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# Aromatase deficiency in a Chinese adult man caused by novel compound heterozygous *CYP19A1* mutations: Effects of estrogen replacement therapy on the bone, lipid, liver and glucose metabolism

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ABSTRACT

*Objectives:* Aromatase deficiency is a rare disorder resulting in estrogen insufficiency in humans. It has been reported in remarkably few men with loss-of-function mutations in the *CYP19A1* gene encoding the aromatase, a cytochrome P450 enzyme that plays a crucial role in the biosynthesis of estrogens from androgens. We investigated a non-consanguineous family including an adult man with clinical features of aromatase deficiency, and studied the effects of estrogen replacement in the man.

*Methods:* We investigated the clinical and biochemical phenotype, performed *CYP19A1* mutational analysis in the family and 50 unrelated persons, studied the effects of *CYP19A1* mutations on aromatase protein structure, functionally characterized the mutations by cell-based aromatase activity assays, and studied the effects of estrogen replacement on the bone, lipid, liver and glucose metabolism.

*Results:* The man with clinical features of aromatase deficiency had novel compound heterozygous *CYP19A1* mutations (Y81C and L451P) that were not found in 50 unrelated persons. Three-dimensional modeling predicted that Y81C and L451P mutants disrupted protein structure. Functional studies on the basis of *in vitro* expression showed that Y81C and L45P mutants significantly decreased the aromatase activity and catalytic efficiency. Estrogen replacement in the man increased bone mineral density, accelerated bone maturation, improved lipid profile and liver steatosis, and improved glucose levels but not insulin resistance.

*Conclusions:* We have identified two novel *CYP19A1* missense mutations in an aromatase-deficient man. Estrogen replacement in the man shows great impact on recovering the impairments in the bone, lipid, liver and glucose metabolism, but fails to improve insulin resistance.



## 1. Introduction

Aromatase, an enzyme of the cytochrome P450 superfamily, plays a critical role in the biosynthesis of estrogens (C18 steroids) from androgens (C19 steroids) in vertebrate species (Simpson et al., 2002). In humans, aromatase is encoded by the *CYP19A1* gene located at chromosome 15q21.2 (Chen et al., 1988). In fertile women the ovary represents the major source of circulating estrogens, whereas in men the testes accounts for up to 15% of circulating estrogens. The remaining 85% comes from peripheral aromatization of circulating androgen precursors in different tissues, including the adipose tissue, brain, skin, endothelium and bone (Gennari et al., 2004).

Aromatase has important biological functions. Aromatase deficiency, which leads to estrogen deficiency, exerts major effects on bone metabolism, adiposity, lipid profiles, liver function, glucose metabolism, insulin sensitivity and sexual behavior (Santen et al., 2009).

*Abbreviations:* ALT, aminoleucine transferase; ArKO, aromatase knockout; AST, aspartate aminotransferase; AUC, area under the curve; β-CTX, c-telopeptide of type I collagen; BLAST, Basic Local Alignment Search Tools; BMI, body mass index; CHO, Chinese hamster ovary; DEXA, dual-energy X-ray absorptiometry; dNTP, deoxynucleotide triphosphate; FSH, follicle-stimulating hormone; GGT, γ-glutamy transferase; HDL, high density lipoprotein; HOMA, homeostasis model assessment; Km, Michaelis constant; LDL, low density lipoprotein; LH, luteinizing hormone; NCBI, National Center for Biotechnology Information; NHLBI-ESP, Exome Sequencing Project of the National Heart, Lung, and Blood Institute; OGTT, oral glucose-tolerance test; PolyPhen2, Polymorphism Phenotyping, version 2; QUICKI, quantitative insulin sensitivity check index; SIFT, Sorting Intolerant From Tolerant; Vmax, maximum reaction rate.

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The aromatase knockout (ArKO) mouse presents with elevated gonadotropins and testosterone levels, loss of bone mass, obesity, dyslipidemia, liver steatosis, insulin resistance and hyperglycemia, defects in folliculogenesis and spermatogenesis, infertility and impaired sexual behavior (Fisher et al., 1998; Honda et al., 1998; Jones et al., 2000; Nemoto et al., 2000; Robertson et al., 1999).

Aromatase deficiency is an extremely rare disorder in humans. To date, only a small number of cases of women (Bouchoucha et al., 2014; Hauri-Hohl et al., 2011; Zirilli et al., 2008) and men (Morishima et al., 1995; Carani et al., 1997; Deladoey et al., 1999; Herrmann et al., 2002; Maffei et al., 2004, 2007; Lanfranco et al., 2008) have been reported, and most of them are Caucasians. Affected women present with ambiguous genitalia at birth, elevated androgens and undetectable estrogens, primary amenorrhea, and failure of breast development at puberty. Men with aromatase deficiency usually present after puberty with continuing linear growth, tall stature, unfused epiphyses, delayed bone age, eunuchoid skeletal proportions, genu valgum, decreased bone mineral density, overweight or obese, dyslipidemia, liver steatosis, insulin resistance, and impaired fertility. Interestingly, one man and one unrelated woman with estrogen resistance caused by mutations in the estrogen receptor α (*ESR1*) gene have been reported, and their clinical presentations are similar to that of aromatase-deficient men and women (Quaynor et al., 2013; Smith et al., 1994). Due to the observations of women and men with mutations in the *CYP19A1* and *ESR1* genes, as well as the studies of animal models such as ArKO mice and estrogen receptor-knockout mice (Jones et al., 2006), the importance of estrogens in the male has been appreciated recently.

We present here a case of a 24-year-old man from a non-consanguineous family with clinical features of aromatase deficiency caused by novel compound heterozygous mutations in the *CYP19A1* gene, and the effects of estrogen replacement therapy in the man on the bone, lipid, liver and glucose metabolism.

## 2. Methods

### 2.1. Ethics

The study was approved by the Institutional Review Board of the Peking Union Medical College Hospital. Written informed consents were obtained from the patient and family, and 50 unrelated persons.

### 2.2. Hormonal and biochemical measurements

Serum estradiol, testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by chemiluminescent immunoassays (Bayer Diagnostics, East Walpole, USA). Osteocalcin and 24 h urine hydroxyproline were measured by radioimmunoassay (Beijing North Institute of Biological Technology, Beijing, China). Serum c-telopeptide of type I collagen (β-CTX) was determined by chemiluminescent immunoassays (E170; Roche Diagnostics, Basel, Switzerland). Alkaline phosphatase, calcium, phosphate, hemoglobin A1c, total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, aminoleucine transferase (ALT), aspartate aminotransferase (AST), γ-glutamy transferase (GGT) were assayed by a multichannel automatic biochemical analyzer (AU5800; Beckman Coulter, Mishima, Japan).

### 2.3. X-ray, bone densitometry and ultrasound

X-ray of hands, wrists, lumbar spine and knees were performed. Bone mineral density was assessed at the lumbar spine (L2–4) and femoral neck using dual-energy X-ray absorptiometry (DEXA) (Lunar Prodigy; GE Healthcare, Madison, USA). The T-score was defined as the deviation from the mean bone mineral density of healthy young adults of the same sex and ethnicity. The Z-score was defined as the deviation from the mean bone mineral density of age, sex and ethnicity matched population. Ultrasound of the liver was performed.

### 2.4. Oral glucose-tolerance testing

The patient underwent a 75-g oral glucose-tolerance test (OGTT) after fasting for 12 h. Plasma glucose and serum insulin levels were measured in blood samples obtained at 0, 30, 60, 120 and 180 minutes after oral glucose loading. Insulin resistance was estimated from the OGTT result and the fasting insulin level, the area under the curve (AUC) for insulin, the homeostasis model assessment (HOMA) of insulin resistance (Wallace et al., 2004), the Matsuda index (Matsuda and DeFronzo, 1999) and the quantitative insulin sensitivity check index (QUICKI) (Katz et al., 2000). Insulin secretion was estimated on the basis of the disposition index, which adjusts insulin secretion for the insulin sensitivity as measured by multiplying the insulinogenic index by the Matsuda index, with the insulinogenic index calculated as the ratio of incremental insulin to incremental glucose response during the first 30 minutes of the OGTT.

### 2.5. DNA sequence analysis

Genomic DNA was extracted from peripheral blood leukocytes of the patient, his parents and his brother, and 50 unrelated persons using QIAGEN DNA extraction kit (QIAGEN, Hilden, Germany). All nine coding exons and proximal splice sites of the *CYP19A1* gene (NCBI Reference Sequence: NM_000103.3) were amplified by PCR with *CYP19A1*-specific primers (Supplementary Table S1) (Mullis et al., 1997). PCR was carried out in 50 μg reactions containing 100 ng genomic DNA, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 20 pmol of each primer and 1.2 U Taq-DNA polymerase for 35 cycles. DNA sequences of both the sense and antisense strands of each PCR product were sequenced in duplicate with an ABI3730 automated sequencer (Applied Biosystems, Foster City, USA), followed by analysis with the sequence of the *CYP19A1* gene using Basic Local Alignment Search Tools (BLAST) software. Sequence changes were assessed for the occurrence of polymorphisms in 50 unrelated persons and in the exome sequence data from approximately 6500 unrelated persons that were obtained from the Exome Sequencing Project of the National Heart, Lung, and Blood Institute (NHLBI-ESP) (http://evs.gs.washington.edu/EVS/).

### 2.6. Prediction of missense variants, protein sequence alignments and three-dimensional structure modeling

The Sorting Intolerant From Tolerant (SIFT) (Kumar et al., 2009) and the Polymorphism Phenotyping, version 2 (PolyPhen2) (Adzhubei et al., 2010) programs were used to predict whether a missense mutation affects protein function. Sequences of aromatase orthologs and paralogs were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) (Supplementary Table S2), and aligned using the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk). Three-dimensional homology modeling of human aromatase mutants was based on the crystal structure of Cytochrome P450 19A1 (Protein Data Bank code 3EQM) (Ghosh et al., 2009) with the use of the PyMOL Molecular Graphics System (http://www.pymol.org).