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Case report

Rare nonclassic type of Congenital adrenal hyperplasia due to 21-hydroxylase deficiency and genotype-phenotypic correlation

Yanru Hou, Yian Li, Jiajia Ai, Li Tian *

Reproductive Medicine Center, Peking University People's Hospital, Peking University Health Science Center, Beijing, China

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ABSTRACT

Objective: To explore the correlation between different CYP21A2 pathogenic gene mutations and clinical phenotypes in Congenital adrenal hyperplasia (CAH) patients. Moreover, combined with the specific phenotypes of patients in the clinic, diagnosis and treatment suggestions should be made for CAH patients.

Methods: In this study, a genetic status of a Chinese family in three generations of 21-hydroxylase deficiency was comprehensively presented, and the pathogenic genes in the family were found and traced in detail. We measured CYP21A2 gene in this family by Sanger sequencing and MLPA. The trophoblast cells of female proband's embryos were detected by PGT-M which used Copy-Number Variations of a Single Human Cell and high throughput sequencing. The CYP21A2 gene mutation site in each embryo were detected by Sanger sequencing, whole genome sequencing and single nucleotide polymorphism (SNP).

Results: There are many related pathogenic genes of CAH in this family. The female proband showed a compound heterozygous mutation in the CYP21A2 gene, including a CYP21A1P/A2 fusion gene (CH-8) (classical phenotype) and a new mutation c.1034T > C (p. L354S) (unknown clinical significance). In the proband's family, a heterozygous gene mutation of c.1034T > C and a CYP21A1P/A2 fusion gene (CH-8) was carried by her father and mother, respectively. Meanwhile, the husband of the proband also has a genetic family with related disease. Both the husband and his father carried the CYP21A2 gene c.844G > T heterozygous mutation, while his mother had no related mutation in the CYP21A2 gene. Furthermore, PGTM gene detection was carried out on the four blastocysts of the proband's offspring through IVF. The results showed that embryos T1, T2 and T4 all carried CYP21A1P/A2 fusion gene (CH-8), as well as embryo T3 carried c.1034T > C heterozygous mutation of maternal origin.

Conclusion: This case is a family report showing a complete genetic map of the proband and her family, describing the genetic process of different pathogenic genes in detail and clearly corresponding to the patient's different phenotypes. It is speculated that the pathogenesis of CAH is caused by different mutations in the CYP21A2 gene and their interactions, which may affect the different phenotypes of CAH patients.

1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of genetic diseases mainly caused by mutations in the CYP21A2 gene encoding the

* Corresponding author.
E-mail address: tlhyrsci@126.com (L. Tian).

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adrenal steroid 21-hydroxylase enzyme (P450c21), leading to defects in adrenal steroidogenesis. It is an autosomal recessive hereditary disease [1]. The mutation of 21-hydroxylase enzyme leads to a decrease in cortisol production, resulting in an increase in the secretion of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), leading to adrenal cortex hyperplasia [2]. As a result, more adrenal androgens are found in excess. CAH can be distinguished clinically in two forms, "classic" and "non-classic" (NCCAH) [3], and the clinical distinction of them depends on the severity of the clinical expression of this deficiency [4]. NCCAH is usually asymptomatic before five years of age and is diagnosed during puberty, especially in patients visiting a fertility clinic. The main characteristics of NCCAH are anovulatory cycle and/or high androgen concentration, which are related to infertility. NCCAH patients often seek medical attention at reproductive medicine center due to infertility, as there is a significant phenotypic overlap between NCCAH and polycystic ovarian syndrome (PCOS), leading to misdiagnosis (1). During the process of assisted reproductive therapy for NCCAH patients, there may be an increase in circulating progesterone concentrations during the follicular phase, as well as anovulatory cycles related to excessive androgens, resulting in abnormal tubal motility, cervical thickness and endometrial receptivity [5].

For adult women with NCCAH, if there is no need for fertility, the main treatment methods for hyperandrogenism are oral contraceptives and anti-androgen therapy. However, for patients who can't tolerate this treatment, glucocorticoid therapy is more preferred for hyperandrogenic symptoms and menstrual cycle management [3,6].

The incidence of classical CAH is 1:10000–1:20000 live births, whereas the incidence of NCCAH is 1:1000 live births [7,8]. The overall prevalence of NCCAH in women with symptoms of androgen excess is approximately 4% [9]. Due to racial differences, the prevalence of 21OHD NCCAH ranges from 1:1000 to 1:2000 [10].

Genetic test is an important diagnostic tool for the diagnosis of NCCAH. It can be used when biochemical results are uncertain or genetic counseling is required before pregnancy in order to identify the CYP21A2 genotyping and the heterozygous carriers. CYP21A2 genotyping is a valuable complement to biochemical investigations. To date, more than 200 mutations of the CYP21A2 gene have been reported in different studies [11], and although there is good agreement between clinical phenotype and patient genotype [3,12,13], much energy is still focused on the clinical phenotype of compound CYP21A2 pathogenic mutations gene and their interactions. In this study, a new CYP21A2 mutations gene was detected by multiple genetic tests (Gene sequencing, MLPA, CNVs and SNP), Moreover, the correlation between clinical manifestation and gene phenotype was found by studying the new gene mutation sites. Interestingly, this early misdiagnosis case provides us with the opportunity to learn about NCCAH from genetics and the clinic and the possibility to explore the correlation between multiple gene mutations and clinical manifestations. This study further provides a reference for the future clinical work and scientific research direction of CAH.

2. Methods

2.1. Clinical phenotype

The patient was misdiagnosed in other hospitals in the early stage, and came to our center with PCOS and infertility. Then, she was given ovulation induction treatment for several cycles; the follicles were well developed but did not lead to pregnancy, and the treatment failed many times. Therefore, IVF treatment was required by the patient. During the process of controlled ovarian stimulation (COS), the follicles developed well, and the hormone level showed that the E2 level increased, but the P remained at an abnormally high level of 3.29–5.98 ng/ml. Oocyte Pick Up (OPU) led to 16 eggs from which we obtained 9 blastocysts, all of which were frozen. In the process of COS, the abnormal high progesterone status persists, which is different from the hormone level of the normal PCOS population. Therefore, it was suggested that the patient should to be performed a genetic testing, and the results showed that the patient (proband) had NCCAH. To identify the source of the pathogenic gene and guide pregnancy, a comprehensive genetic analysis of other family members (including the embryos of the offspring) were subsequently carried out.

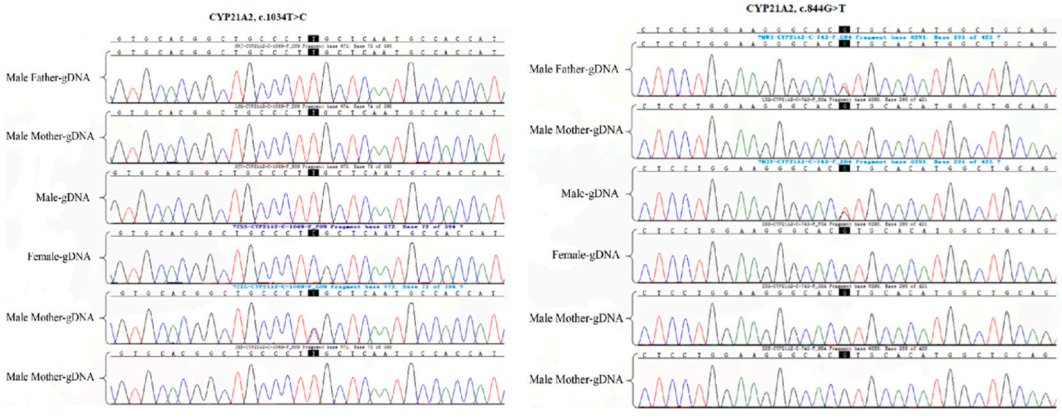

Fig. 1. Detection of CYP21A2 gene mutation site in the family by Sanger sequencing. The female proband and her father had a CYP21A2 mutation gene c.1034T > C (p. L354S), moreover, the husband of the proband and the male father carried the CYP21A2 gene c.844G > T.

2.2. Genetic tests

The CYP21A2 gene mutation sites were measured by Sanger sequencingand the CYP21A1P/A2 fusion gene were detected by Multiplex ligation-dependent probe amplification (MPLA) in the family. Furthermore, the trophoblast cells of female proband's embryos were detected by PGT-M(Preimplantation genetic testing for monogenetic disease) which using Copy-Number Variations of a Single Human Cell and high-throughput sequencing. The CYP21A2 gene mutation sites and the source of CYP21A1P/A2 fusion gene in each embryo were detected by Sanger sequencing, whole genome sequencing (WGS) and single nucleotide polymorphism (SNP).

3. Results

The Sanger sequencing showed that the female proband and her father had a CYP21A2 mutation gene c.1034T > C (p. L354S), moreover, the husband of the proband and the male father carried the CYP21A2 gene c.844G > T (Fig. 1). The detection of CYP21A2

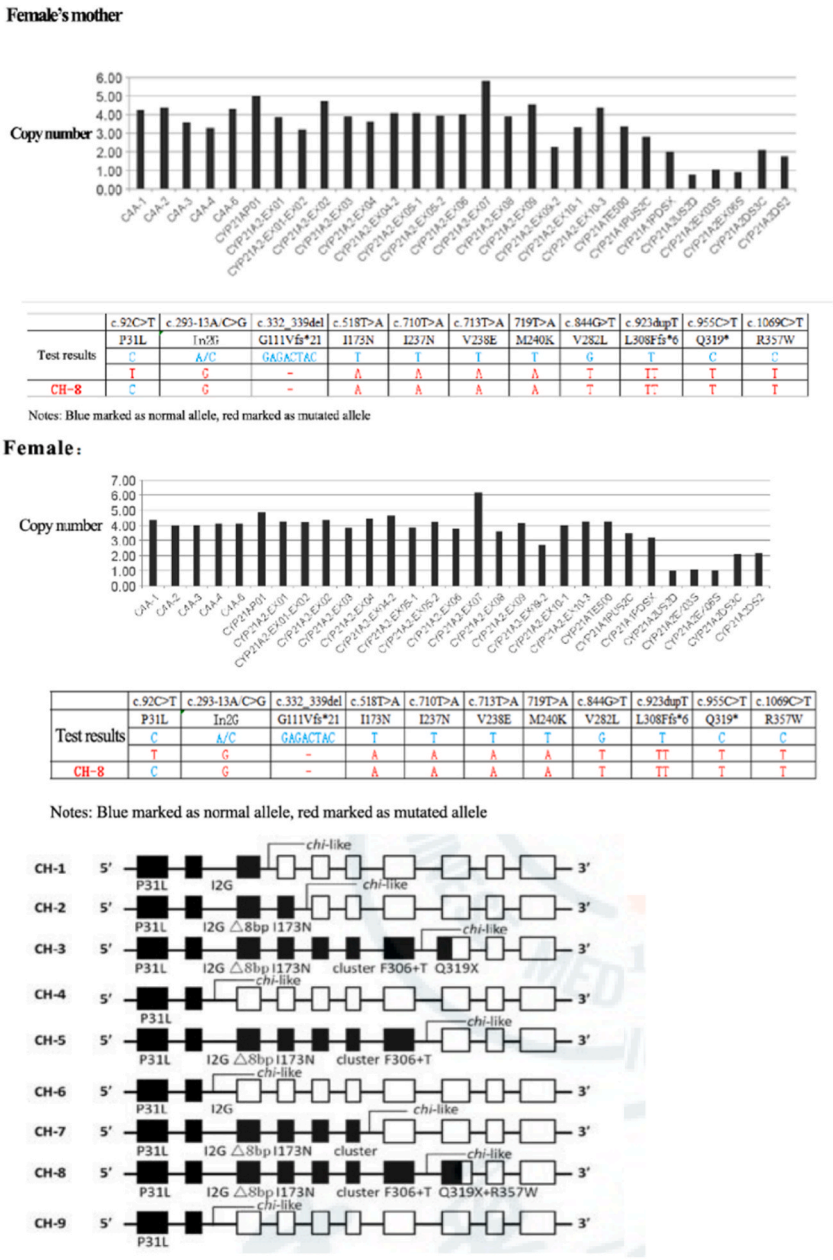

Fig. 2. Detection of CYP21A2 fusion gene in the family by MLPA, which presented CYP21A2 mutation gene site. The female proband and her mother carry CYP21A2, CYP21A1P/A2 fusion gene (CH-8), and MLPA showed exon1-7 deletion.

fusion gene in the family by MLPA, which presented that the female proband and her mother carried CYP21A2, CYP21A1P/A2 fusion gene (CH-8), and MPLA showed exon1-7 deletion (Fig. 2). Furthermore, the trophoblast cells of female proband's embryos were detected by PGT-M. The trophoblast cells of each embryo were detected by CNVs and high-throughput sequencing, and The CYP21A2 gene mutation sites and the source of CYP21A1P/A2 fusion gene in each embryo were detected by Sanger sequencing, WGS and SNP. The results showed that embryos T1, T2 and T4 all carried CYP21A1P/A2 fusion gene (CH-8), as well as embryo T3 carried c.1034T > C heterozygous mutation of maternal origin (Figs. 3 and 4).

The CYP21A2 genetic analysis showed that the proband was heterozygote for CYP21A2 and CYP21A1P/A2 fusion genes (CH-8). Another new heterozygote for the c.1034T > C (p. L354S) mutation at different sites of the same gene was detected. Further detection of the proband's family revealed that the pathogenic gene mutation carried by the proband's father was CYP21A2, c.1034T > C, and the pathogenic gene carried by her mother was CYP21A2, and CYP21A1P/A2 fusion gene (CH-8). The proband's husband simultaneously had a genetic family with related diseases. The pathogenic gene carried by this male patient and his father was CYP21A2, c.844G > T. His mother did not carry the CYP21A2-related gene mutation (Fig. 5). Because of the very rare CAH pathogenic gene family aggregation, PGTM gene detection was performed on the four blastocysts of the proband and her spouse through IVF. The results showed that the T1, T2 and T4 embryos all carried CYP21A2 and CYP21A1P/A2 fusion genes (CH-8), and the T3 embryo carried CYP21A2 and c.1034T > C from the proband, respectively (Fig. 5).

MLPA showed the deletion of exons 1–7 in CYP21A1P/A2 fusion genes and the clinical manifestation of the mutation was the classic type of CAH, with the enzyme activity was 0. The new point mutation c.1034T > C (p. L354S), has not been reported in previous studies, and its clinical significance is unknown. The gene mutation of CYP21A2, c.844G > T (V282 L), detected by the male, is pathogenic and belongs to the nonclassical type, with enzyme activity reduced by 20–60%. Considering that the proband's clinical symptoms were mild and her enzyme activity was not completely lost, it is speculated that the gene mutation of c.1034T > C results in less loss of enzyme activity. Four blastocysts were thawed and survived, and PGT was performed. According to the PGTM test results of embryos, considering sex, the test data showed that T3 > T4 > T1 > T2. Meanwhile, the transfer sequence was comprehensively evaluated in combination with other clinical indicators, such as embryo rating. Then, the patient waited for the results of PGT and

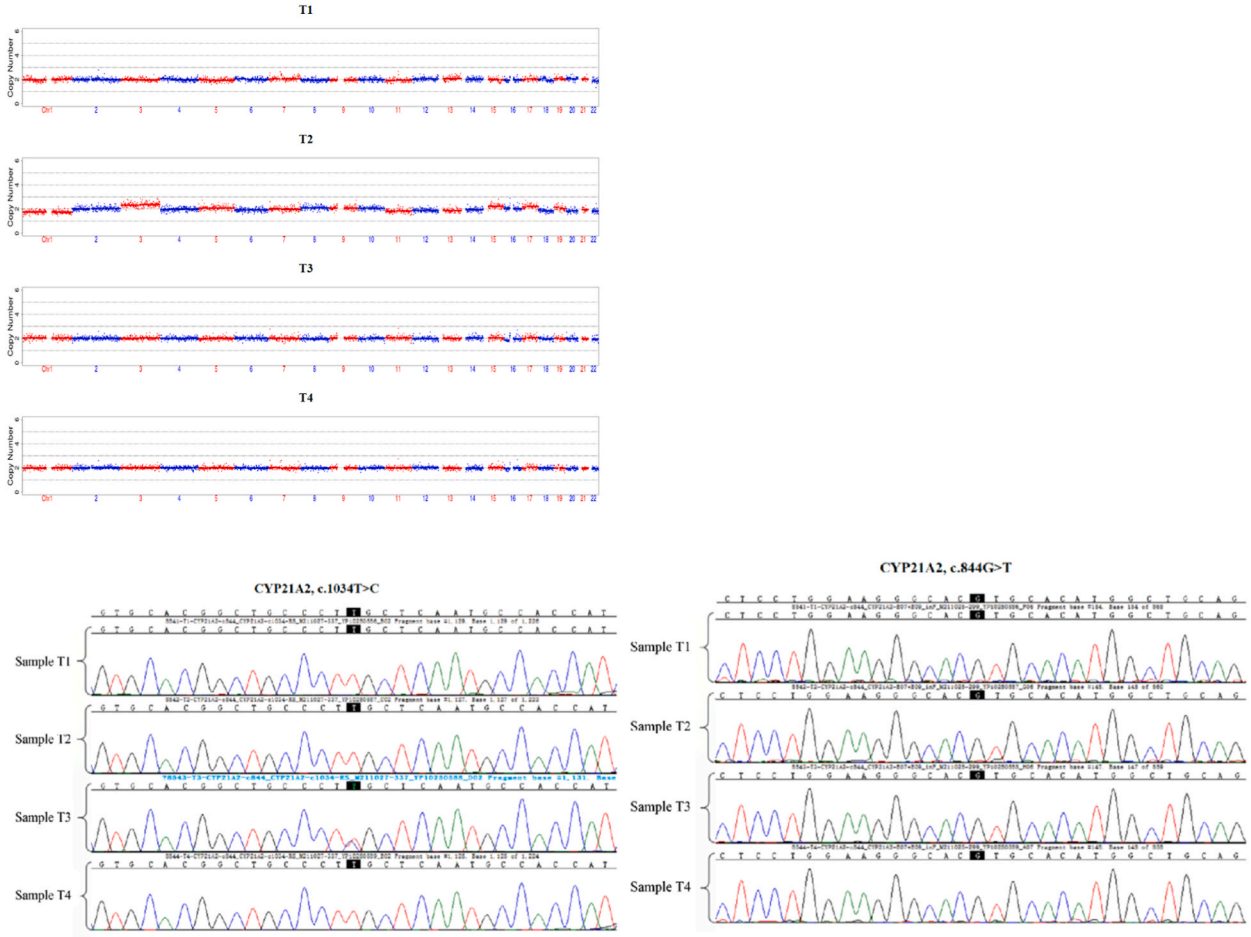

Fig. 3. Detection of trophoblast cells of each embryo by CNVs and high-throughput sequencing, and CYP21A2 mutation gene site of each embryo by Sanger sequencing. The embryo T3 carried c.1034T > C heterozygous mutation of maternal origin.

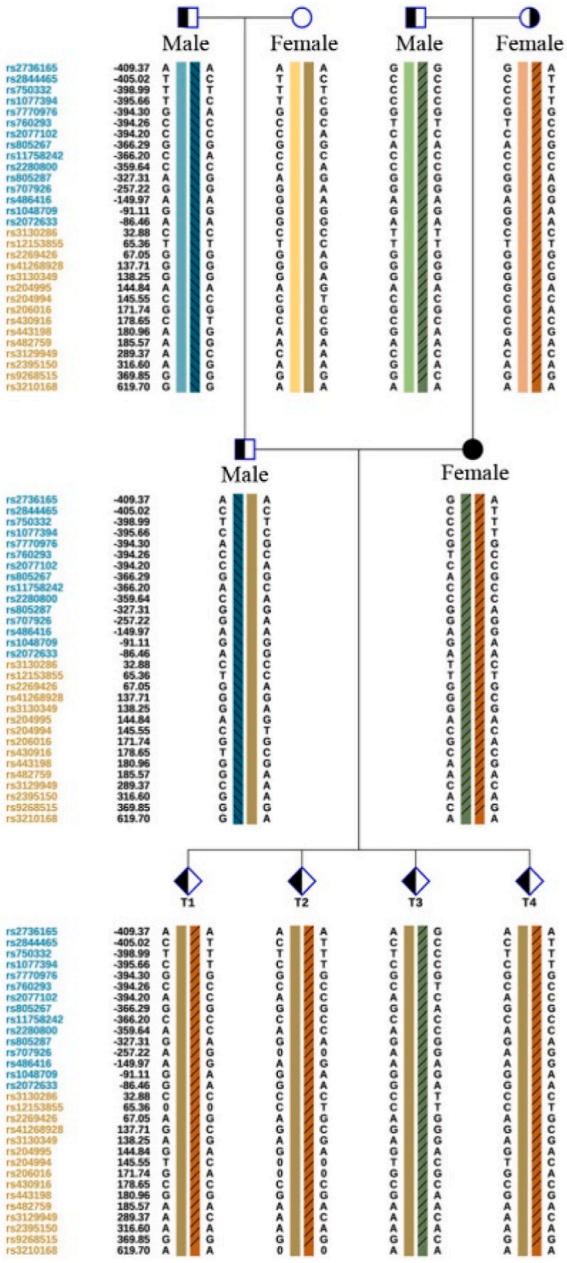

Fig. 4. Detection of CYP21A2 gene mutation site and source of CYP21A1P/A2 fusion gene in each embryo by WGS + SNP. The embryos T1, T2 and T4 all carried CYP21A1P/A2 fusion gene (CH-8).

received glucocorticoid treatment. Unexpectedly, she became pregnant naturally. At present, she was treated with prednisone acetate orally. The intrauterine pregnancy was 32 weeks, and the pregnancy continued. The patient underwent noninvasive DNA testing, and the fetus was normal. The patient refused to continue amniocentesis or umbilical cord blood puncture for fetal genetic testing. At present, the patient is under close follow-up.

4. Discussion

21OHD CAH is an autosomal recessive disease. The most common form of CAH is due to 21-hydroxylase deficiency resulting from mutations in CYP21A2 gene. A study in 2022 shows that a high prevalence of CYP21A2 gene mutations among 16 families of CAH patients. The presence of six most common gene mutations were Intron 2 (c.293-13A/C > G), c.844G > T, c.1019G > A, c.92C > T, c.955C > T and c.518T > A in CYP21A2 gene [14]. Previous studies have shown that more than 200 mutations of the CYP21A2 gene

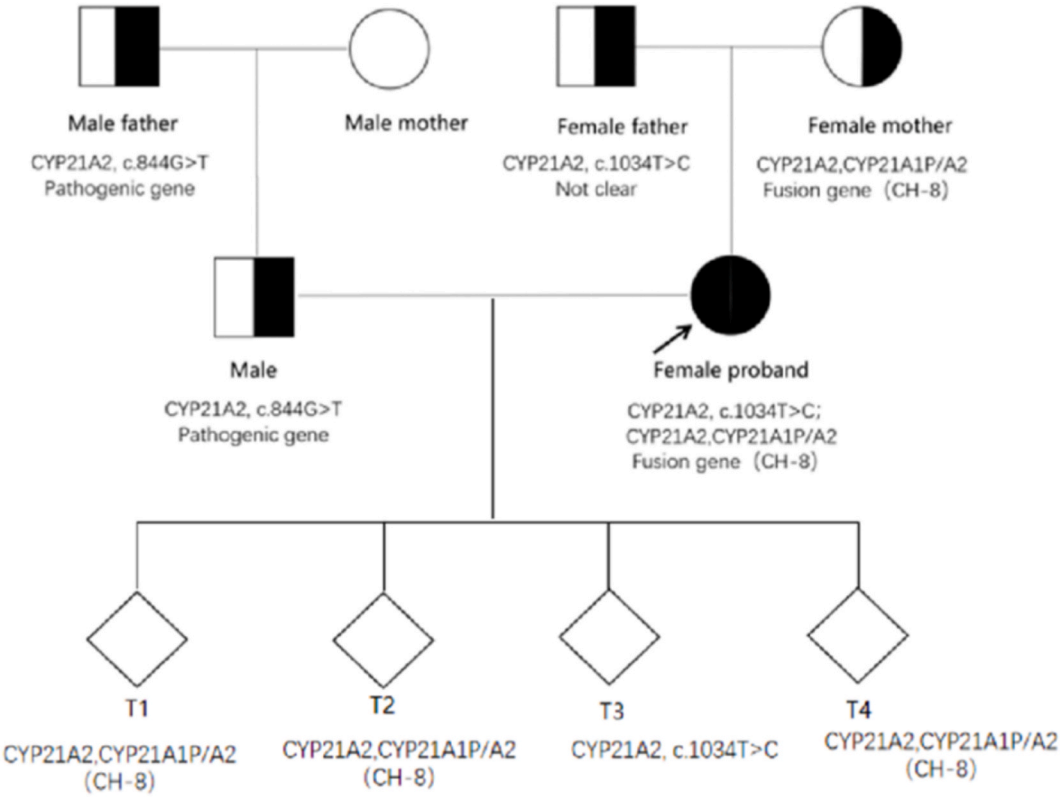

Fig. 5. Family map of CYP21A2 pathogenic gene mutations.

have been reported in different studies [11]. In this study, a genetic status of a Chinese family in three generations of 21-hydroxylase deficiency was comprehensively presented, and the pathogenic genes in the family were found and traced in detail. And a new CYP21A2 mutations gene was detected by multiple genetic tests. After an analysis on the correlation between clinical manifestation and gene phenotype of this family, the new gene mutation site was further known.

4.1. Diagnosis and differential diagnosis of NCCAH

The patients are often masculinized and experience salt wasting due to the excessive synthesis of androgens, which manifests as female masculinity and male precocious puberty. In clinical practice, it belongs to the management of the endocrinology professional subfamily. Some endocrinology-related studies have suggested that, for 21OHD, the diagnosis relies on 17OHP elevations, which span a gradient reflective of the spectrum of enzymatic defects. Both neonatal and clinically prompted screening for 21OHD consists of 17OHP measurement, and values $>$ 200 ng/dL (6 nmol/L) are suggestive of the diagnosis. Patients with classic 21OHD typically have 17OHP concentrations above 10,000 ng/dL. In patients with modest 17OHP baseline elevations of 200–1000 ng/dL (6–30 nmol/L), a cosyntropin stimulation test is the current standard examination method, and 17OHP values $>$ 1000 ng/dL can be diagnosed [15]. However, in actual work, because a considerable proportion of the disease is caused by atypical clinical symptoms, female patients often go to the reproductive clinic for infertility. Thus, misdiagnosis or missed diagnosis often occurs in the clinical diagnosis and treatment process.

The proband in this case was diagnosed with PCOS and primary infertility. Ovulation induction treatment failed many times in other hospitals, and IVF was required for this treatment. Due to the persistent abnormal high progesterone status during COS, the abnormality caused by metabolism was not excluded and finally diagnosed by genetic testing. This suggests that, in clinical work, NCCAH and PCOS are often confused because of their phenotypic commonality. Some studies [5] have suggested that the clinical features of NCCAH and PCOS include hirsutism, ovulation and menstrual disorder. It is difficult to distinguish these two syndromes based on clinical reasons alone. In addition, both NCCAH and PCOS are associated with obesity, insulin resistance and dyslipidemia. Therefore, once other diseases similar to CAH are excluded, such as diseases related to ovulation reduction or anovulation and/or androgen excess (hyperprolactinemia, thyroid disease, polycystic ovary syndrome and androgen producing tumor, etc.), NCCAH screening can be considered.

4.2. Correlation between different genotypes and phenotypes

4.2.1. Clinical significance of genetic mutation types and phenotypes in the female proband

The pathogenic gene CYP21A2 and its highly homologous pseudogene CYP21A1P of 21-OHD are arranged in tandem on the short arm of chromosome 6 (6p21.3) [16]. When the CYP21A2 gene and the pseudogene CYP21A1P form the CYP21A1P/A2 fusion gene, since CYP21A1P does not encode 21 hydroxylase, it may lead to the copy number deletion of different lengths of the CYP21A2 gene and further damage the activity of the 21-hydroxylase encoded by the CYP21A2 gene [17]. Currently, 9 types of fusion genes have been reported internationally, which include gene mutations of the classic CAH type [18]. In the classical chimera group, six different linkage sites have been reported, which are named CH-1, CH-2, CH-3, CH-5, CH-6 and CH-7 [19,20]. In addition, CH-8 is a relatively novel mutation type, and its gene mutation includes the In2G mutation. If the connecting site of the pseudogene occurs upstream of In2G, it is called an "uncommon" chimera, and its 21-hydroxylase activity is slightly impaired [21,22]. Combined with the fact that the female proband in this case carried the CYP21A2, CYP21A1P/A2 fusion gene (CH-8) and CYP21A2, c.1034T > C (p. L354S), it can be speculated that the different phenotypes of CAH patients may be affected by the different types of chimeras of the CYP21A1P/A2 fusion gene on 21-hydroxylase; that is, the chimeras can maintain the partial activity of 21-hydroxylase. Alternatively, different types of gene mutations and the interaction between them, that is, the severity of the disease, may be related to the pathogenic mutation carried by another allele of the nonfusion gene; thus, the phenotype of the disease presents as NCCAH that is more conducive to the growth and development of the body (the proband only showed menstrual disorder, infertility and continuous increase of progesterone) rather than a single and absolute clinical phenotype.

4.2.2. Clinical significance of genetic mutation types and phenotypes of other family members (including offspring)

Because NCCAH has atypical symptoms, the detection rate of NCCAH may be lower than the actual incidence rate in clinical practice, especially in male patients. Therefore, it is rare to report on the basis of family. Liu Lijun et al. [23] reported that the siblings of a family in Henan, China all suffered from CAH. Although the parents of the two sisters were not consanguineously married, both were heterozygous for the CYP21A2 gene (both parents were carriers), which ultimately led to the early onset of CAH and obvious symptoms in both sisters. In this family, the proband's father carried CYP21A2, c.1034T > C, and the female mother carried the CYP21A2, CYP21A1P/A2 fusion gene (CH-8). The proband's husband and his father were carriers of the CAH pathogenic gene. Because CAH is an autosomal recessive disorder, there is a 1/4 probability of CAH in children per birth; if the proband's spouse is the same carrier, there is a 1/2 probability of CAH in children per birth. Therefore, the proband's family carried out genetic testing on their parents and genetic counseling to assess the risk of disease in their offspring. Because the clinical phenotype of CAH varies greatly among patients of different sexes, the proband's family was encouraged to adopt IVF and PGT testing to achieve eugenics when necessary.

According to the genetic testing report of this case, the CYP21A2 gene mutation sites of the four embryos were all from the female proband, while the pathogenic gene of the male was not transmitted to the offspring. Among the four embryos of the offspring, three contained the same fusion gene (CH-8) mutation of the proband, and one (T3) contained the c1034 point mutation. Therefore, in combination with the current research on CAH-related pathogenesis genes, it is recommended to use T3 embryo for transplantation. However, since the patient naturally became pregnant after treatment of the primary disease and refused to perform genetic testing for the fetus due to personal wishes, it will also necessary to closely follow up the situation of the newborn for a long time, including the examination of the newborn's external genitalia as a routine examination item. If external genitalia deformity, clitoris hypertrophy or penis hypertrophy occurs; the sex of the external genitalia is difficult to distinguish; or the areola and pigmentation of the external genitalia of the newborn are abnormal, the disease should be highly suspected. If the sex of the newborn is female, genetic testing should be recommended for the newborn and her spouse, and relevant guidance for subsequent childbirth should be provided. In addition, the clinical significance of CYP21A2 c.1034T > C was further suggested by the follow-up of the incidence and genetic testing of the offspring.

5. Conclusion

This case is a family report showing a complete genetic map of the proband and her family, describing the genetic process of different pathogenic genes in detail and clearly corresponding to the patient's different phenotypes. It is speculated that the pathogenesis of CAH is caused by different mutations in the CYP21A2 gene and their interactions, which may affect the different phenotypes of CAH patients. However, the one-to-one correspondence between the specific phenotype of a single individual and the CYP21A2 mutation has not yet been clearly determined, and subsequent reports of more disease-related content are needed. In clinical work, it is also necessary to pay attention to the diagnosis and treatment of CAH patients with multiple types of gene mutations and strive to achieve early identification to avoid misdiagnosis and missed diagnosis of patients due to the NCCAH phenotype.

Ethics statement

Ethics Committee of Peking University People's Hospital that approved the study and the committee's reference number was 2021PHB083-001. And all participants gave informed consent.

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Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

CRediT authorship contribution statement

Yanru Hou: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Yian Li:** Investigation, Data curation. **Jiajia Ai:** Methodology, Investigation, Data curation. **Li Tian:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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