Ding *et al. Reproductive Biology and Endocrinology* (2024) 22:140
https://doi.org/10.1186/s12958-024-01309-4

Reproductive Biology and Endocrinology

RESEARCH Open Access

Identification of novel variants and candidate genes in women with 46,XX complete gonadal dysgenesis

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Abstract

Background 46,XX complete gonadal dysgenesis (46,XX-CGD) is a rare disorder of sexual development (DSD) characterized by primary amenorrhea and a lack of spontaneous pubertal development in individuals with a 46,XX karyotype despite the presence of female internal and external genitalia due to failure of bilateral ovarian development. The condition is genetically heterogeneous, and in most cases, its etiology is unknown. Determining the genetic cause would provide insights into potential targets for genetic diagnosis and counseling.

Methods To clarify the molecular mechanisms of 46,XX complete gonadal dysgenesis in the population of China, whole-exome sequencing (WES) was performed on DNA samples from patients with 46,XX-CGD. In silico analysis was conducted to predict the pathogenicity of the variants.

Results We recruited 20 patients with 46,XX-CGD and identified 8 variants in 6 genes, including three homozygous variants in *MCM9*, *POF1B*, and *PSMC3IP*; compound heterozygous variants in *TWNK*; and three heterozygous variants in *TP63* and *INSRR*, from 7 patients. These variants included 3 recurrent variants and 5 novel variants.

Conclusions This study identified several novel variants, broadening the variant spectrum of 46,XX-CGD. 46,XX-CGD is a genetically heterogeneous condition, and WES is a powerful tool for determining its genetic etiology. The results of this study will aid researchers and clinicians in genetic counseling and suggest that WES is valuable for detecting 46,XX-CGD, which may lead to early interventions for patients.

Keywords DSD, 46,XX-CGD, Pathogenic variants, WES, Genetic etiology

Introduction

Disorders of sex development (DSDs) are defined as congenital conditions in which the development of chromosomal, gonadal, or anatomic sex is atypical, affecting approximately 1 in 4500–5000 individuals. The new Chicago consensus proposed in 2006 subdivided DSDs into three categories: 46,XY DSD, 46,XX DSD, and sex chromosome DSDs [1]. 46,XX-CGD without characteristics of Turner syndrome (TS) is a rare form of DSD that is characterized by primary amenorrhea, a lack of spontaneous pubertal development, and underdeveloped (streak) or absent gonads in individuals with a 46,XX karyotype

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despite the presence of female internal and external genitalia. These symptoms are due to the failure of bilateral ovarian differentiation during the embryonic period. Patients present with hypergonadotropic hypogonadism, and hypoplastic internal and external genitalia, including the fallopian tubes, uterus, and vagina. Gonadal hypoplasia manifests as streaked gonads, and menstruation can occur during artificial cycles. These patients often present to the hospital with pubertal breast failure or primary amenorrhea [2].

Normal ovarian development and maintenance depend on a precisely timed cascade of events, including gonadogenesis, oogenesis, folliculogenesis, and oocyte maturation and ovulation [3]. Therefore, a variety of potential causes can lead to ovarian dysfunction. Genetic variants can only explain a small fraction of the cause [4]. Genetic variants in several known genes involved in early gonadal development, such as *FOXL2* [5], *NR5A1* [6], and *WNT4* [7], have been implicated in 46,XX -CGD. Taking advantage of next-generation sequencing approaches, new variants in genes important for oocyte meiosis, homologous recombination, and DNA damage and repair, such as *STAG3*, *SYCE1*, *MCM8*, *MCM9*, *SPIDR*, *MARF1*, *PSMC3IP*, *BRCA2* and *ESR2* [8] involved in estrogen signaling are constantly being discovered. However, the etiology of 75–90% of cases remains unknown [9]. The genetic heterogeneity and rarity of the disorder suggest that additional pathogenic variants and genes remain undiscovered.

In this study, we performed WES of DNA samples obtained from 20 unrelated patients with 46,XX-CGD and identified novel variants in known genes and proposed candidate genes in 7 patients. The findings from this study expand the genetic spectrum of 46,XX-CGD. These findings will help clarify the molecular mechanisms underlying 46,XX-CGD and provide insights into potential targets for genetic diagnosis and counseling.

Materials and methods

Patient recruitment and clinical evaluation

Patients diagnosed with 46,XX-CGD were recruited from the Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility Center of the Peking Union Medical College Hospital from 2022 to 2024. The diagnosis of 46,XX-CGD was based on clinical features, including primary amenorrhea, a lack of spontaneous pubertal development, and elevated serum levels of FSH (FSH>40 IU/L). Additionally, all patients presented with juvenile female external genitalia and a 46,XX karyotype. Breast development was recorded based on the Tanner stage. All hormone analyses in this study were performed at the same laboratory in the hospital. Serum estradiol (E2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels were measured via an automated Elecsys Immunoanalyzer (Beckmann). Enzyme deficiencies associated with steroid synthesis, such as 17α-hydroxylase deficiency (17OHD), cytochrome P450 oxidoreductase deficiency (PORD), and steroidogenic acute regulatory (StAR) deficiency, were excluded from this study. Informed written consent was obtained from all participants or their legal guardians if the patient was under 18 years of age. The study protocol was reviewed and approved by the Peking Union Medical College Hospital Ethics Committee (No. JS-2510).

Whole-exome sequencing and assessment of variants

Genomic DNA was extracted from the peripheral leukocytes of all patients using a QIAamp DNA Blood Mini Kit (Qiagen, Germany). WES was performed on the Illumina HiSeq 2500 platform (Illumina, USA) with a paired-end read length of 150 bp. The SureSelect Human All Exon V6 Kit (Agilent Technologies, USA) was used for exome capture. The sequencing reads were aligned to the human reference genome (GRCh37) via the Burrows–Wheeler Aligner (BWA). Variants were called using the Genome Analysis Toolkit (GATK) and annotated with ANNOVAR. Variants were filtered based on quality, population frequency, and predicted impact on protein function. Variants with a minor allele frequency (MAF)>1% in public databases (1000 Genomes Project, Exome Aggregation Consortium, and Genome Aggregation Database) were excluded. Pathogenicity was predicted via in silico tools such as SIFT, PolyPhen-2, PROVEAN, and Mutation Taster. Variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines. Segregation analysis was performed on available family members to confirm inheritance patterns.

Structure prediction

Orthologous proteins of TP63, POF1B, and PSMC3IP were obtained from the Ensemble Genome Browser and aligned via the Clustal Omega multiple sequence alignment tool (EMBL-EBI) to evaluate evolutionary conservation. The missense variants of uncertain significance, p.Val642Gly in TP63, p.Lys311Thr in POF1B, and p.Gln26Pro in PSMC3IP, were modeled via SWISS-MODEL based on the structures of TP63 (PDB ID: Q9H3D4), POF1B (PDB ID: Q8WVV4.1) and PSMC3IP (PDB ID: Q9P2W). Structural analyses were performed via PyMOL to observe the effects of missense variants on the spatial structure of the protein.

Results

Clinical features

All 20 patients were clinically diagnosed with 46,XX-CGD according to standard criteria (detailed in the

Methods section). All of these patients presented with primary amenorrhea or lack of spontaneous breast development and visited a hospital at 13 to 16 years of age. Based on the physical examination results, none of the patients showed obvious abnormalities in physical development. The ovaries of all patients could be detected as underdeveloped by pelvic ultrasound examination. However, all patients presented with hypergonadotropic hypogonadism with an elevated level of FSH (50.2–105.77 IU/L), accompanied by a decreased level of E2 (<5–51 pg/ml). All patients had a poor level of breast development, with Tanner Grades I-II. The clinical characteristics of the patients with positive sequencing results are shown in Table 1.

Identification of novel variants

To characterize the genetic pathogenesis of 46,XX-CGD, we performed WES on the DNA samples of the 20 patients. The variants with a minor allele frequency of less than 1% in public databases (1000 Genomes Project, Exome Aggregation Consortium, and Genome Aggregation Database) were retained. We identified 8 variants in 6 genes, with 3 recurrent variants and 5 novel variants, including three homozygous variants in *MCM9*, *POF1B*, and *PSMC3IP*; compound heterozygous variants in *TWNK*; and three heterozygous variants in *TP63* and *INSRR* from 7 patients (Table 1). Based on the ACMG guidelines, 4 variants were classified as pathogenic (P) or likely pathogenic (LP) variants and 4 variants were defined as variants of uncertain significance (VUSs). The homozygous variant c.1151-1G>A of *MCM9* was confirmed in P1 and inherited from unaffected parents, consistent with an autosomal recessive inheritance pattern. P2 harbored two compound heterozygous variants in *TWNK* (c.1814delT and c.-722G>T), which were inherited from unaffected parents. The heterozygous variants c.1928G>A and c.1925T>G in *TP63* were de novo variants. The variants c.2217–2 A>C in *INSRR* and C.932 A>C in *POF1B* were identified in patients and their fathers. The heterozygous variant c.77 A>C in *PSMC3IP* was identified in P7. The inheritance patterns of each disease are described in Table 1.

Analysis of the novel variants identified in patients with 46,XX-CGD

We evaluated the 8 variants via in silico analysis. First, the frequency of the variants was assessed. Data from the gnomAD database, a rich and informative database that contains exome data from the ExAc and 1000G databases, and data from many other databases suggest that all of these variants are rare and that seven are not present in the database. These results indicate that the frequency of these variants in the population is extremely low, which is compatible with the low incidence of 46,XX-CGD.

Table 1 Summary of identified variants

	Age of presentation	FSH	LH	E2	Breast	Gene	Nucleotide Change	Amino Acid Change	Zygosity	ACMG Classification	Allelic Frequency	Inheritance pattern	Previous reported	PolyPhen-2	PROVEAN	SIFT	Mutation Taster	Splice AI
P1	13	89.1	31	<25	I	*MCM9*	c.1151-1G> A	Splice site	Hom	LP(PVS1+PM2_Supporting)	0	AR/Parental	Yes	-	-	-	-	Splice-altering(0.92)
P2	15	50.2	35.5	<15	II	*TWNK*	c.1814delT	p. Val605Glyfs Ter10	Het	LP(PVS1+PM2)	0	AR/Matenal	New	-	-	-	D	-
							c.-722G>T	5'UTR	Het	Vus(PM2)	0.02%	AR/ Paternal	New	-	-	-	-	-
P3	14	105.77	53.61	<5	II	*TP63*	c.1928G> A	p. Arg643Gln	Het	LP(PS2+PM2+PP3)	0	AD/De novo	Yes	D (0.979)	N (-1.1)	D (0.006)	D	-
P4	15	63.07	21.43	<15	II	*TP63*	c.1925T> G	p.Val642Gly	Het	Vus(PM2)	0	AD/De novo	New	D (1)	D (-2.53)	D (0.002)	D	-
P5	16	59.16	33.1	51	II	*INSRR*	c.2217–2 A>C	Splice site	Het	LP(PVS1+PM2)	0	-/Paternal	New	-	-	-	-	Splice-altering
P6	16	73.1	21.9	<15	II	*POF1B*	C.932 A>C	P.Lys311Thr	Hom	VUS(PM2-Supporting+PP3)	0	XLK/Paternal	Yes	D (0.971)	D (-3.26)	D (0.000)	D	-
P7	15	97.63	26.78	20	II	*PSMC3IP*	c.77 A> C	p. Gln26Pro	Hom	Vus(PM2+PP3)	0	AR/-	New	D (0.952)	D (-4.14)	D (0.019)	D	-

PolyPhen http://genetics.bwh.harvard.edu/pph2/; D: Probably damaging (> = 0.957), P: possibly damaging (0.453 < =pp2_hdiv<=0.956) B: benign(pp2_hdiv<=0.452); SIFT/Provean http://provean.jcvi.org/protein_batch_submit.php?species=human. D: Damaging (sift<=0.05); T: tolerated (sift> 0.05)/ D: Deleterious(-14< provean<-2.5); T: tolerated (-2.5< provean<14), MutationTaster http://www.mutationtaster.org/. A: disease causing_automatic, D: disease_causing, N: polymorphism, P: polymorphism_automatic. Splice AI:0-1(The larger the score, the greater the probability of being altered by splicing)

Moreover, the variants were predicted to be deleterious by online prediction tools including Polyphen-2, PROVEAN, SIFT, and MutationTaster. Spice AI predicted two splice variants to be splice-altering (Fig. 1A, B). The multiple sequence alignment analysis revealed that the three variants, p.Val642Gly in TP63, p.Lys311Thr in POF1B, and p.Gln26Pro in PSMC3IP, affected the strictly conserved domains among vertebrate orthologs, including eight distantly related species (Fig. 1C, D, and E), indicating that the location of the amino acid changes may affect the function of the protein. To investigate the effect on the proteins of three missense variants defined as VUSs, schematic structures of the wild type (left) and the mutant (right) amino acids are shown in Fig. 1F, G, and H. Compared with the 3D structure of wild type protein, the spatial structure of the peptide chain around the mutation amino acid and the hydrogen bond distance at the side chain changed, which may lead to protein misfolding, resulting in pathogenicity.

Discussion

In this study, we performed WES of blood DNA samples from 20 patients and identified 8 variants in 6 genes from 7 patients associated with 46,XX-CGD, including three homozygous variants in *MCM9*, *POF1B*, and *PSMC3IP*; compound heterozygous variants in *TWNK*; and three heterozygous variants in *TP63* and *INSRR*. The five variants c.1814delT and c.-722G>T in *TWNK*, c.1925T>G in *TP63*, c.2217–2 A>C in *INSRR*, and c.77 A>C in *PSMC3IP* were first reported in 46,XX-CGD, which not only expanded the spectrum of 46,XX-CGD but also enriched the genetic architecture of the pathogenesis. Our study further supports the notion that genetic variants of several genes are important in the pathogenesis of 46,XX-CGD and may be the main reason for sporadic cases of unknown etiology.

The MCM9 protein is required for DNA repair via homologous recombination [10]. Previous studies have shown that defects in *Mcm8* or *Mcm9* lead to defects in homologous recombination and gametogenesis and that *Mcm9*(-/-) female mice are infertile and completely devoid of follicles in their ovaries [11]. In recent years, it has also been reported that homozygous variants of *MCM9* can lead to primary amenorrhea and ovarian failure in women, which are accompanied by abnormal or normal height [12, 13]. P1 presented with primary amenorrhea at the age of 13. Her stature was underdeveloped at 145 cm, placing her height in the third percentile according to the height data for children and adolescents in 2023. Using WES, a previously reported splice variant (c.1151-1G>A) of the *MCM9* gene was identified and assessed as LP (PVS1+PM2_Supporting) by ACMG. The *MCM9* c.1151-1G>A variant changes an intron 8 splice acceptor site conserved at the nucleotide level across species and in silico analysis predicted a new acceptor splicing site in the downstream 1 bp, which caused a frameshift mutation and premature termination (Fig. 1A). This variant is not present in the gnomAD database. Therefore, *MCM9* is an attractive candidate pathogenic gene for ovarian dysgenesis.

The variants in *TWNK* are associated with Perrault syndrome type 5 (OMIM:616138), a rare autosomal recessive disorder characterized by sensorineural hearing loss and ovarian dysgenesis in women [14, 15]. Fewer than 30 patients with Perrault syndrome type 5 have been reported worldwide. Female patients present with severely impaired endocrine function, which is characterized by primary amenorrhea, a lack of secondary sexual characteristics and hypogonadism, and streaked ovaries [16]. In some cases, patients present with a variety of neurological symptoms, including sensorineural hearing loss, ataxia, nystagmus, hyporeflexia, and sensory axonal neuropathy with distal sensory disturbances [17]. Two novel variants of uncertain significance in the *TWNK* gene were identified in P2, who presented with ovarian dysgenesis. The patient has been followed up until now at the age of 18 years and is free of neurological or hearing impairment. The only symptom is ovarian dysgenesis, which requires continued follow-up and indicates a possible genotype-phenotype correlation. The nonsense variant c.1814delT (p.Val605GlyfsTer10) in the helicase structural domain, located in the last exon of *TWNK*, was confirmed to be of maternal origin via Sanger sequencing. The variant had no frequency in the gnomAD database population and was predicted to be deleterious in silico, which was assessed as LP by ACMG. The c.-722G>T variant located in the 5'UTR was of maternal origin. The mutation frequency in the gnomAD database was 0.021%, and the function prediction tools cannot predict the impact of the variant, which was evaluated VUS, and its pathogenicity needs to be further investigated via functional tests.

TP63 is specifically expressed in oocytes of primordial follicles and plays a decisive role in oocyte survival after DNA damage [18]. Variants in *TP63* that affect the C-terminal transactivation inhibitory domain (TID) have been reported in different populations of patients with syndromic or isolated 46,XX-CGD [19, 20]. Chengzi Huang et al. identified six *TP63* C-terminal TID-related variants in patients who presented with primary or secondary amenorrhea, accounting for 0.78% (8 of 1030) of isolated primary hypogonadism cases in a large Chinese population, which was confirmed by in vitro functional studies and animal experiments [21]. In this study, two adjacent variants were detected in the C-terminal TID domain of the *TP63* gene in P3 and P4 with 46,XX-CGD, including a known pathogenic variant (c.1928G>A, p.Arg643Gln) and a novel variant (c.1925T>G, p.Ty642Asp). The

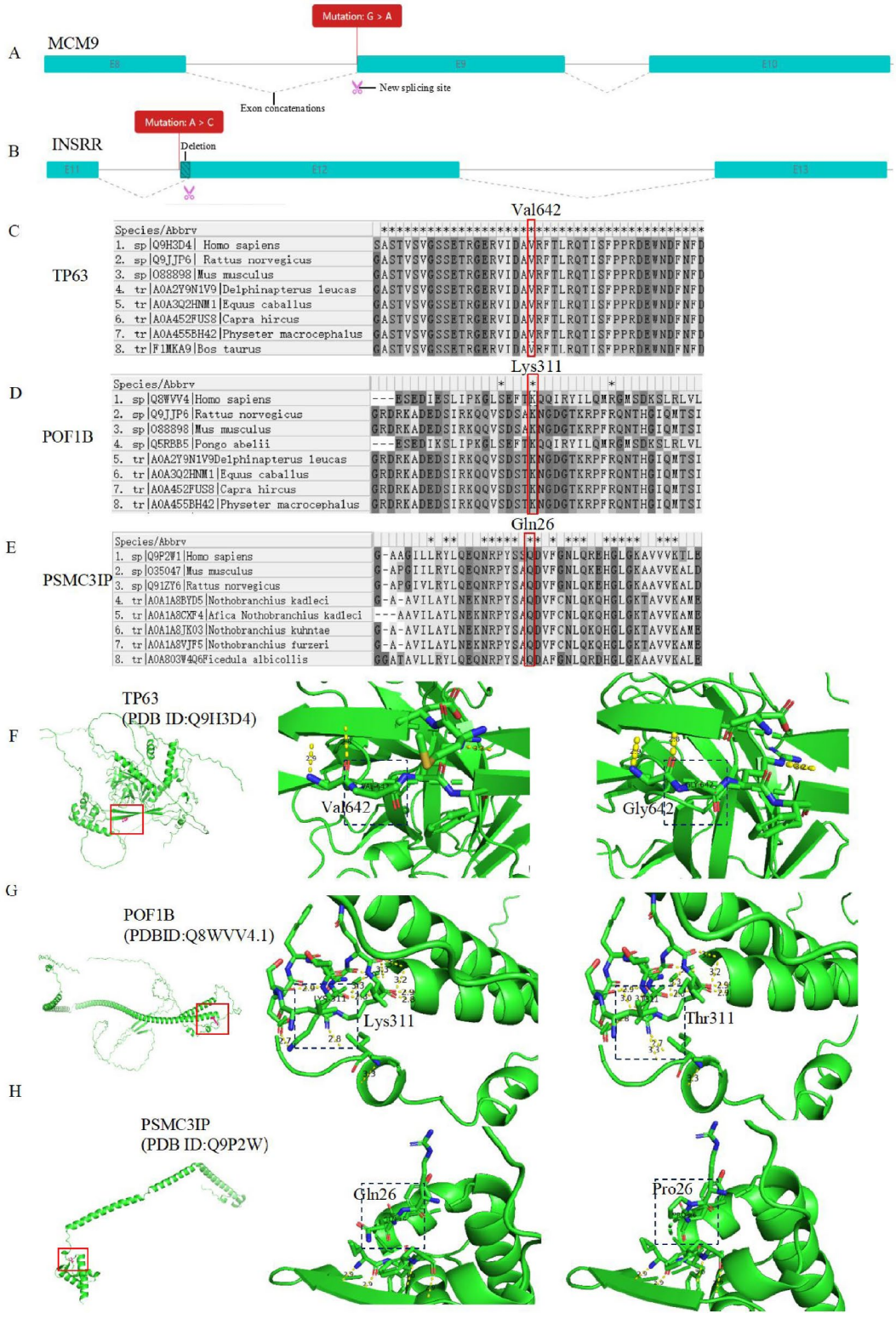

Fig. 1 In silico analysis of the variants. **A**, **B**. The effect of the splicing variant in MCM9 and INSRR. The scissor symbol indicates the new splicing site. Blue oblique boxes represent deletions of bases. The broken lines represent the concatenations between exons. **C**, **D**, **E**. Sequence alignments illustrate the conservation of the affected amino acid (Val642 in TP63, Lys311in POF1B, and Gln26 in PSMC3IP) across eight diverse species, with the variant position marked by the red box. **F**, **G**, **H**. The schematic 3D structures of the wild type (left) and the mutant amino acids(right) were modeled using its wild-type template, with the Hydrogen bond marked by yellow lines. The specific differences are circled in black

sequence of the p.Tyr642Asp variant is highly conserved across various species, and its deleteriousness is predicted in silico. Protein structure analysis revealed significant changes in the spatial structure and surrounding hydrogen bonds, indicating that the p.Tyr642Asp variant is a promising variant of 46,XX-CGD, but further verification via functional tests is needed.

The *INSRR* gene encodes the insulin receptor (INSR)-related receptor. Pitetti J-L et al. described the critical role of the insulin receptor (INSR) and the IGF type I receptor (IGF1R) in adrenal development and primary sex determination in a mouse model study. The INSR/IGF1R variant gonads remain undifferentiated, regardless of genetic sex [22]. P5 carried a heterozygous c.2217−2 A>C (splicing) variant of the *INSRR* gene. Splice AI predicted that it might affect splicing (PVS1). The variant resulted in an 8 bp deletion downstream, which created a novel receptor splice site that resulted in a frameshift mutation and premature termination, as shown in Fig. 1B. The variant had no frequency in the gnomAD database population and was evaluated as a VUS. The variant was inherited from the father without obvious abnormalities. *INSRR* gene function may differ by sex or exhibit incomplete penetrance, which needs to be confirmed by further functional studies. Its potential involvement in 46,XX-CGD provides a new avenue for research.

The *POF1B* gene is located at Xq21.1 and encodes an actin-binding protein. This gene was first identified in 1996 in a 17-year-old primary ovarian insufficiency (POI) patient with secondary amenorrhea [23]and subsequently reported to be associated with ovarian dysgenesis, with in vitro functional experiments suggesting that POF1B may play a role in germ cell division [24]. The variant C.932 A>C of *POF1B* has been reported in a 21-year-old patient who presented with POI [25]. Bioinformatics analyses predicted that the variant was deleterious. The amino acid sequences are highly conserved among multiple species. The difference in our study is that P6 presented with ovarian dysgenesis characterized by primary amenorrhea. These findings reinforce the importance of POF1B in ovarian development.

PSMC3IP encodes a nuclear protein-binding α- and β-estrogen receptor as well as receptors for glucocorticoids, thyroid hormones, androgens, and progesterone. It acts as a coactivator of hormone-dependent transcriptional activation [26]. The biallelic variants in this gene have been described in four independent families with ovarian dysgenesis families whose members present with primary amenorrhea and streaked gonads [27–30]. Sirchia subsequently reported that biallelic variants in *PSMC3IP* are associated with secondary amenorrhea, expanding the phenotypic spectrum [31]. A novel missense variant (c.77 A>C, p.Lys26Gln) in *PSMC3IP* was detected in P7 presented with ovarian dysgenesis in the study. Bioinformatics analysis predicted that the variant would be deleterious. The affected amino acid residue, Lys26, is highly conserved across eight species. It is proposed to be a candidate variant for 46,XX-CGD.

Large-scale exome sequencing data have been used to explore the pathogenic genes of POI, expanding the variant spectrum and genetic architecture. POI can be syndromic, manifesting as highly variable somatic abnormalities in addition to reproductive phenotypes or non-syndromic phenotypes, and the inheritance pattern can be monogenic, digenic, or multigenic [32]. This study is the first to focus on patients with 46,XX-CGD and identify several novel variants. Consistent with the genetic pattern of POI, the *MCM9* and *TWNK* genes are associated with syndromic 46,XX-CGD. However, in our study, the patient with the *TWNK* variant has not developed neurological abnormalities to date. This presentation warrants further follow-up and could be explained by specific variant sites and different types of variants. The inheritance patterns presented in the study were only monogenic, which may be explained by the small sample size, and the continuous collection of new cases is needed. In addition, the deleteriousness of the variants was only predicted in silico, and functional studies in vivo and in vitro have not been conducted, necessitating further in-depth research.

Conclusion

This study included 20 46,XX-CGD patients, and variants were identified in 7 of these patients. The identification of these novel variants and candidate genes expands the genetic spectrum of 46,XX-CGD and provides new insights into its genetic basis. Approximately 35% (7/20) of the sporadic cases in the study had potentially pathogenic variants that could be identified, indicating the utility of WES in determining the genetic pathogenesis of the disease. These findings enhance our understanding of the genetic etiology of this condition and highlight the importance of genetic testing in affected individuals.

Abbreviations

DSD	Disorders of sex development
46,XX-CGD	46,XX complete gonadal dysgenesis
WES	Whole exome sequencing
17OHD	17α-hydroxylase deficiency
PORD	Cytochrome P450 oxidoreductase deficiency
StAR	Steroidogenic acute regulatory
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
E2	Estradiol
ACMG	American College of Medical Genetics and Genomics
P	Pathogenic
LP	Likely pathogenic
VUS	Variants of uncertain clinical significance
TS	Turner syndrome

Acknowledgements
We would like to thank all the patients who agreed to participate in this study.

Author contributions
LD collected and analyzed the data. LD, and QT contributed to the writing, review, and/or revision of the manuscript. PZ, SD, and DZ contributed to administrative, technical, or material support. All authors read and approved the final manuscript.

Funding
This research was funded by grants from the Peking Union Medical College Hospital· Public Welfare Project for Rare Disease Service Improvement.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Peking Union Medical College Hospital Ethics Committee and was conducted according to the Declaration of Helsinki principles (No. JS-2510). Written informed consent was obtained from all adults or legal guardians of adolescents under 18 years old.

Consent for publication
Written informed consents were obtained from all enrolled patients or their parents.

Competing interests
The authors declare no competing interests.

Received: 28 August 2024 / Accepted: 26 October 2024
Published online: 11 November 2024

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