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Comprehensively identifying and validating the implications of *NR5A1* and *DHX37* variants for 46,XY disorders of sex development diagnosis

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Abstract

Background The clinical phenotype and pathogenic mechanism of 46,XY disorders of sex development (DSD) are complex, and several pathogenic variants are identified by next-generation sequencing. However, these variants currently require additional interpretation and validation prior to their application in 46,XY DSD diagnosis and clinical guidance.

Results Here, we identified three genetic variants in two 46,XY DSD patients by whole exome sequencing screening and Sanger sequencing validation. The pathogenicity of three genetic variants was identified by in silico analysis and functional experiments. One patient carrying the reported pathogenic variant (c.319 C > T) of *NR5A1* showed a phenotype of 46,XY complete gonadal dysgenesis, which was different from the reported 46,XY partial gonadal dysgenesis. These findings suggested that the variant (c.319 C > T) of *NR5A1* contributes to the clinical phenotypic heterogeneity of 46,XY DSD. The other patient carried two genetic variants, among which the c.1252 C > T variant of *NR5A1* produced truncated protein and lost the transcriptional activation of NR5A1 to the targeted genes. The other c.769G > A variant of *DHX37* had no significant effect on the expression level and cellular localization of DHX37, and the downstream signaling pathway of DHX37. Moreover, the in silico and structural analysis identified the c.769G > A variant of *DHX37* as a deleterious variant that may affect DHX37 function. According to the American College of Medical Genetics and Genomics guidelines and relevant literature reports, combined with the patient's clinical phenotype and pedigreed analysis, it is proposed that the likely pathogenic variant identified in this patient is the c.1252 C > T variant of *NR5A1*. Nonetheless, the potential pathogenicity of the *DHX37* (c.769G > A) variant of this patient also merits further investigation and consideration.

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Conclusions Our results have expanded the clinical phenotype spectrum and genetic diagnosis spectrum of 46,XY DSD, which will contribute to the accurate diagnosis and treatment guidance for 46,XY DSD patients and provide evidence-based genetic counseling for 46,XY DSD family fertility.

Keywords 46,XY disorders of sex development, *NR5A1*, *DHX37*, Genetic diagnosis, Clinical phenotypic heterogeneity

Introduction

Disorders/differences of sex development (DSD) in humans are a genetically heterogeneous condition characterized by an incongruity between the individual's chromosomal karyotype and their gonadal phenotype or anatomical sex [1]. 46,XY DSD is a subgroup of DSD whose etiology is complex and difficult to diagnose, including the individuals with gonadal dysgenesis (GD) and disorders of androgen synthesis or action, among which GD includes 46,XY complete/partial gonadal dysgenesis (46,XY CGD/46,XY PGD) [2]. Complete female external genitalia, lack of secondary sexual traits and testicular development, and bilateral streak gonads are representative phenotypes of 46,XY CGD. 46,XY PGD is the different degrees of masculinization of external genitalia and the dysgenetic testis [3]. Testicular regression syndrome (TRS) is a subgroup of 46,XY GD and refers to the initial formation of testicular tissue during embryonic development followed by complete or partial disappearance of both or one testicular tissue. Most TRS individuals present as part of the GD clinical profile, such as micropenis, undervirilized external genitalia, and lack of bilateral or unilateral gonadal tissue [4]. Due to the wide range of clinical phenotypes and complex etiology of human DSD, even with next-generation sequencing (NGS) technology, less than 50% of DSD individuals can obtain a definitive genetic diagnosis [5, 6]. Pathogenic variants of sex determining region Y (*SRY* MIM 480000), nuclear receptor subfamily 5 group A member 1 (*NR5A1* MIM 184757) and mitogen-activated protein kinase kinase kinase 1 (*MAP3K1* MIM 600982) genes are the most prevalent causes of 46, XY GD [7–9]. With the wide application of NGS technology in the diagnosis of DSD, the novel genes related to DSD have been identified in recent years, such as DEAH-box RNA helicase 37 (*DHX37* MIM 617362), which was originally associated with human neurodevelopmental disorder [7–9]. The diagnostic contribution of *DHX37* variants to the whole 46,XY GD group is about 10% – 15%, and the diagnostic contribution to the TRS subgroup is about 20%, which is similar to the diagnostic contribution of *SRY* and *NR5A1* variants [10]. Since *DHX37* was found to be associated with DSD, several clinical phenotypes associated with *DHX37* variants have also been identified including 46,XY CGD, 46,XY PGD and TRS [11–13]. In addition to the discovery of novel DSD-related genes, NGS technology can also be used to find or expand the clinical manifestations of known DSD-causing genes such as *NR5A1* [14, 15]. It is believed that NGS technology including exome sequencing and genome sequencing, especially the in-depth application of rapid genome sequencing technology, will provide timely and accurate genetic diagnosis for more DSD cases [16]. The definitely genetic diagnosis of 46,XY DSD individual is not only an important basis for individual treatment guidance and family fertility genetic counseling of DSD, but also helpful to understand the pathogenic mechanism of various genes in the process of human gonadal determination and formation.

In 46,XY males, the testes are determined by a complex gene network that promotes testis formation while inhibiting ovarian development [17]. A single harmful variant in any of the genes in this complex gene regulatory network can affect the function of gene-encoded protein, thus leading to human DSD. Family studies of DSD patients have also confirmed the monogenic inheritance of human DSD [18]. Over the past decade, with the in-depth study of DSD families and the application of NGS technology, it has been found that different members in DSD families carrying the same pathogenic variants but showing different degrees of clinical phenotypes, or even being carriers with normal phenotypes. Conversely, the similar clinical phenotypes of DSD patients may also be caused by different genetic variants [19]. These findings suggest that a single genetic variant is not sufficient to cause such a wide range of clinical phenotypes of DSD. Emerging studies have proposed the digenic or oligogenic inheritance of DSD through NGS sequencing results and in silico analysis [19, 20]. *NR5A1* is the pathogenic gene leading to a very wide range of clinical phenotypes of DSD, and therefore *NR5A1*-caused DSD is considered to be the most consistent with the digenic or oligogenic inheritance of DSD. Previous studies have reported that one 46,XY GD patient with ambiguous genitalia carrying a digenic inheritance of a pathogenic variant of *NR5A1* (p.Arg313Cys) and a missense variant of *MAP3K1* (p.Gln237Arg), among which the missense variant of *MAP3K1* is a rare variant, thus it is speculated that this missense variant also leads to abnormal testicular development [21]. A large sample study found that 47.14% of 46,XY DSD patients carried multiple DSD-related genetic variants (at least two genetic variants). 80% of 46,XY DSD patients with the *NR5A1* variants had other DSD-related genetic variants [22]. Two patients with 46,XY DSD carrying both *NR5A1* and *DHX37* variants have also been reported [10]. These 46,XY DSD patients

who carry di/oligogenic variants usually have one genetic variant that is pathogenic variant and the other is usually a variant of unknown significance (VUS). Therefore, the pathogenicity of these di/oligogenic variants necessitates comprehensive functional interpretation and clinical validation to enable precise genetic diagnosis for 46,XY DSD patients.

In this study, we aim to use whole exome sequencing and Sanger sequencing as well as in silico analysis and functional experiments to clarify the pathogenic variants or pathogenic mechanisms of 46,XY DSD patients. The novel pathogenic variants or clinical phenotypes associated 46,XY DSD discovered in this study will be helpful for the diagnosis and treatment of 46,XY DSD patients in the future.

Materials and methods

Study subjects and sample collection

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China, Ethics Committee Approval Numbers: XJTU1AF2020LSK-102 and XJTU1AF2024LSYY-141). This study was performed in accordance with the principles of Declaration of Helsinki. Written informed consent was obtained from all the participants and parents of patients who participated in this study. We recruited two DSD patients and their family from the First Affiliated Hospital of Xi'an Jiaotong University and collected relevant clinical data. The external genital phenotypes of patients with DSD were evaluated and classified according to the Prader grading system [23]. A Prader score of 3 or less is considered a severe clinical phenotype, and a Prader score greater than 3 is considered a relatively mild clinical phenotype [24]. Hormone testing data and gonadal conditions for the two patients were not obtained. One hundred healthy donors including 50 male and 50 female, were also recruited for the study.

Human peripheral blood lymphocyte culture and chromosomal karyotype analysis

Peripheral blood of patients and their relatives with heparin sodium anticoagulant was extracted and inoculated into lymphocyte culture medium. Incubate the cells at a temperature of 37 °C for a duration of 72 h, followed by cell harvesting. Chromosome specimens were prepared and karyotype analysis was performed after G-banding. Twenty divisions were counted in each sample and five karyotypes were analyzed.

Whole exome sequencing (WES) screening and Sanger sequencing validation

Genomic DNA (1 μg) was isolated from the peripheral blood (200 μL) of DSD patients, their family and healthy donors according to the Qiagen DNA Blood Midi/Mini Kit (Qiagen, Hilden, Germany) instructions. Isolated genomic DNA (50 ng) was interrupted to 200 bp around by fragmentation enzymes. The DNA fragments were then end repaired, and the 3'end was added one A base. Secondly, the DNA fragments were ligated with barcoded sequencing adaptors, and fragments about 320 bp were collected by XP beads. After polymerase chain reaction (PCR) amplification, the DNA fragments were hybridized and captured by Nano WES (Berry genomics) according to the manufacturer's protocol. The hybrid products were eluted and collected, and then subjected to PCR amplification and the purification. Next, the libraries were quantified by real-time quantitative PCR. Finally, Novaseq6000 platform (Illumina, San Diego, USA), with 150 bp pair-end sequencing mode, was used for sequencing the genomic DNA of the DSD patients. Raw image files were processed using CASAVA for base calling and generating raw data. To confirm the variants identified by WES, fragments covering the variant site in the target gene were amplified by PCR followed by Sanger sequencing. PCR was conducted with Premix Taq™ Hot Start Version/TaKaRa LA Taq® with GC Buffer (Takara, Osaka, Japan) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final hold at 72 °C for 5 min. PCR products were purified and sequenced using an ABI 3730 DNA Analyzer with the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). All Sanger sequencing primers used to validate the variants were listed in Supplemental Table 1. Genetic analysis was not performed on the healthy parents and healthy twin brother of patient no.2 due to unavailability of genetic testing.

In silico analysis and structural prediction

The sequencing reads were aligned to the human reference genome (hg19/GRCh37) using Burrows-Wheeler Aligner tool and PCR duplicates were removed by using Picardhttp://picard.sourceforge.net. Verita Trekker® Variants Detection System by Berry Genomics and the third-party software GATKhttps://software.broadinstitute.org/gatk/ were employed for variant calling. Variant annotation and interpretation were conducted by ANNOVAR and the Enliven® Variants Annotation Interpretation System authorized by Berry Genomics. Annotation databases mainly included human population databases and disease and phenotype databases, such as ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org), 1000 Genomes Project (1KGP, http://www.internationalgenome.org), Online Mendelian Inheritance in Man (OMIM, http://www.omim.org). The automatic PVS1 interpretation tool AutoPVS1 (https://

autopvs1.bgi.com/) performed evidence strength evaluation of the PVS1 criterion for the identified genetic variants. The damaging effect of obtained variants is analyzed using a variety of predictive software including combined annotation dependent depletion (CADD), Mutation Taster, MutPred2, protein analysis through evolutionary relationships (PANTHER), polymorphism phenotyping v2, (PolyPhen-2), protein variation effect analyzer (PROVEAN) and sorting tolerant from intolerant (SIFT). Clustal X and ESPript were used to analyze and display the conserved amino acid sequence, respectively. The secondary structure of protein was predicted by PSIPRED protein analysis workbench, and SWISS-MODEL and I-TASSER were employed to predict the three-dimensional structure of protein. Protein stability, flexibility and biochemical properties were analyzed by I-Mutant, PredyFlexy and DNAStar, respectively.

Cell culture and plasmid construction

Human embryonic kidney 293T cells obtained from Cell Resource Center (Beijing, China) were cultured in DMEM medium (Gibco, Thermo Fisher Scientific, Suzhou, China) supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China). Human *NR5A1* Gene ORF cDNA clone expression plasmid (N-Flag tagged), human *DHX37* Gene ORF cDNA clone expression plasmid (C-Flag tagged), N-Flag tagged negative control vector and C-Flag tagged negative control vector were purchased from Sino Biological Inc. (Beijing, China). All mutant plasmids were constructed by Fast Mutagenesis System (TransGen Biotech Inc., Beijing, China). Wild type plasmids, mutant plasmids, and control plasmids were transiently transfected into 293T cells using lipofectamine™ 3000 reagent (Life Technologies, Carlsbad, USA) according to the reagent protocol.

Western blot

Total cellular protein was extracted by using the radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and quantified by using the bicinchoninic acid (BCA) kits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the isolation of proteins, which were then transferred onto the nitrocellulose membranes. After the membrane was blocked with non-fat milk, the various primary antibodies (Proteintech, Wuhan, China) were incubated overnight at 4℃, and then the secondary antibodies were incubated. The horseradish peroxidase (HRP) chemiluminescent reaction was used for protein band detection.

Immunofluorescence staining

Cells transfected with plasmids were fixed with 4% paraformaldehyde, and then were permeated with 0.2% TritonX-100. Cells were blocked with 3% bovine serum albumin (BSA) and then incubated with the primary anti-FLAG antibody, followed by the secondary antibody labeled with fluorescein isothiocyanate (FITC) or Cy3 (Proteintech). The nuclei were re-stained with 4',6-diamidino-2-phenylindole (DAPI) and the images were recorded by a laser confocal microscopy (Leica, Germany).

Dual-luciferase reporter gene assay

The *NR5A1* responsive minimal promoters of its targeted gene human cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*, − 110 bp − +49 bp) were inserted into the pGL3-basic plasmid by using restriction enzyme sites. Luciferase activity was detected by using a Double-Luciferase Reporter Assay Kit (TransGen Biotech), and all data were normalized for Renilla luciferase activity.

Statistical analysis

All data were statistically analyzed using GraphPad Prism software. All functional experiments were independently repeated three times. A *P* value less than 0.05 was considered statistically significant.

Results

Clinical phenotype and genetic analysis of the 46,XY DSD patients

In this study, the gender of proband in Chinese family no.1 was female and the proband's main complaint was primary amenorrhea (Fig. 1A; Table 1). Clinical examination revealed that patient no.1 had normal female external genitalia, but ultrasound examination revealed that patient no.1 had no uterus. Chromosomal karyotype examination showed that the karyotype of patient no.1 was normal male 46,XY karyotype (Fig. 1B). The detailed clinical phenotypes of the patient no.1 are shown in Table 1.

The Chinese family no.2 in this study had two twins, in which the patient no.2 was a newborn (Fig. 1C). Clinical examination revealed atypical external genitalia, micropenis, hypospadias, and the absence of a vagina. Ultrasound showed bilateral cryptorchidism and no uterus was explored. The karyotype of the patient no.2 was a normal male 46,XY karyotype (Fig. 1D). The detailed clinical phenotypes of the patient no.2 are shown in Table 1. The other twin had normal male external genitalia. Therefore, the patient no.1 and patient no.2 in this study can be preliminarily diagnosed as 46,XY DSD based on their clinical phenotypes.

To further clarify the clinical subtypes and pathogenic genes of 46,XY DSD patients in this study, WES was performed in patient no.1 and patient no.2. WES revealed a nonsense variant of *NR5A1* (c.319 C > T, p.Gln107Ter) gene in the blood sample of patient no.1 (Fig. 2A). Pedigree verification of the patient no.1 showed that the

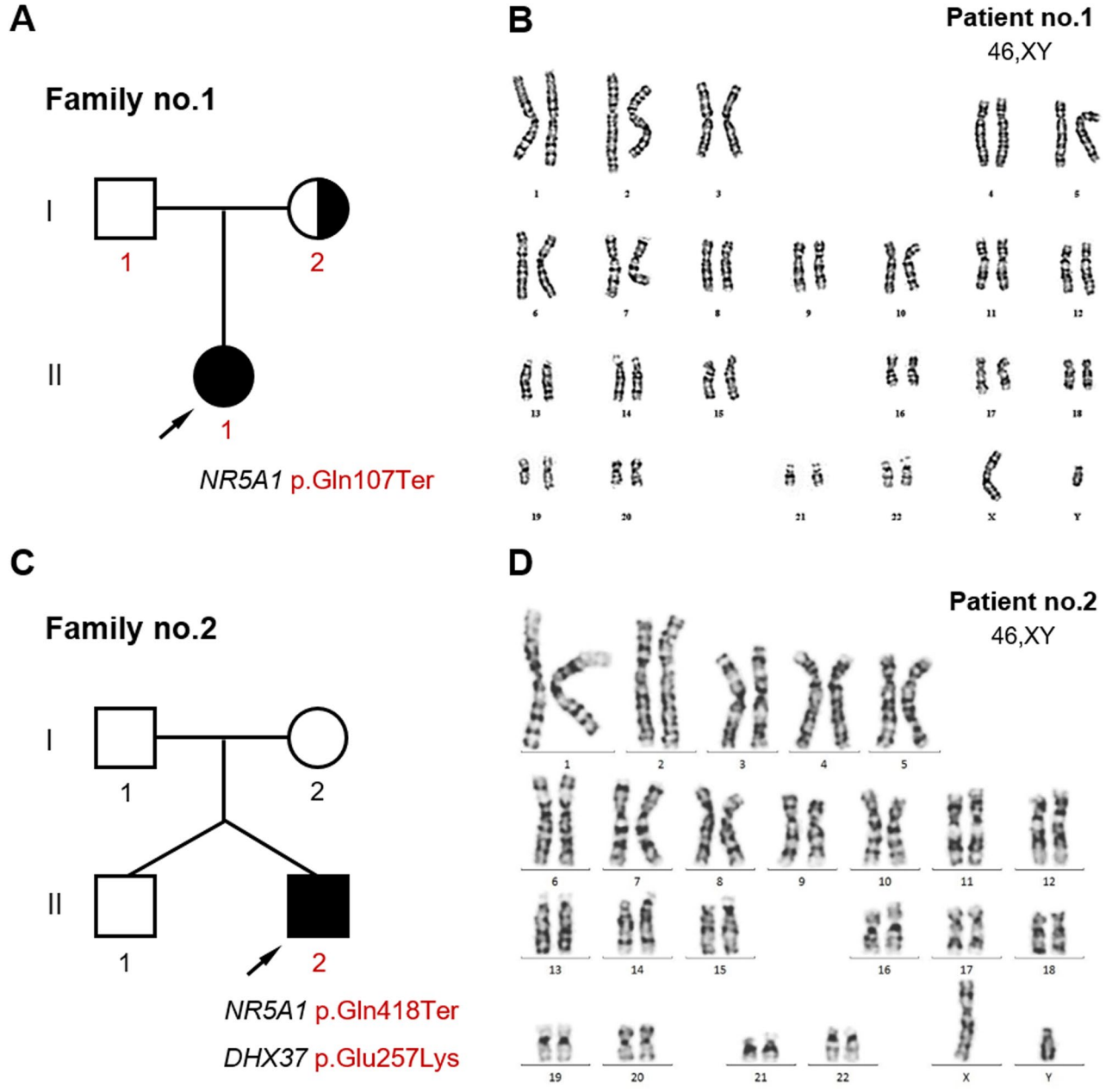

Fig. 1 Pedigree chart and karyogram of 46,XY DSD patients in this study. **A** The pedigree chart of patient no.1. The hollow square represents the normal male and the semi-solid black circle represents the carrier. The solid black circle represents the patient no.1 with a female gender. **B** The karyogram of patient no.1. The karyotype of patient no.1 was 46,XY. **C** The pedigree chart of patient no.2. The hollow square represents the normal male and the black square represents the patient no.2. The hollow circle represents the normal female. **D** The karyogram of patient no.2. The karyotype of patient no.2 was 46,XY. The red-labeled individuals indicated that the genetic testing has been performed

Table 1 Clinical information and phenotypes of two patients diagnosed as 46,XY DSD in this study

Patient ID	Age (years)	Social gender	Karyotype	External genitalia	Internal genitalia
1	17	Female	46,XY	Female external genitalia	Absent uterus
2	Birth	Male	46,XY	Severe hypospadias, micropenis and bilateral cryptorchidism	Absent uterus and vagina

Abbreviation:DSD Disorders/differences of sex development

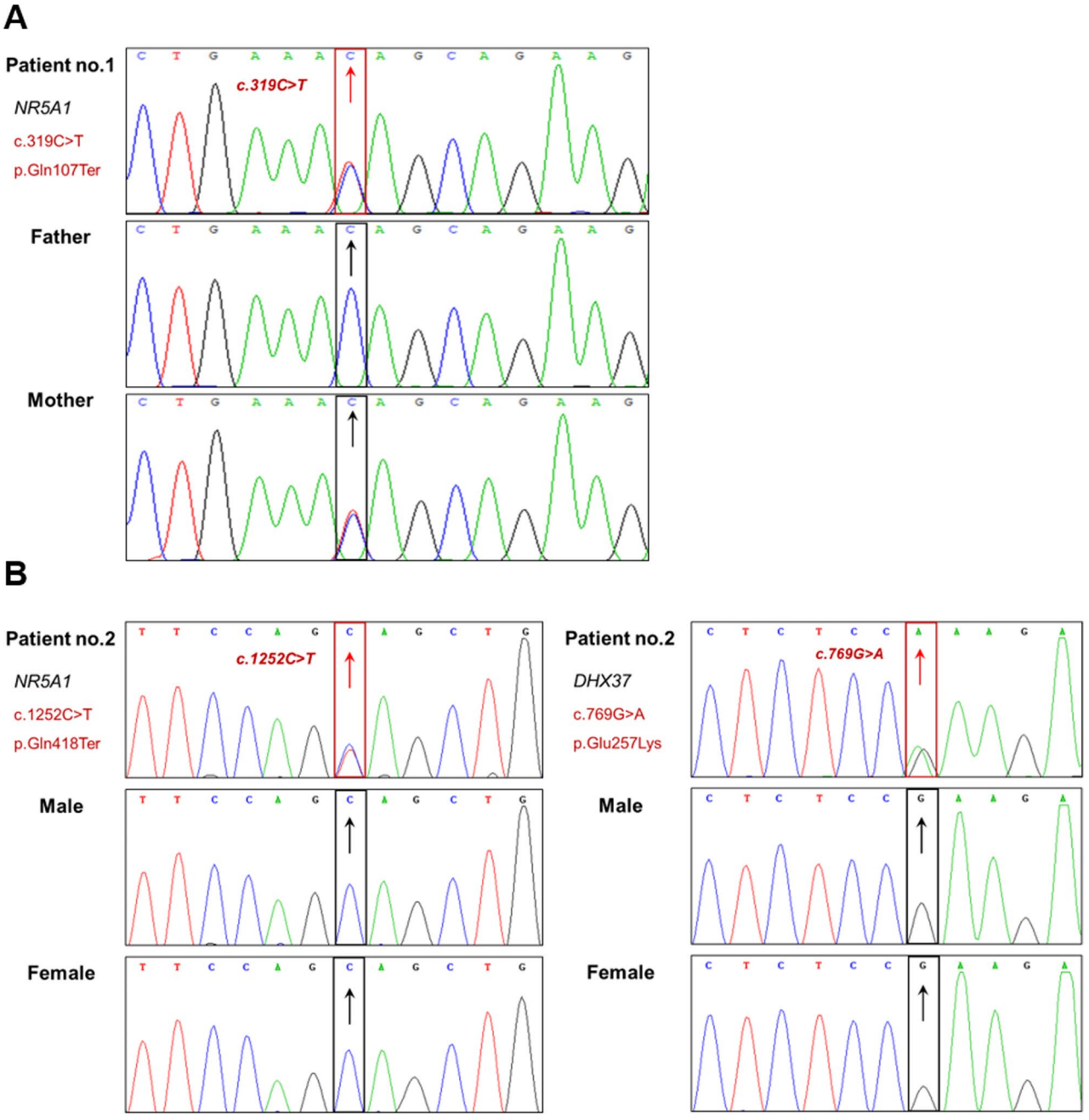

Fig. 2 Sanger sequencing verification of three genetic variants identified by WES. **A** The *NR5A1* gene c.319 C > T variant was confirmed in the patient no.1 and her parents. **B** The c.1252 C > T variant of *NR5A1* gene and the c.769G > A variant of *DHX37* gene were confirmed in the patient no.2. The.1252 C > T variant of *NR5A1* gene and the c.769G > A variant of *DHX37* gene were not detected in the healthy donors ($n = 100$)

heterozygous variant was inherited from her mother, who was the carrier of this heterozygous variant (Figs. 1A and 2A). The c.319 C > T variant of *NR5A1* gene in patient no.1 was not found in both gnomAD and 1000 Genomes Project databases. The pLI (probability of being loss-of-function intolerant) score of *NR5A1* gene in the gnomAD database is 0.948, approaching the critical threshold of 1, which indicates that the gene is likely intolerant to loss-of-function variants. The automated classification tool AutoPVS1 for PVS1 interpretation determined that the PVS1 evidence strength for this variant (c.319 C > T) is classified as very strong and predicted to undergo nonsense-mediated mRNA decay (NMD). Previous literature has shown that the c.319 C > T variant of *NR5A1* gene has been found in two patients with 46,XY DSD, and transient expression experiments suggest that this variant leads to impaired the biology function of NR5A1 [24, 25]. According to the American College of Medical Genetics

and Genomics (ACMG) guidelines and the application of the guidelines proposed by ClinGen Sequence Variation Interpretation Working Group, as well as the screening of genetic variants associated with phenotypes of sexual dysgenesis in HPO database and OMIM database, it was suggested that the c.319 C > T variant of *NR5A1* gene was a pathogenic variant in patient no.1.

WES also detected a nonsense variant of *NR5A1* (c.1252 C > T, p.Gln418Ter) gene in the blood sample of patient no.2. The c.1252 C > T variant of *NR5A1* gene in patient no.2 was not found in both gnomAD and 1000 Genomes Project databases. We also detected the c.1252 C > T variant of *NR5A1* gene in the population, and we did not detect the variant in the 100 healthy donors ($n = 100$, Fig. 2B). The automated classification tool AutoPVS1 for PVS1 interpretation determined that the PVS1 evidence strength for this variant (c.1252 C > T) is classified as strong. According to the ACMG guidelines and the application of the guidelines proposed by ClinGen Sequence Variation Interpretation Working Group, as well as the screening of genetic variants associated with phenotypes of sexual dysgenesis in HPO database and OMIM, it was suggested that the c.1252 C > T variant of *NR5A1* gene was a likely pathogenic variant in patient no.2.

Furthermore, WES detected a missense variant in the *DHX37* gene (c.769G > A, p.Glu257Lys) associated with the clinical phenotypes of patient no.2. The ClinVar database describes this variant as of uncertain significance, and the condition for detection of the variant is the neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies (NEDBAVC). The allele frequency of this variant in the gnomAD or 1000 Genomes Project was 0.00001. We also did not detected the c.769G > A variant of *DHX37* gene in 100 healthy donors ($n = 100$, Fig. 2B). Furthermore, patient no.2 as a newborn did not exhibit the abnormal phenotypes in the neurodevelopment. Therefore, these results suggest that the likely pathogenicity of the variants detected in patient no.2 remains to be further identified.

In silico analysis and structural prediction for the effects of *NR5A1* and *DHX37* variants

To evaluate the pathogenicity of *NR5A1* and *DHX37* variants in patient no.2 of this study, we validated the variants by using in silico analysis and functional experiments. The nonsense variant found in patient no.2 was located in the last exon of the *NR5A1* gene and was predicted not to trigger NMD [26, 27]. As a result, this variant causes the formation of a stop codon at amino acid 418 of the NR5A1 protein, producing a truncated protein. Compared with the wild type NR5A1 protein (461 amino acids), 43 amino acids of the truncated NR5A1 protein have been deleted. According to the protein structural analysis, the truncated NR5A1 protein will lack a key activation function domain AF-2 (Fig. 3A). It was found that the function of the AF-2 domain is to assist in the recruitment of transcriptional coactivators to the promoter regions of NR5A1-regulated targeted genes [28]. Thus, NR5A1 lacking the AF-2 domain will lose their normal transcriptional activation capability. The three-dimensional structure of wild type and mutant NR5A1 was constructed by homology modeling method. The results showed that the three-dimensional structure of the truncated protein formed by the nonsense variant of NR5A1 was similar to that of the wild type protein, but lacking a α-helix in the truncated NR5A1 protein. (Fig. 3B).

Another missense variant carried by patient no.2 was located in the RecA1 domain of the RNA helicase *DHX37* gene (p.Glu257Lys), which is an important motif for RNA helicases to bind ATP and RNA (Fig. 4A). Amino acid conservation analysis showed that the amino acids at site 257 of DHX37 were highly conserved among different species (Fig. 4B). Protein secondary structural analysis showed that this missense variant of DHX37 resulted in the alteration of α-helix and β-sheet of DHX37 protein (Fig. 4C). Protein three-dimensional structural analysis showed that the acidic amino acid of wild type DHX37 protein was replaced by a basic amino acid (Fig. 5A). Meanwhile, the variant of the acidic amino acid glutamic acid at the 257 position of DHX37 protein to the basic amino acid lysine results in a change in the charge number of DHX37 protein (Table 2). The analysis of I-Mutant and PredyFlexy showed that this variant resulted in a decrease in protein stability and an increase in protein flexibility (Fig. 5B). The damaging of p.Glu257Lys missense variant of DHX37 was predicted by using CADD, Mutation Taster, MutPred2, PANTHER, PolyPhen-2, PROVEAN, and SIFT, and the results showed that the variant was damaging or probably damaging (Table 3). Altogether, these findings suggest that the missense p.Glu257Lys variant may affect the conformation of DHX37 protein and thus impair its function by in silico analysis.

Expression analysis and cellular localization of *NR5A1* and *DHX37* variants

To further clarify the pathogenicity of *NR5A1* (p.Gln418Ter) and *DHX37* (p.Glu257Lys) variants, the functional assays were used to detect the expression level and cellular localization of NR5A1 and DHX37 protein. Compared with the wild type NR5A1 protein (molecular weight 55 kDa), NR5A1 protein with 418 site variant led to a decrease in molecular weight of NR5A1 protein (< 55 kDa for p.Gln418Ter, Fig. 6A). These results suggested that the p.Gln418Ter nonsense variant in *NR5A1* gene lead to the production of truncated NR5A1 protein.

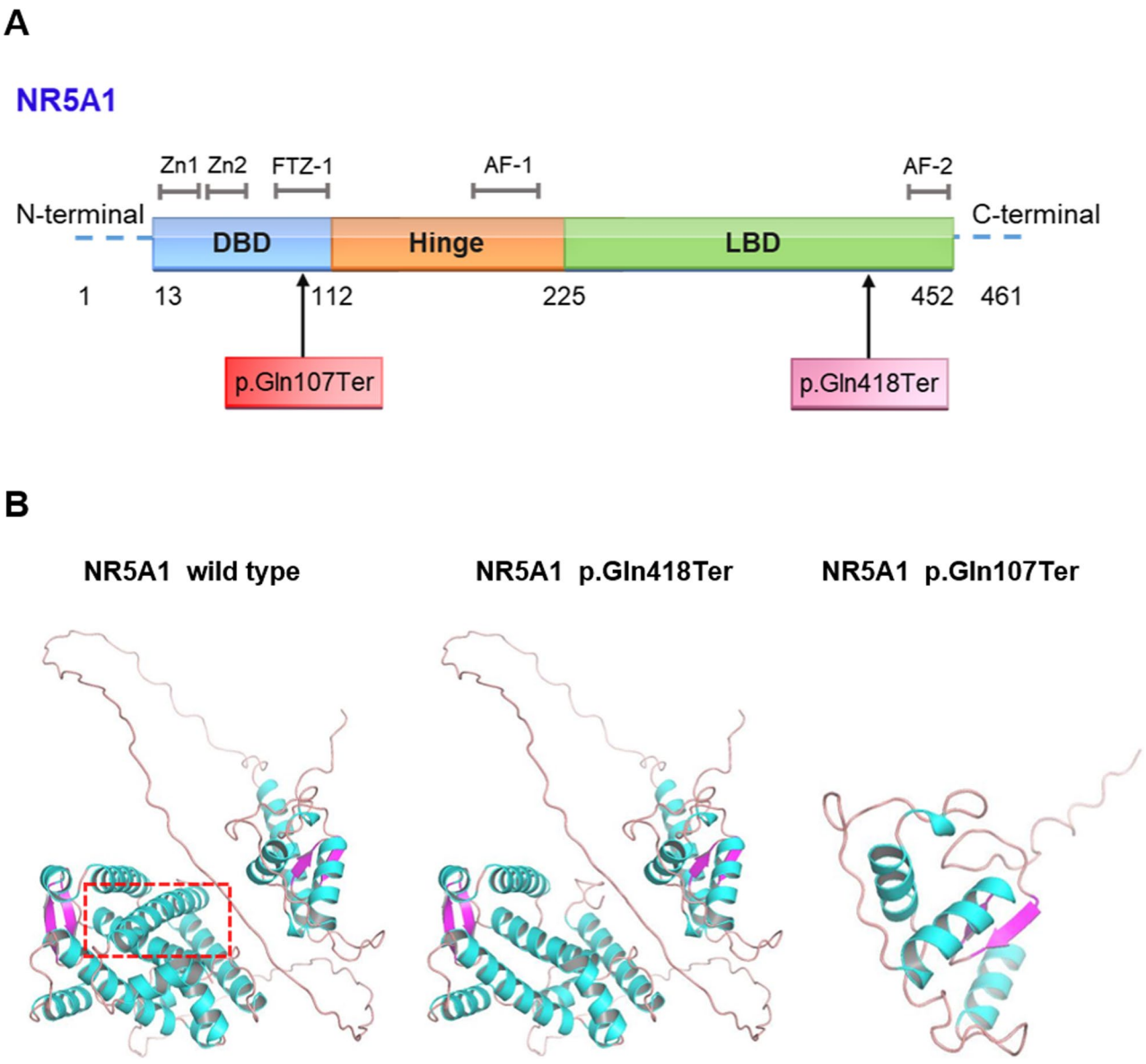

Fig. 3 Schematic diagram of the location of *NR5A1* variants in the NR5A1 protein functional domain. **A** Schematic diagram of the location of the NR5A1 p.Gln107Ter and p.Gln418Ter variants in the protein functional domain. DBD: DNA binding domain, Hinge: hinge region. LBD: ligand binding domain, Zn1 and Zn2: two zinc fingers, FTZ-1: FTZ-F1 box, AF-1 and AF-2: two functional activation domains. **B** Homology modeling method predicts the effects of p.Gln418Ter or p.Gln107Ter amino acid variants on the tertiary structure of NR5A1 protein. The red dashed rectangle indicates the domain of the mutant NR5A1 protein (p.Gln418Ter) that is absent

Furthermore, we also observed the cellular localization of wild type and mutant NR5A1 proteins in cells. As shown in Fig. 6B, the wild type NR5A1 protein exhibited the strictly nuclear localization. However, the mutant NR5A1 protein (p.Gln418Ter) showed a non-exclusive nuclear localization. Therefore, these results indicated that the truncated NR5A1 protein produced by the p.Gln418Ter nonsense variant in *NR5A1* gene impair the nuclear localization of NR5A1 protein, leading to an impact on their function. In addition, we also detected the expression level and cellular localization of DHX37 protein. As shown in Fig. 7A, there was no significant difference in the protein expression level of wild type DHX37 protein and mutant DHX37 protein (p.Glu257Lys). Furthermore, both wild type DHX37 protein and mutant DHX37 protein (p.Glu257Lys) were strictly localized in the nucleus (Fig. 7B). These results suggested that the p.Glu257Lys missense variant of *DHX37* gene did not affect the expression level and cellular localization of DHX37 protein.

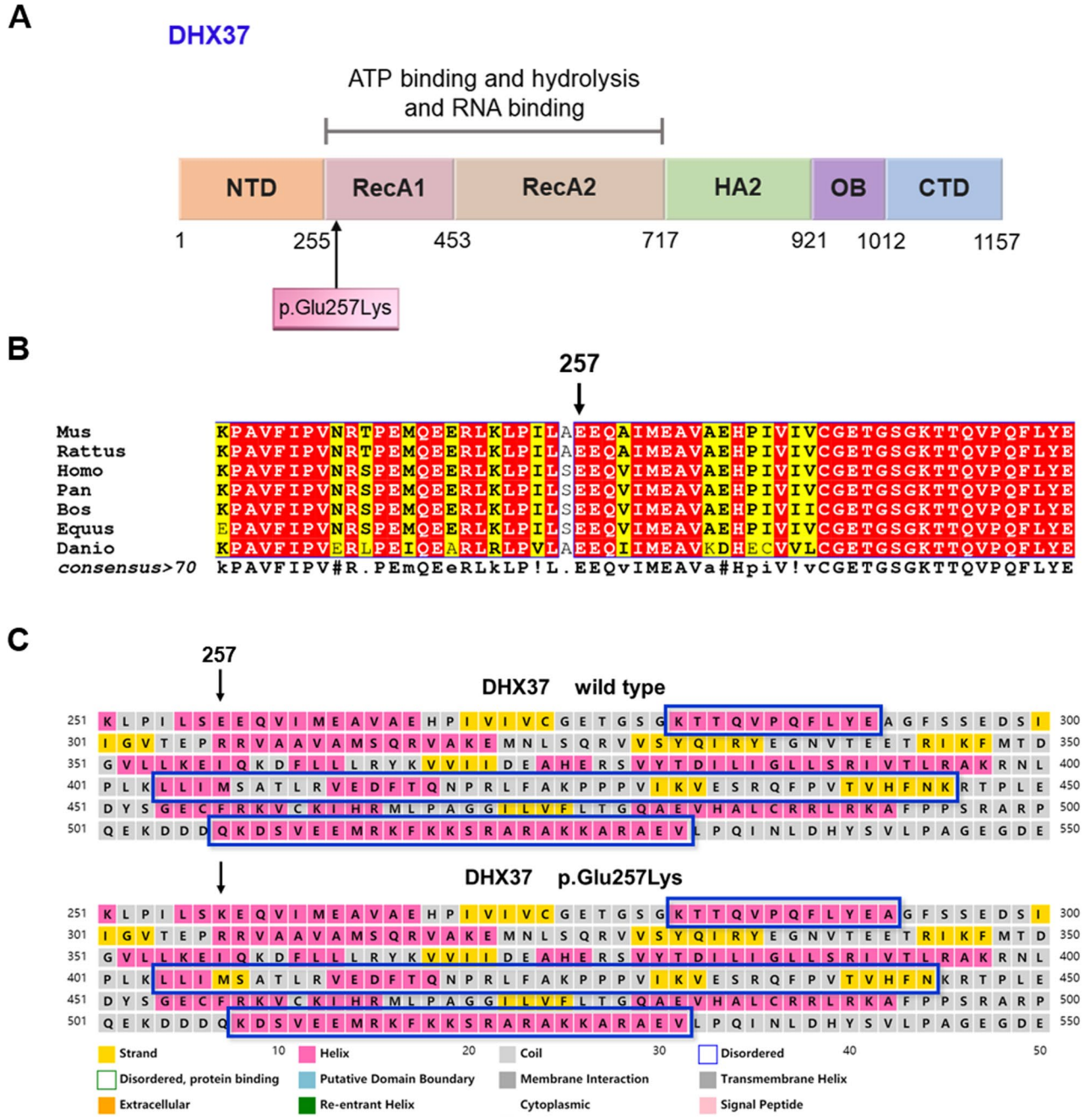

Fig. 4 Analysis of DHX37 missense variant (p.Glu257Lys) site and DHX37 amino acid sequence. **A** Schematic diagram of the location of the p.Glu257Lys variant in the DHX37 protein functional domain. NTD: N-terminal domain, RecA1: RecA-like domain 1, RecA2: RecA-like domain 2, HA2: helicase-associated domain 2, OB: oligonucleotide-binding fold domain, CTD: C-terminal domain. **B** Amino acid sequence conservation analysis of DHX37 (p.Glu257Lys) variant. **C** Protein secondary structural analysis of DHX37 (p.Glu257Lys) variant. The blue rectangular boxes indicate areas where there are discrepancies in the secondary structure between the wild type DHX37 and mutant DHX37 (p.Glu257Lys)

In vitro functional assays for *NR5A1* and *DHX37* variants

To identify the effect of truncated NR5A1 protein on its gene function, the transactivation experiments were performed. Our results showed that the transactivation capability of wild type *NR5A1* vector on the target gene *CYP11A1* promoter (– 110 bp – +49 bp) is a dose-dependent manner. Compared with the wild type *NR5A1* vector, the mutant *NR5A1* (Gln418Ter) vector decreased the transactivation capability of NR5A1 on the *CYP11A1* promoter (– 110 bp – +49 bp) from 100% to 25% – 45%. In addition, when the wild type *NR5A1* vector were co-transfected with the mutant *NR5A1* (p.Gln418Ter) vector, the mutant *NR5A1* (p.Gln418Ter) vector did not significantly inhibit or reduce the transactivation capability of

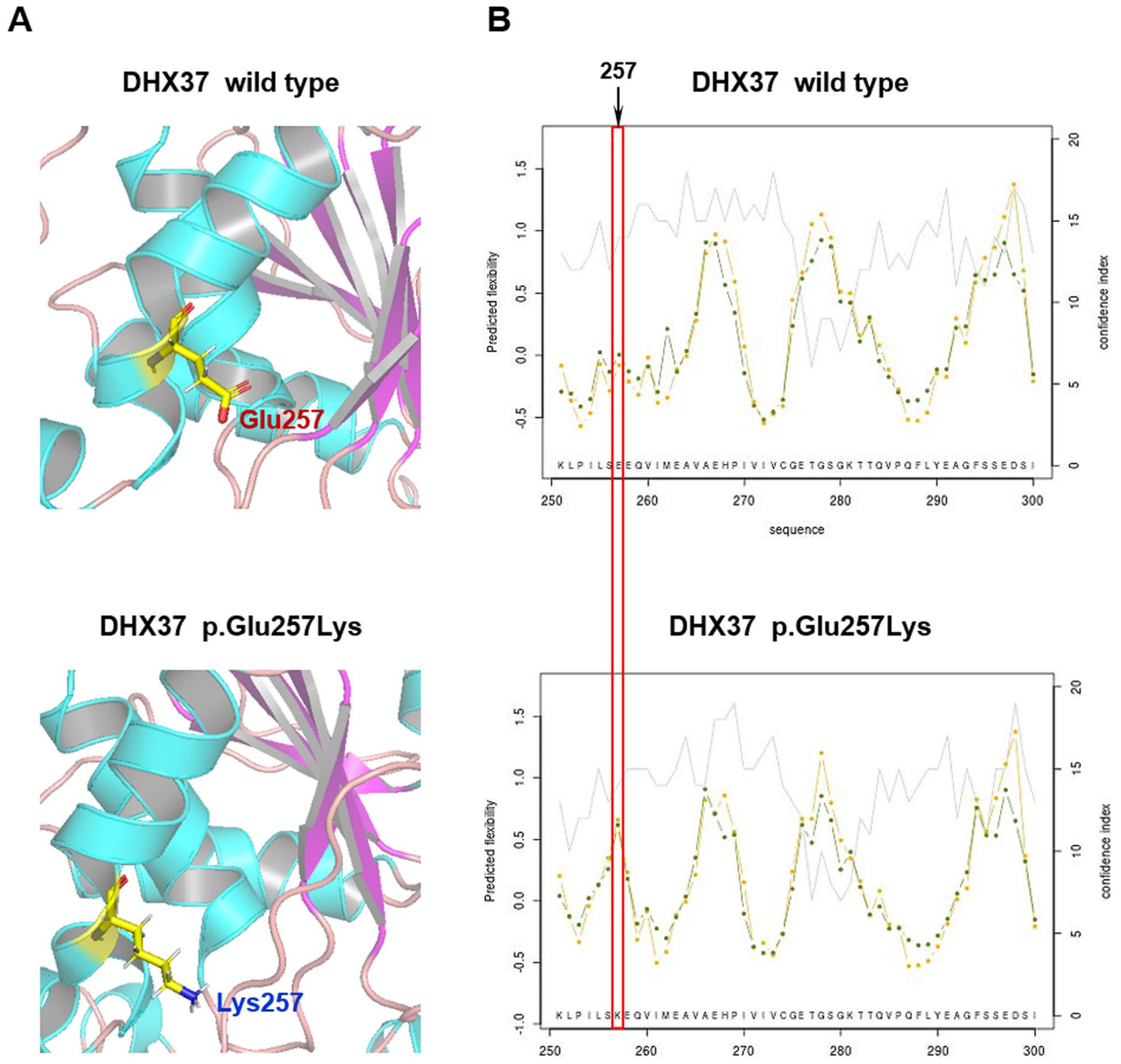

Fig. 5 Effects of the p.Glu257Lys variant on DHX37 protein conformation. **A** Protein three-dimensional structural analysis of DHX37 (p.Glu257Lys) variant were analyzed by I-TASSER. **B** Protein flexibility analysis of DHX37 (p.Glu257Lys) variant were analyzed by PredyFlexy

Table 2 Bioinformatics analysis for biochemical properties of DHX37 protein

	Molecular weight (Da)	Amino acids	Strongly basic amino acids	Strongly acidic amino acids	Hydrophobic amino acids	Polar amino acids	Isoelectric point	Charge (pH7.0)
Wild type	129545.71	1157	164	157	394	239	8.099	9.520
p.Glu257Lys	129544.76	1157	165	156	394	239	8.239	11.516

Abbreviation:DHX37 DEAH-box RNA helicase 37

Reference sequence human DHX37 protein (RefSeq NP_116045.2, NCBI)

NR5A1 on the *CYP11A1* promoter (– 110 bp – +49 bp) (Fig. 6C). This result indicated that the truncated protein produced by the p.Gln418Ter variant of *NR5A1* could not affect the function of wild type NR5A1 protein. Taken together, these results suggest that the p.Gln418Ter variant identified in this study produces a stop codon that prematurely terminates the protein translation that produces the truncated NR5A1 protein, leading to a loss of

Table 3 Bioinformatics prediction of *DHX37* genetic variant

Type of genetic variant	Site	Predicted software	Predicted results	Predicted value
Missense variant	c.769G > A p.Glu257Lys	CADD	Likely deleterious	26 (≥ 20)
		Mutation Taster	Disease causing	56 (0.0–215)
		MutPred2	Loss of helix	0.623 (> 0.5)
		PANTHER	Probably damaging	1500 (> 450)
		PolyPhen2	Probably damaging	0.992 (0.00–1.00)
		PROVEAN	Deleterious	-3.89 (< -2.5)
		SIFT	Damaging	0.003 (< 0.05)

Abbreviations: CADD Combined annotation dependent depletion, *DSD* Disorders/differences of sex development, *DHX37* DEAH-box RNA helicase 37, *PANTHER* Protein analysis through evolutionary relationships, *PolyPhen2* Polymorphism phenotyping v2, *PROVEAN* Protein variation effect analyzer, *SIFT* Sorting tolerant from intolerant

Reference sequence human DHX37 transcript (RefSeq NM_032656.4, NCBI), human DHX37 protein (RefSeq NP_116045.2, NCBI). The numbers in parentheses indicate the reference values

function of the NR5A1 protein. Previous studies have shown that the missense variants of *DHX37* could cause nucleolar stress, which results in the accumulation of pro-ovary WNT/β-catenin that disrupts testis determination, and also leads to the activation of p53 stabilization that promotes testicular regression [29]. To further clarify the role of *DHX37* missense variant (p.Glu257Lys) on its downstream signaling pathways, we detected the β-catenin and p53 signaling pathways. Compared with the cells transfected with wild type *DHX37* vector, the expression levels of β-catenin and p53 in cells transfected by *DHX37* variants (p.Glu257Lys) vector were unchanged (Fig. 7A). These results indicated that the *DHX37* missense variant (p.Glu257Lys) does not affect the β-catenin and p53 downstream signaling pathways.

Discussion

NR5A1 gene is an important nuclear receptor transcription factor during gonadal development, regulating many target genes related to reproductive development and steroidogenesis, thus its genetic variants is also one of the most common genetic causes for human DSD [30]. Unlike other pathogenic genes of DSD such as *SRY*, *NR5A1* variants lack a clear correlation with DSD clinical manifestations. In 46,XY DSD individuals, *NR5A1* variants can lead to a very wide range of clinical phenotypes, among which the variants at different loci can lead to similar clinical phenotypes, and the variants at the same locus can also lead to different clinical phenotypes [31]. Previous studies have reported that carrying the same *NR5A1* variant in the same family can lead to different clinical manifestations or even normal phenotypes, indicating that the *NR5A1* variant has different degrees of incomplete penetrance [32]. The p.Gln107Ter nonsense variant of the *NR5A1* gene reported in this study has also been reported in previous studies, and these patients carrying the p.Gln107Ter variant of *NR5A1* gene were from China and Germany [24, 25]. Functional experiments showed that the p.Gln107Ter variant of *NR5A1* gene would impair NR5A1 protein localization and the transcriptional activation of NR5A1 for its targeted genes [25]. It is worth noting that our patient no.1 presents a completely different clinical phenotype from the patient reported in Germany. The German patient was born with abnormal external genitalia included a normal phallus, penoscrotal hypospadias, bilateral cryptorchidism, and absence of uterus (Prader 4 grade). Our patient no.1 presented with normal female external genitalia and primary amenorrhea during adolescence (Prader 0 grade). Clinical examination confirmed that the patient no.1 had no uterus. Our patient no.1 carries the same *NR5A1* variant as the previously reported German patient, but the two patients present different subgroups of 46,XY DSD (Table 4).

Our patient no.2 also carries the nonsense variant (p.Gln418Ter) of *NR5A1* gene, which are of the same variant type as those found in patient no.1 and the previously reported German patient. However, these variants are located in the different locations of *NR5A1* gene. Although the clinical phenotypes of patient no.2 are different from that of patient no.1 (Prader 0 grade), the clinical phenotypes of patient no.2 (Prader 4 grade) are similar to that of the German patient previously reported (Prader 4 grade). Structural analysis of NR5A1 protein demonstrated that compared to the p.Gln418Ter variant, the p.Gln107Ter variant resulted in a loss of most functional domains within NR5A1 protein (Fig. 3B). The p.Gln107Ter variant in the *NR5A1* gene is predicted to trigger NMD. The ClinGen database assigns *NR5A1* a haploinsufficiency (HI) score of 3, indicating the sufficient evidence for haploinsufficiency, thus supporting the application of the PVS1-strong criterion to this variant (Table 5). The p.Gln418Ter variant in the *NR5A1* gene is located within the terminal exon and is predicted to escape NMD, thus requiring downgrading of the PVS1 criterion to moderate strength or PM1 (lacking a key domain AF-2 of NR5A1) (Table 5). Functional experiments revealed that similar to the p.Gln107Ter variant, the p.Gln418Ter variant also impaired the cellular localization and transcriptional activation function of NR5A1

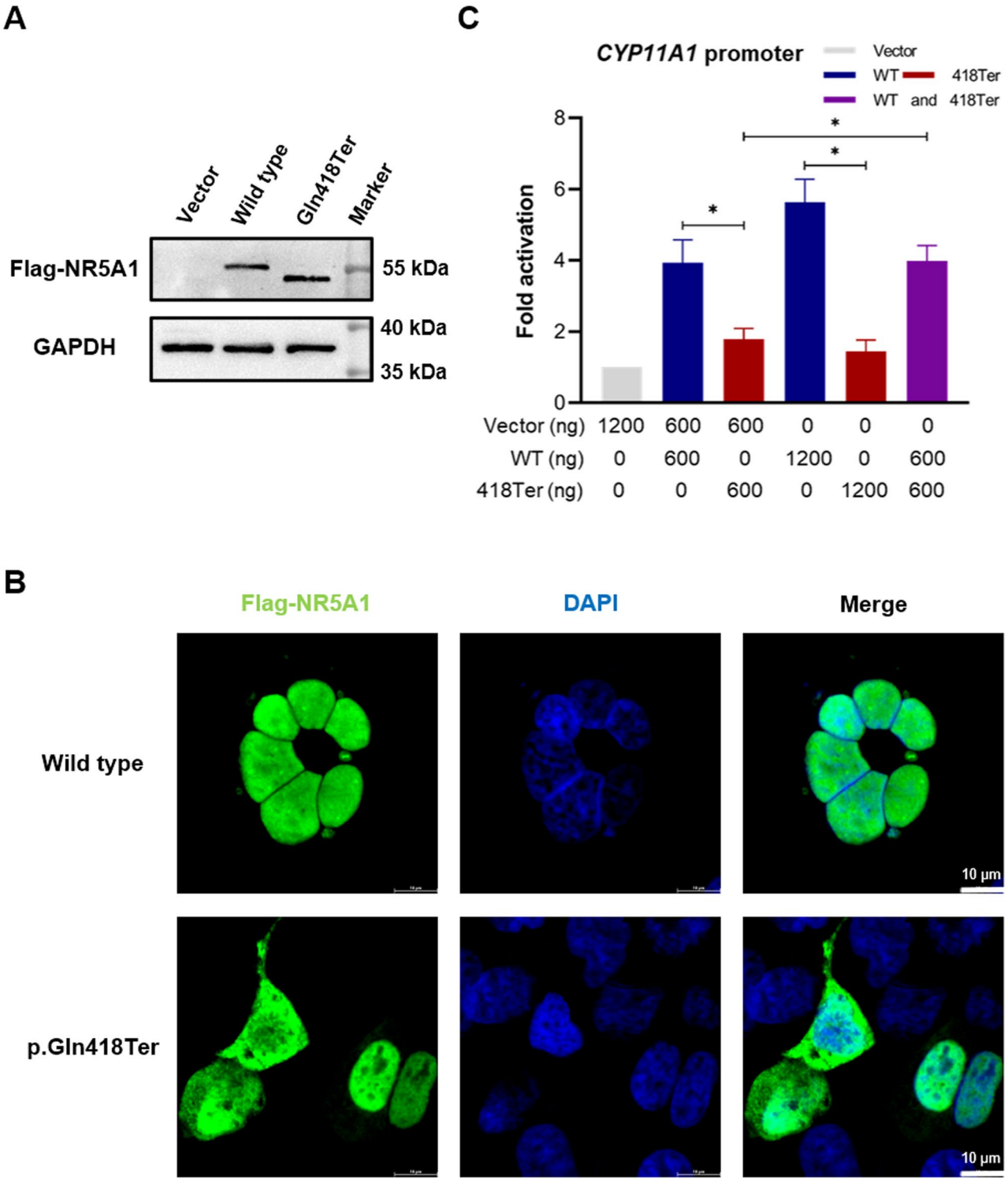

Fig. 6 Effect of p.Gln418Ter variant on the expression and function of NR5A1 protein. **A** NR5A1 protein expression and molecular weight of wild type vector (Wild type), mutant p.Gln418Ter vector (Gln418Ter) and empty vector (Vector) transfected into cells were evaluated by Western blot assay. **B** Cellular localization of wild type NR5A1 protein (Wild type) and mutant NR5A1 protein (p.Gln418Ter) transfected into cells was identified by cellular immunofluorescence assay. The white scale represents 10 μm. **C** Effect of wild type NR5A1 (WT) and mutant p.Gln418Ter NR5A1 (418Ter) on transcriptional activation of *NR5A1* target gene *CYP11A1* promoter (− 110 bp − +49 bp). Results are expressed as a ratio of empty vector (Vector) activity, which is considered to be 1. Data represent the mean of four independent experiments, and each performed in triplicate and bars represent the SEM. * indicates a statistically significant difference ($P < 0.05$)

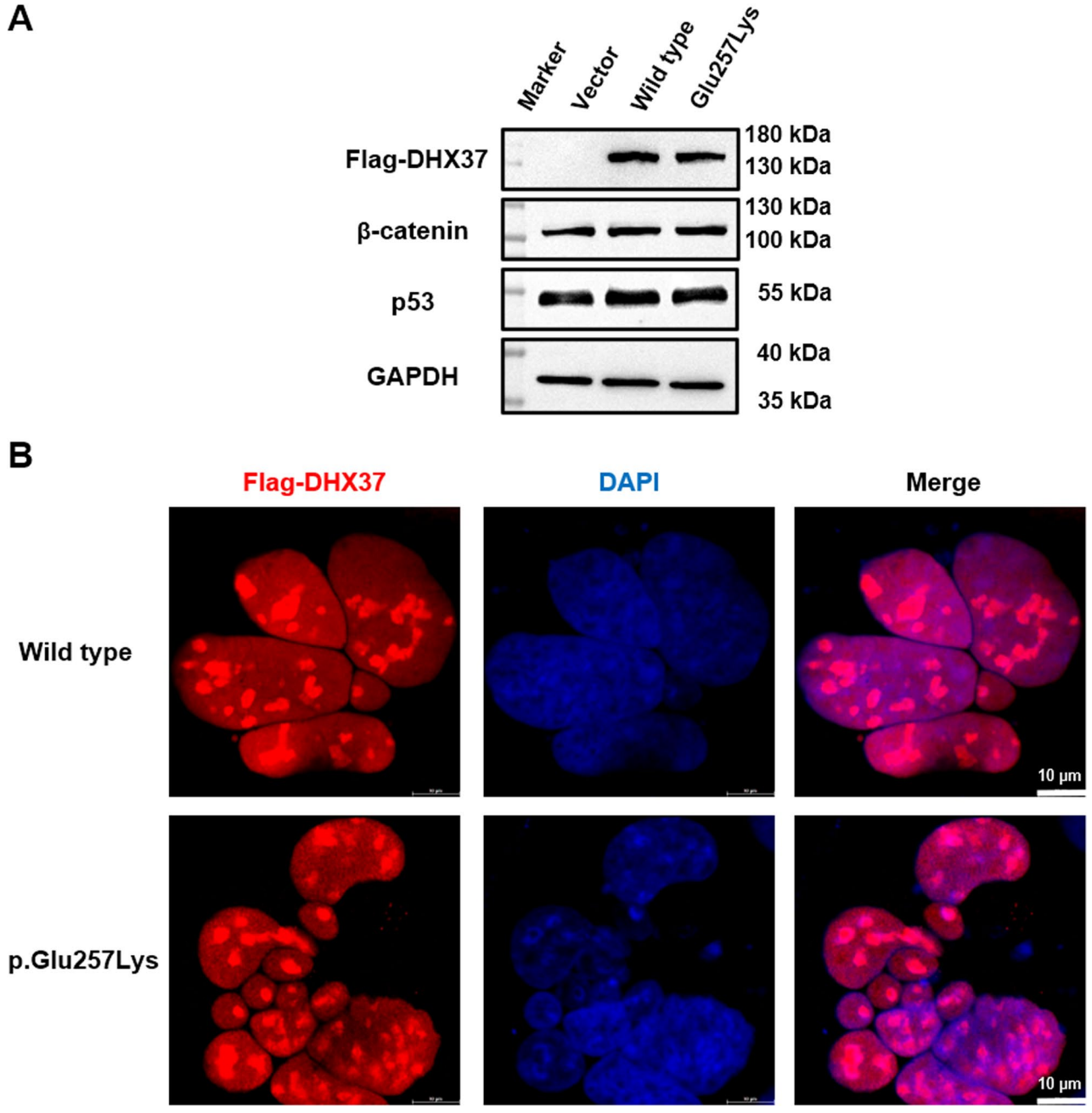

Fig. 7 Effect of p.Glu257Lys variant on the expression and function of DHX37 protein. **A** Expression of various proteins of wild type DHX37 vector (Wild type), mutant DHX37 vector (Glu257Lys) and empty vector (Vector) transfected into cells were evaluated by Western blot assay. **B** Cellular localization of wild type DHX37 protein (Wild type) and mutant DHX37 protein (p.Glu257Lys) transfected into cells was identified by cellular immunofluorescence assay. The white scale represents 10 μm

protein on its targeted genes (PS3). According to the above analysis, it is speculated that the patient carrying the p.Gln107Ter variant may exhibit more severe clinical phenotypes, consistent with our findings for patient no.1 (Prader 0 grade, Table 4). However, it should be noted that the German patient with the same pathogenic variant (p.Gln107Ter) had the relatively mild clinical phenotypes (Prader 4 grade, Table 4). These observations highlight the clinical phenotypic heterogeneity of *NR5A1* pathogenic variants leading human 46,XY DSD and emphasize further investigation required for elucidating its underlying mechanisms.

Studies using NGS to diagnose DSD patients have found that many patients with DSD have two or more likely pathogenic variants associated with sex development [33]. In our study, patient no.2 has two variants of

Table 4 Comparative analysis of two 46,XY DSD cases in this study and those previously reported

	German patient	Patient no.1	Brazilian patient no.1	Brazilian patient no.2	Patient no.2
Age of diagnosis	Birth	17 years	0.5 month	12 years	Birth
Clinical diagnosis	46,XY PGD	46,XY CGD	46,XY PGD	46,XY PGD	46,XY PGD
Prader	4	0	4	4	4
Genetic diagnosis	*NR5A1* p.Gln107Ter	*NR5A1* p.Gln107Ter	*NR5A1* p.(Ser4Ter) *DHX37*p.(Val999Met)	*DHX37* p.(Val999Met)	*NR5A1* p.Gln418Ter *DHX37* p.Glu257Lys
Amino acid change	*NR5A1* Missing 354 AA	*NR5A1* Missing 354 AA	*NR5A1* Missing 457 AA *DHX37* Np AA→Np AA	*DHX37* Np AA→Np AA	*NR5A1* Missing 43 AA *DHX37* Ac AA→Ba AA
ACMG classification	P	P	*NR5A1* P *DHX37* LB	*DHX37* LB	*NR5A1* LP *DHX37* VUS

Abbreviations:AA Amino acids, *Ac* Acidic, *ACMG* American College of Medical Genetics and Genomics, *Ba* Basic, *CGD* Complete gonadal dysgenesis, *DSD* Disorders/differences of sex development, *DHX37* DEAH-box RNA helicase 37, *LB* Likely benign variant, *LP* Likely pathogenic variant, *Np* Nonpolar, *NR5A1* Nuclear receptor subfamily 5 group A member 1, *P* Pathogenic variant, *PGD* Partial gonadal dysgenesis, *VUS* Variant of unknown significance

Reference sequence human DHX37 transcript (RefSeq NM_032656.4, NCBI), human DHX37 protein (RefSeq NP_116045.2, NCBI), human NR5A1 transcript (RefSeq NM_004959.5, NCBI), human NR5A1 protein (RefSeq NP_004950.2, NCBI)

Table 5 Clinical genetic diagnosis of two 46,XY DSD patients in this study

Patient ID	Gene	Nucleotide change	Amino acid change	Variant type	Allele frequency	ACMG	Pathogenicity
1	*NR5A1*	c.319 C > T	p.Gln107Ter	Nonsense variant	0 (gnomAD) 0 (1KGP)	PVS1(strong)+PS3 + PM2-supporting	Pathogenic variant
2	*NR5A1*	c.1252 C > T	p.Gln418Ter	Nonsense variant	0 (gnomAD) 0 (1KGP)	PVS1(moderate)/ PM1 + PS3+PM2-supporting	Likely pathogenic variant
	DHX37	c.769G > A	p.Glu257Lys	Missense variant	0.00001 (gnomAD) 0.00001 (1KGP)	PM1 + PM2-supporting +PP3	VUS

Abbreviations:ACMG American College of Medical Genetics and Genomics, *DSD* Disorders/differences of sex development, *DHX37* DEAH-box RNA helicase 37, *NR5A1* Nuclear receptor subfamily 5 group A member 1, *VUS* Variant of unknown significance, *1KGP* 1000 Genomes Project

Reference sequence human DHX37 transcript (RefSeq NM_032656.4, NCBI), human DHX37 protein (RefSeq NP_116045.2, NCBI), human NR5A1 transcript (RefSeq NM_004959.5, NCBI), human NR5A1 protein (RefSeq NP_004950.2, NCBI)

DSD-related genes. One is a nonsense variant of *NR5A1* gene, the other genetic variant carried by patient no.2 is a missense variant of *DHX37* gene. DHX37 is a highly conserved DEAH-box family of RNA helicases including two highly conserved RecA1 and RecA2 domains. Up to date, the reported missense variants of *DHX37* gene associated with 46,XY DSD are mainly located in the RecA1 and RecA2 domains of DHX37 protein [34]. This p.Glu257Lys missense variant of the *DHX37* gene in patient no.2 was also located in the RecA1 domain of DHX37 protein, and the amino acid (glutamic acid) at this site was highly conserved across various species (PM1). The p.Glu257Lys missense variant was described in the ClinVar database as being associated with NEDBAVC and of uncertain significance, and the p.Glu257Lys missense variant exhibited very low allele frequency (0.00001, PM2-supporting). Previous investigations have revealed that the *DHX37* pathogenic variants related to DSD are predominantly heterozygous, whereas those related to NEDBAVC are mainly either homozygous or compound heterozygous [35]. Consistent with previous studies, the p.Glu257Lys variant found in this study were determined to be heterozygous missense variants, and the clinical phenotype of patient no.2 also exhibited the clinical phenotypes of TRS or PGD, which is consistent with the clinical phenotypes of 46,XY DSD individuals carrying *DHX37* pathogenic variants. Structural analysis and physicochemical analysis showed that the substitution of acidic amino acid glutamic acid by basic amino acid lysine at position 257 of DHX37 protein would affect its charge and structure, and multiple bioinformatics software predicted that the p.Glu257Lys variant would impair the function of DHX37 protein (PP3). According to the ACMG guidelines the p.Glu257Lys variant of *DHX37* gene can be assessed as a VUS (Table 5). To further elucidate the significance of p.Glu257Lys variant, we used functional experiments to study this variant, and we found that the p.Glu257Lys variant had no significant effect on DHX37 protein expression. Both wild type DHX37 and mutant DHX37 proteins (p.Glu257Lys variant) were located in the nucleus, which is similar to the previous reports that both wild type DHX37 protein and three mutant DHX37 proteins (p.Thr304Met, p.Arg334Leu and p.Gly1030Glu) exhibited the nucleolar localization [9]. Since DHX37 plays an important biological role in ribosome biogenesis, the missense variants of *DHX37* can interfere with ribosome biogenesis, and the defects in ribosome biogenesis may trigger nucleolar stress responses. Studies have found that nucleolar stress causes cell cycle arrest and apoptosis through the activation of p53 signaling pathway, which can lead to testicular regression [36]. Nucleolar stress also interferes with testis determination

by activating the Wnt/β-catenin signaling pathway that can promotes ovarian development [37]. Recent studies have found that the p.Ser408Leu variant of *DHX37* can lead to the upregulation of β-catenin protein [38]. We also detected the β-catenin and p53 signaling pathways that related to the biological function of DHX37 protein, and the results showed that the p.Glu257Lys variant of DHX37 did not affect the expression of downstream signaling molecules β-catenin and p53. Although our current functional assays have not detected a significant impact of the p.Glu257Lys variant on protein function, further investigation is warranted given the localization of p.Glu257Lys variant within the RecA1 domain (a region involved in ATP binding/hydrolysis or RNA binding) of DHX37 (Fig. 4A). Additional studies are required to explore the potential effects of p.Glu257Lys variant of *DHX37* on substrate (ATP or RNA) binding affinity. In addition, single-cell expression analysis of the developing XY mouse gonad for *DHX37* and selected sex-determining genes (*SRY*, *SOX9* and *SOX8*) revealed the co-expression of *DHX37* and *SOX9* within in a proportion of cells [9]. However, the molecular mechanisms or interaction molecules through which the *DHX37* gene regulates human gonadal development remain largely elusive. Future work should focus on identifying the molecules and pathways that interact with *DHX37* gene, thereby clarifying the role of its variants in human DSD.

We have yet to acquire the genetic testing results for the patient no.2's family, but the twin brother of patient no.2 has normal male external genitalia, and its parents exhibit normal phenotypes and possess normal fertility. If the p.Glu257Lys variant of *DHX37* gene is confirmed to be *de novo*, the pathogenicity of this variant is increased, and it can be classified as a likely pathogenic variant. Additionally, when the p.Glu257Lys variant of DHX37 gene is inherited from their parents, the mother of patient no.2 as a carrier, typically remains unaffected. Previous studies have reported that the female carrying pathogenic variants of the *DHX37* are generally unaffected [29]. If the father and twin brother of patient no.2 are carriers of the *DHX37* variant, but they are phenotypically normal. This is consistent with previous studies that have reported that the asymptomatic father carries the pathogenic variant of *DHX37* (p.Arg308Gln) [8]. These findings suggest that the presence of incomplete penetrance of *DHX37* gene associated 46,XY DSD. A recent study found that the p.(Val999Met) missense variant of *DHX37* was identified in two unrelated 46,XY PGD patients from Brazil. One of them carries two variants in both *NR5A1* and *DHX37* genes, the *de novo* p.(Ser4Ter) nonsense variant of *NR5A1*, and the p.(Val999Met) in *DHX37* inherited by the asymptomatic father of this patient from Brazil. Although the p.(Val999Met) variant of *DHX37* was a likely benign variant by ACMG rating, the other unrelated 46,XY PGD Brazilian patient was also identified this variant (Table 4). This suggests that the pathogenicity of the p.(Val999Met) variant cannot be ignored [10]. The 46,XY PGD patient from Brazil in above study carrying both *NR5A1* and *DHX37* variants was suggested as a digenic inheritance. Similarly, the potential pathogenicity of p.Glu257Lys variant of *DHX37* with ACMG rating VUS carried by 46,XY PGD patient no.2 in our study cannot be ignored. Additional cases will be required in the future to evaluate the potential pathogenicity of p.Glu257Lys variant of *DHX37*.

In conclusion, we identified three DSD associated variants in two 46,XY DSD patients through whole exome sequencing and Sanger sequencing validation. The functional impact of *NR5A1* p.Gln418Ter variant and *DHX37* p.Glu257Lys variant was explored using in silico analysis and in vitro functional experiments. These findings broaden the spectrum of pathogenic genetic variants related 46,XY DSD clinical phenotypes, facilitating accurate diagnosis of DSD patients in the future.

Abbreviations

ACMG	American College of Medical Genetics and Genomics
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
CADD	Combined Annotation Dependent Depletion
CGD	Complete Gonadal Dysgenesis
CTD	Carboxy Terminal Domain
DBD	DNA Binding Domain
DHX37	DEAH-box RNA Helicase 37
DSD	Disorders/differences of Sex Development
GD	Gonadal Dysgenesis
gnomAD	Genome Aggregation Database
HA2	Helicase Associated domain 2
HPO	Human Phenotype Ontology
HRP	Horseradish Peroxidase
LBD	Ligand Binding Domain
MAP3K1	Mitogen Activated Protein Kinase Kinase Kinase 1
NGS	Next Generation Sequencing
NR5A1	Nuclear Receptor subfamily 5 group A member 1
OB	Oligonucleotide Binding fold domain
OMIM	Online Mendelian Inheritance in Man
PANTHER	Protein Analysis Through Evolutionary Relationships
PCR	Polymerase Chain Reaction
PGD	Partial Gonadal Dysgenesis
PolyPhen2	Polymorphism Phenotyping v2
PROVEAN	Protein Variation Effect Analyzer
RIPA	Radio Immunoprecipitation Assay
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIFT	Sorting Intolerant From Tolerant
SRY	Sex determining Region Y
TRS	Testicular Regression Syndrome
VUS	Variant of Unknown Significance
WES	Whole Exome Sequencing

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
MX and XW supervised and designed the study. CL and BY performed the in vitro functional experiments and in silico analysis. CL, BY, XL, XW and MX drafted and revised the manuscript. SZ, XL, PL, and WC collected clinical samples and data. All authors reviewed and approved the final manuscript.

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Data availability
The datasets generated and/or analysed during the current study are available in the ClinVar repository (https://submit.ncbi.nlm.nih.gov/clinvar/). The relevant accession number is SCV005911640 (https://www.ncbi.nlm.nih.gov/clinvar/variation/3891321/?term=SCV005911640).

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China, Ethics Committee Approval Numbers: XJTU1AF2020LSK-102 and XJTU1AF2024LSYY-141). This study was performed in accordance with the principles of Declaration of Helsinki. Informed consent was obtained from all the participants and parents of patients who participated in this study.

Consent for publication
Written informed consent was obtained from all participants and the parents of minors for the publication of their personal or clinical details along with any identifying images in this study.

Competing interests
The authors declare no competing interests.

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