped to understand the genetic events leading to the 17OHD and to gather more infor-

mation on the genotype phenotype correlation. Analysis of WT and mutant constructs showed that expression of P450c17 in HEK293T cells was significantly reduced in the p.His373Leu construct compared to WT, while expression in c.1148delA-transfected HEK293T cells was not detectable either in terms of protein level or enzyme activity (Fig. 3). The p.Glu383fsX36 mutation appears to prevent production of a flag-tagged product due to premature termination, and to abolish enzyme activity. The 17 α -hydroxylase and 17,20-lyase activities were evaluated by measuring the conversions of progesterone to 17-OHP and the conversion of 17-PREG to DHEA, respectively. After incubation with 2.0 μ M progesterone, the concentrations of 17-OHP generated by the WT construct was 744 nmol/L. The 17 α -hydroxylase activity of the c.1118A>T mutant was reduced to 21.9 nmol/L and the 17,20-lyase activity of the c.1118A>T mutant was negligible (1.3 nmol/L), while the 17,20-lyase activity of the c.1148delA mutant was almost zero (Fig. 3C). As a result, enzyme activity measured in both mutants disappeared completely in both 17 α -hydroxylase and 17,20-lyase.

Three-dimensional computer modeling of the heme-binding site of the P450c17 enzyme showed that the catalytic activity of the enzyme is lost when His is replaced by Leu at amino acid position 373. The imidazole side chain of His is a common ligand for the metal protein and is part of the catalytic site of many enzymes [34]. It is believed that His has the ability to switch between proton and non-proton states, which allows it to participate in acid-base catalysis, but Leu does not bind heme because it lacks an imidazole side chain. As shown in Fig. 4, since the His is changed at position 373, the mutant enzyme does not form a complete heme-containing structure. The absence of His at position 373 is predicted to render the protein unable to form a hydrogen bond and thus it may have little activity. The c.1148delA mutant structure does not contain a heme group and does not have enzyme activity in spite of the presence of His at position 373.

Our patient had no hypertension and hypokalemia. Since no hypertension was observed in the patient, we expected that the activity of 17 α -hydroxylase would be conserved somewhat in *in vitro* study. However, when the individual vectors with the same mutations observed in the patient were introduced into HEK293T cells, not only the enzyme activity but also the protein expression could not be observed. Further research is needed to determine whether the *CYP17A1* mutation is associated with the phenotype as well as karyotype.

In summary, our patient is a compound heterozygote (p.His373Leu and p.Glu383fsX36) and 17 α -hydroxylase/17,20-

lyase activity was completely lost in an *in vitro* functional study of the two mutant *CYP17A1* expression vectors. This result provides the evidence that the enzymatic function of p.Glu383fsX36 is impaired by the mutation and accounts for the clinical manifestations of the patient with the compound heterozygous *CYP17A1* mutations.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Conception or design: E.Y.M., M.J.K., S.L., S.M. Acquisition, analysis, or interpretation of data: E.Y.M., J.L., E.S.K., S.M. Drafting the work or revising: E.Y.M., S.Y.K., S.M. Final approval of the manuscript: E.Y.M., J.H.H., S.M.

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