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# Targeted long-read sequencing identifies missing pathogenic variant in unsolved 11β-hydroxylase deficiency

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## Abstract

**Background** 11β-hydroxylase deficiency (11β-OHD), caused by homozygosity or compound heterozygosity *CYP11B1* variants, is the second most common cause of congenital adrenal hyperplasia (CAH). Due to the high degree of sequence identity between *CYP11B1* and *CYP11B2*, chimeric genes, and complex structural variants (SVs), the conventional approach to gene testing for 11β-OHD is facing challenges. The study aimed to clarify the underlying genetic causes of two siblings of a Chinese family with 11β-OHD.

**Methods** Peripheral blood samples and clinical information were collected from subjects and their family members. Sex steroid concentrations were measured using LC-MS/MS. Long-range PCR-based next-generation sequencing (NGS), PCR assay and target long-read sequencing were used to detect the pathogenic variants.

**Results** Early onset hypertension, increased serum levels of adrenocorticotropin (ACTH), progesterone, testosterone, and decreased cortisol and potassium were detected in both affected siblings. Long-range PCR-based NGS identified a heterozygous missense variant (NM_000497.4:c.281 C > T, p.P94> L) in *CYP11B1* gene in the two siblings. PCR detected no chimeric *CYP11B2/CYP11B1* gene. We finally identified a second pathogenic variant in *CYP11B1* gene via target long-read sequencing (T-LRS). This novel variant was a deletion-insertion variant and located chr8:143957269–143,957,579 (hg19) with the insertion of 'ACAG' (NM_000497.4:c.954 + 78_980delinsACAG), which was in trans with *CYP11B1*: c.281 C > T.

**Conclusions** Our study suggests that the integrated long-range PCR-based NGS and T-LRS seem to be the most reliable and accurate method for 11β-OHD genetic diagnosis and carrier sequencing.

**Keywords** 11β-hydroxylase, Congenital adrenal hyperplasia, CYP11B1, Long-read sequencing

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## Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive genetic disorders of adrenal steroid biosynthesis, in over 90% of cases due to 21-hydroxylase deficiency (21-OHD) [1]. 11β-hydroxylase (CYP11B1) deficiency (11β-OHD) is the second most common type of CAH, accounting for approximately 5–8% of cases [2]. 11β-OHD is relatively rare in China, with an estimated incidence of 1:100,000 to 1:200,000 live births in the general population [3]. A deficiency in 11β-hydroxylase leads to decreased synthesis of cortisol and corticosterone, resulting in overproduction of steroid precursors and excess androgen. Lack of negative feedback by cortisol leads to an increase in ACTH secretion, which causes adrenal cortex hyperplasia and contributes to the production of steroid precursors. Accumulation of 11-deoxycorticosterone (DOC) generates a mineralocorticoid effect, resulting in hypertension and hypokalemia [4]. Generally, 11β-OHD has been classified into two forms: the classic 11β-OHD phenotype is characterized by virilization of external genitalia in newborn females, precocious puberty in both sexes, hypertension, and hypokalemia. A non-classic 11β-OHD form is usually characterized by mild hirsutism, irregular menses, and slight increase of serum androgen [5, 6]. Thus, non-classic 11β-OHD is easily misdiagnosed clinically.

The *CYP11B1* consists of 9 exons and encodes a protein of 503 amino acids [7]. To date, over 226 different variants causing 11β-OHD have been identified and distributed across the entire coding region, including 135 missense/nonsense variants, 30 splicing variants, 18 small deletions, 12 small insertions/duplications, 3 small indels, 2 regulatory variants, 15 gross deletions and 11 complex rearrangements (HGMD Professional 2023.3). It is noteworthy that gross deletions and complex rearrangements are technically challenging to detect due to the limitations of short-read sequencing (SRS) [8]. Recently, long-read sequencing (LRS), or third-generation sequencing has overcome some of the deficiencies of conventional methods [9].

Here, we report a family with two clinically diagnosed 11β-OHD siblings who have only one heterozygous pathogenic missense variant identified by long-range PCR-based NGS. By using Pacbio LRS, we identified a second pathogenic structural variant that was missed by SRS. To our knowledge, this is the first case report of diagnosing 11β-OHD using T-LRS.

## Methods

### Subjects

This study comprised 4 participants: the proband diagnosed with 11β-hydroxylase deficiency, her affected brother, and their healthy father and mother. Clinical phenotypes, biochemical markers, and imaging data were collected from the family, with informed consent obtained from all members for publication.

### Steroid hormone measurement by LC-MS/MS

Steroid hormones were measured using a high performance liquid chromatograph (ExionLC™ AD, SCIEX, USA) and a triple quadrupole mass spectrometer (Triple Quad 4500MD, SCEIX, USA). The preparation of blood samples and the LC-MS/MS experimental procedure was carried out according to the instructions of the Multiple Steroid Hormone Kit (MS014B, Qlife, China). The LC-MS/MS experiment was conducted in the Jinan AXZE Medical Test Laboratory.

### Long-range PCR-based next-generation sequencing

DNA was isolated from peripheral leukocytes obtained from the patient using a commercial kit (TIANGEN, China). The quantity/quality of DNA was assessed using Onedrop OD1000 spectrophotometer and by agarose gel electrophoresis. A specific set of primers were used to detect the chimeric gene (Supplementary Table 1). The targeting sequence of *CYP11B1* and *CYP11B2* gene was captured by amplification enzyme and corresponding long fragment primer, the enriched libraries were sequenced using an Illumina HiSeq X Ten sequencer (Illumina, San Diego, USA) for paired reads of 150 bp, and sequencing result analysis was done in Shanghai WeHealth Biomedical Technology Co., Ltd. Sanger sequencing was used to validate the pathogenic variants by Jinan AXZE Medical Test Laboratory.

### Long-read sequencing

Blood sample was obtained from the patient for DNA isolation. The fragmented DNA was repaired and ligated with Barcode Adapter using rapid DNA ligase. The product added with GXL DNA Polymerase (Takara) and Primer (5'-GCAGTCGAACATGTAGCTGACTCAG GTCAC-3') was subjected to denaturation at 98 °C for 1 min and followed by 7 cycles at 98 °C for 15 s, 68 °C for 10 min, and finally incubated at 68 °C for 10 min. Then the hybrid DNAs were captured with the Congenital Adrenal Hyperplasia (CAH) probe (Boke Bioscience). All purification steps were carried out with Agencourt AMPure XP beads (Beckman Coulter). Qubit dsDNA HS Assay Kit (Life Invitrogen) was used to detect the concentration of the product. The DNA library was generated with SMRTbell Express Template Kit 2.0 (PacBio) and sequenced on the PacBio Sequel II platform. The raw sequencing data was qualified with SMRT Link (version 12.0) to obtain HiFi reads. Circular Consensus Sequence (CCS) reads were automatically generated by the PacBio SMRT analysis module. Single-nucleotide variations (SNVs) were called by DeepVariant (version 1.3.0) and annotated by Vep (version 107). Structure variations

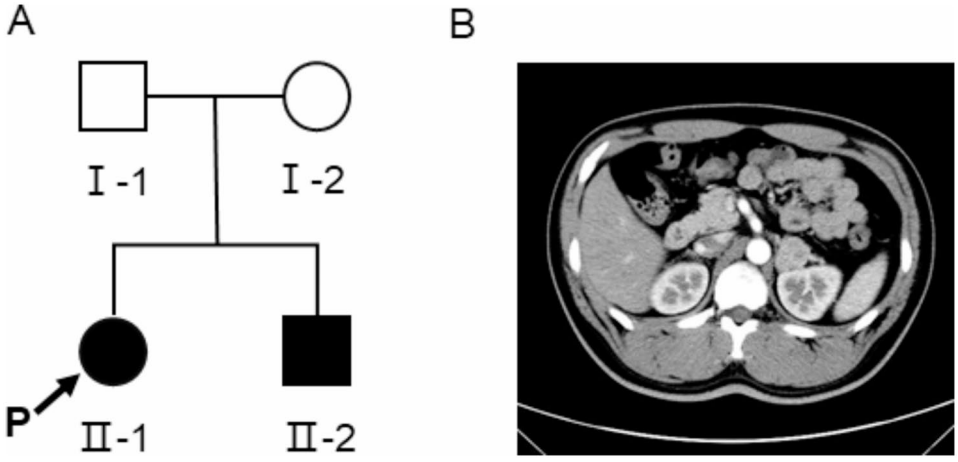


**Fig. 1** Patient family pedigree and computed tomography scan of the proband. **A** The family pedigree shows the proband and similarly affected brother (black shading). **B** CT image showing a 3.0 × 2.4 cm left adrenal nodule

**Table 1** Clinical characteristics of the two patients

| Patient | The proband | | The younger brother | | |
|---|---|---|---|---|---|
| | Pre-intervention | Post-intervention | Pre-intervention | Post-intervention | Reference |
| Blood pressure (mmHg) | 180/130 | 130/80 | 160/105 | 130/75 | < 140/90 |
| ACTH (pg/ml) | 331.7 | 120.7 | 334.3 | 254.0 | 7–63 |
| Cortisol (ug/dl) | 5.48 | 1.89 | 6.15 | 2.0 | 8.7–22.4 |
| LH (mIU/L) | 2.1 | 9.56 | 3.18 | 4.41 | 2.4–12.6 |
| FSH (mIU/L) | 6.4 | 5.02 | 3.04 | 3.70 | 3.5–12.5 |
| Progesterone (ng/ml) | 7.15 | 1.34 | 3.31 | 1.59 | 0.18–2.84 |
| Estradiol (pg/ml) | 63.54 | 95.38 | 77.14 | 43.66 | Female 25–138<br>Male 0–84 |
| Testosterone (ng/ml) | 3.54 | 0.22 | 3.56 | 6.36 | Male 1.82–8.11<br>Female 0.1–1.23 |
| Potassium (mmol/L) | 3.30 | 3.89 | 2.73 | 4.33 | 3.5–5.5 |

ACTH: Adrenocorticotrophic hormone. LH: Luteinizing hormone. FSH: Follicle-stimulating hormone

(SVs) were detected by Sniffles (version 2.0.7) and Cutesv (version 1.0.12). SVs were annotated by AnnotSV (version 3.1.1). Long-range PCR (specific primers are in Supplementary Table 2) and Sanger sequencing were used to validate the deletion. The Long-read sequencing experiment was conducted in Beijing GrandOmics Biosciences Co., Ltd.

## Results

### Clinical presentation of the pedigree

The index case was a 22-year-old female who had a diagnosis of hypertension at the age of 13 (Fig. 1A). There was no obvious masculinization, and her parents stated that there were no obvious abnormalities in vulva development at birth. The patient had undergone spontaneous menarche at the age of 12 and had sparse menstrual flow. On physical examination, the patient's height was 160 cm (paternal height is 165 cm and maternal height is 160 cm), her body weight was 75 kg, and her body mass index (BMI) was 29.3 kg/m$^2$. She had Tanner stage 3 breast development, with Tanner pubic hair stage 4. Adrenal computed tomography scanning revealed a single mass measuring 3.0 cm×2.4 cm in the left adrenal gland (Fig. 1B). The proband's younger brother is now 14 years old and was diagnosed with hypertension at the age of 10. He also had a history of hypokalemia. His height was 163 cm, his body weight was 73 kg, and his body mass index (BMI) was 27.5 kg/m$^2$. Longitudinal growth seemed to stall at the age of 13. Transthoracic echocardiography showed interventricular septum thickening (13 mm).

Laboratory findings are summarized in Table 1. Overall, the serum levels of ACTH, progesterone, testosterone, were markedly elevated in both siblings. Decreased cortisol and potassium were also noted. Meanwhile, 17-hydroxyprogesterone (697 ng/dl, normal range < 233 ng/dl) and androstenedione (811 ng/dl, normal range 31–65 ng/dl) content analyzed by mass spectrometry were increased in the younger brother. Based on the clinical information, a diagnosis of 11β-hydroxylase deficiency was suggested.

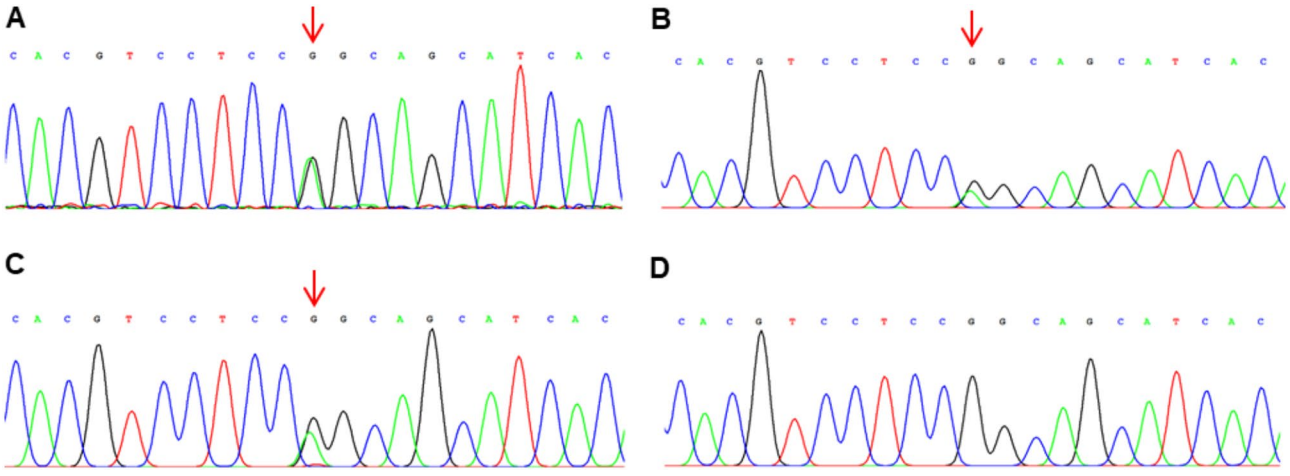


**Fig. 2** Heterozygous *CYP11B1*:c.218 C>T variant identified in **A** the proband, **B** the brother, **C** the father, and **D** the mother, mother is reference, other three are heterozygous for the variant

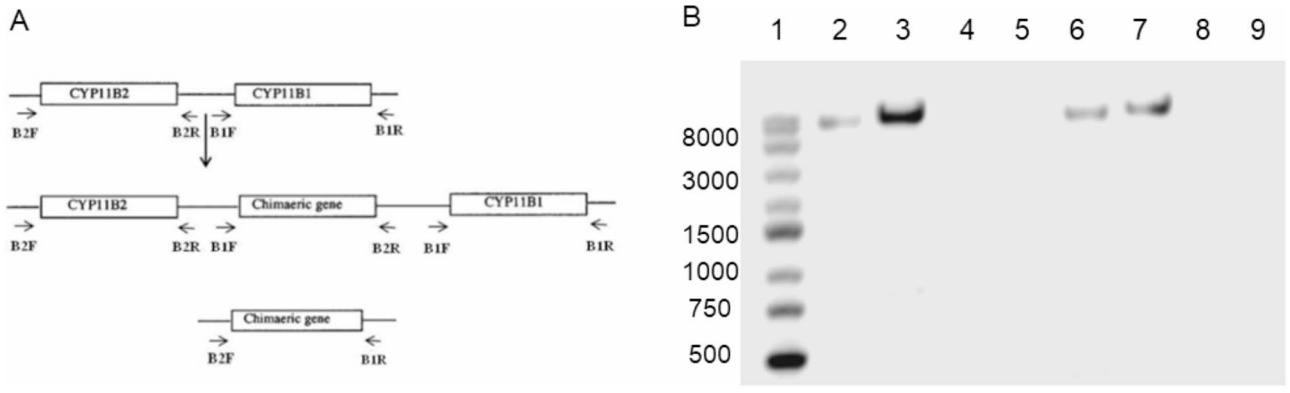


**Fig. 3** Chimeric *CYP11B2/CYP11B1* fusion gene test of the proband. **A** Scheme of the chimeric *CYP11B2/CYP11B1* gene and the location of chimeric *CYP11B2/CYP11B1* primers. **B** The electrophoretogram of PCR products was obtained from the proband and the control using mixed primers. Lane 1: DNA marker, Lane 2: primer 1 F/1R (proband), Lane 3: primer 2 F/2R (proband), Lane 4: primer 1 F/2R (proband), Lane 5: primer 2 F/1R (proband), Lane 6: primer 1 F/1R (control), Lane 7: primer 2 F/2R (control), Lane 8: primer 1 F/2R (control), Lane 9: primer 2 F/1R (control)

### *CYP11B1* variant analysis

In order to clarify the genetic diagnosis of the patient, long-range PCR-based next-generation sequencing was performed. Approximately 95.69% of the bases exhibited a coverage of 20X, with 93% of the reads possessing a phred-like score exceeding 30. Subsequent analysis revealed the presence of a heterozygous missense variant (NM_000497.4: c.281 C>T, p.P94> L) in exon 8 of the *CYP11B1* gene in the patients and their father (Fig. 2A-D). The variant was classified according to modified ACMG/AMP criteria and ClinGen General Sequence Variant Curation Process [10, 11]. This variant has been previously reported in homozygous and compound heterozygous states in at least two individuals affected with 11β-OHD (PM3_Strong). It is not found in gnomAD East Asian (gnomAD v2.1.1, PM2_Supporting). In vitro experimental studies have demonstrated that this variant results in an almost complete loss of enzyme activity (PS3) [12]. A different Likely pathogenic missense variant at this amino acid residue, c.281 C>A (p.P94> L) has been reported (Clinvar ID: 1497921, PM5_Supporting). Based on ACMG/AMP criteria this variant was classified as pathogenic (PS3, PM2_Supporting, PM3_Strong, PM5_Supporting). However, the nature of 11β-hydroxylase deficiency is an autosomal recessive inheritance, so we considered that there should be another pathogenic variant in *CYP11B1* gene. The chimeric *CYP11B2/CYP11B1* gene has been reported to be a less common cause of 11β-OHD [13, 14]. However, no chimeric *CYP11B2/CYP11B1* gene was detected via long-range PCR assay (Fig. 3A-B).

Another possible reason is that the missing variant is difficult to identify by long-range PCR-based SRS, such as structural variants. To rule out this possibility, T-LRS was used to identify another causative variation in the patients. In total, 307.80 Mb raw data from LRS was generated for analysis. The mean sequencing depth for the targeted regions was 400.36, and the average length

of the reads was 3329.66 bp. 97.93% of the reads had a Phred score above 30, and 97.80% of the bases had a 20X coverage. One SV on chromosome 8 in *CYP11B1* gene was identified. This SV was a deletion-insertion variant and located on chromosome 8 (chr8:143957269–143957579) with the insertion of 'ACAG'. This variant covered part sections of intron 5 and exon 6 of *CYP11B1* gene (Fig. 4A). The deletion-insertion variant (c.954+78_980delinsACAG) results in a frameshift and is predicted to cause nonsense mediated decay (NMD) in *CYP11B1* gene where LOF is a known mechanism of disease (PVS1).

It is not present in gnomAD East Asian (gnomAD v2.1.1, PM2_Supporting) and is in trans with pathogenic variant (*CYP11B1*:c.281 C>T, PM3_trans). Based on modified ACMG/AMP criteria (Richards et al. 2015) this variant was classified as pathogenic (PVS1, PM2_Supporting, PM3_trans).

To verify this variant in *CYP11B1* gene, long-range PCR and Sanger sequencing were carried out to confirm the deletion-insertion variation in the family members (Fig. 4B and E, supplemental Fig. 1). Results showed that compared to the father, the mother and the siblings carried this heterozygous SV.

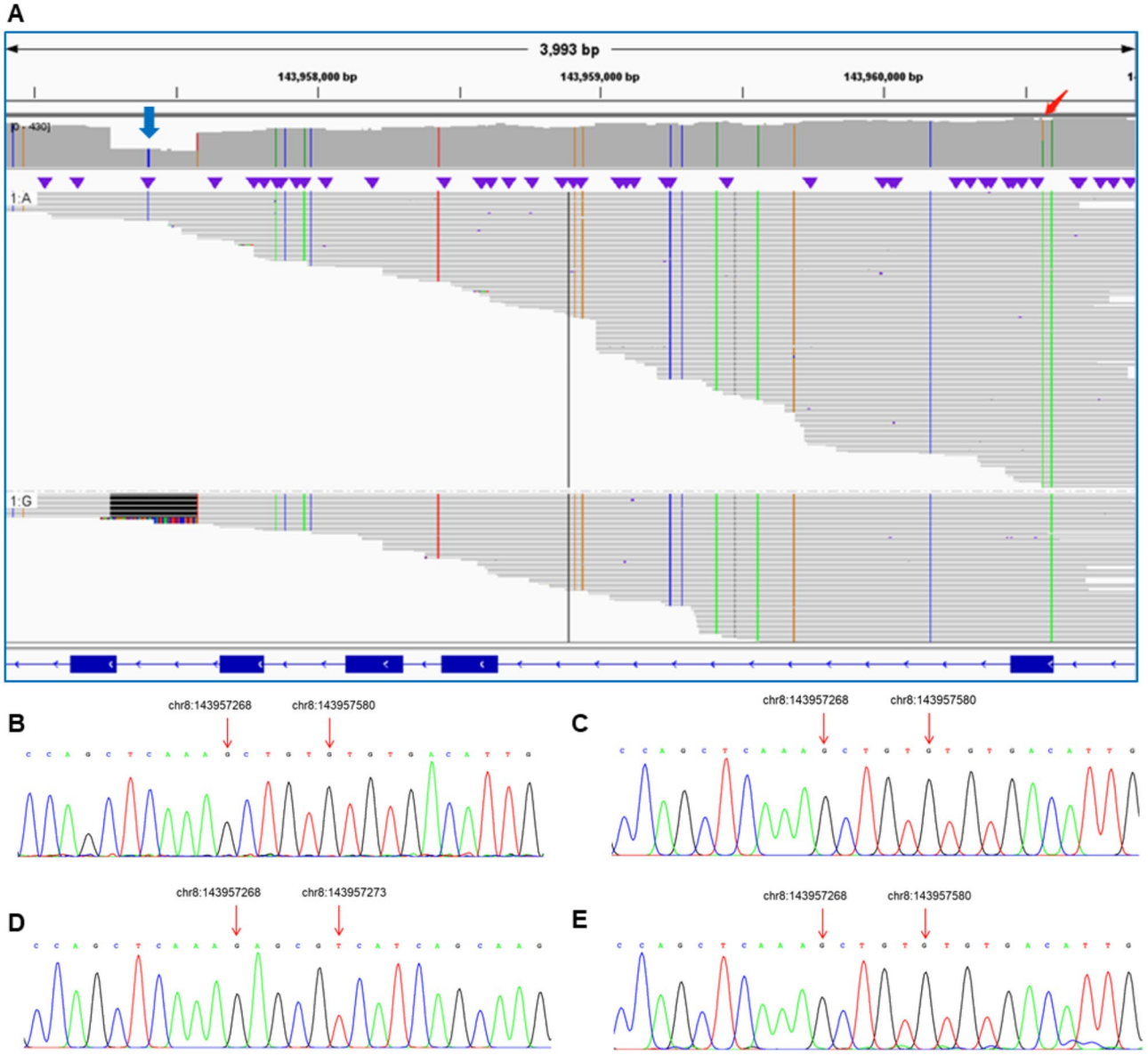


**Fig. 4** Targeted long-read sequencing (T-LRS) was used to identify the missing variant. **A** IGV plot of T-LRS result showing the variations of *CVP11B1* gene. The red arrow represents the c.218 C>T point variant, the blue arrow represents c.954+78_980delinsACAG. **B-E** Heterozygous c.954+78_980delinsACAG identified in B the proband, **C** the brother, **D** the father, and **E** the mother, father is reference, other three are heterozygous for the delins variant

### Follow-up after treatment

The patients were started on dexamethasone supplementation therapy at 0.375 mg once daily to replace the deficient cortisol and reduce excess androgens and mineralocorticoid precursors. To control blood pressure, irbesartan 150 mg and amlodipine 5 mg were prescribed once daily. Following a 6-month treatment period, the blood pressure and biochemical markers of both patients had nearly reverted to normal levels. The proband resumed spontaneous menstrual bleeding as well.

## Discussion

In this study, we described a family with two siblings who first visited our department with early-onset hypertension. The siblings presented with characteristic features of low renin hypertension, hypokalemia, hirsutism, and precocious puberty. Laboratory examinations indicated increased ACTH, 17-OHP, androgens, androstenedione, and decreased cortisol levels. Based on these clinical manifestations and hormone profiles, the diagnosis of 11β-OHD could be confirmed.

CAH due to 11β-OHD is the second most common autosomal recessive disorder. Because of low incidence, missed diagnosis and misdiagnosis easily occur under these circumstances [15, 16]. Genetic diagnosis of 11β-OHD is essential for timely and effective treatment and providing appropriate genetic counseling [17]. 11β-Hydroxylase is encoded by *CYP11B1*, *CYP11B1* and *CYP11B2* share 95% exonic sequence homology and 90% intronic sequence homology. To clarify the molecular diagnosis, *CYP11B1* gene was selectively amplified and sequenced. Long-range PCR-based NGS identified a heterozygous pathogenic *CYP11B1* variant (c.218 C>T, p.P94> L) in two siblings. This variant has been reported several times [12, 18, 19]. Through measurement of 11β-hydroxylase activity, Krone et al. showed p.P94> L variant impaired enzymatic activity [12].

Using SRS, we identified a single pathogenic variant in the *CYP11B1* gene. Due to the autosomal recessive nature of 11β-OHD, it is possible that a pathogenic variant in the other allele was not detected by conventional sequencing methods. According to HGMD database, gross deletions and complex rearrangements comprised over 10% of the variants, which are more likely to be missed by long-range PCR-based NGS. The chimeric *CYP11B2/CYP11B1* gene has been reported many times [13, 20], but no abnormal rearrangements were detected by the PCR assay.

Intragenic or intergenic deletion is another possible cause of 11β-OHD. However, the majority of previous studies regarding SVs in *CYP11B1* gene were multiple exonic deletions [21–23]. The current approaches may perform well for large CNVs, but struggle with single-exon CNVs or small intragenic CNVs (<10 kb) [24]. Recently, LRS was used to identify complex structural abnormalities and clarify missing variants in cases that remained unsolved following whole exome sequencing analysis. LRS such as PacBio sequencing has clear advantages over SRS in SV discovery, de novo assembly, and transcript isoform identification. A few recent studies have investigated the application of LRS in the diagnosis of congenital adrenal hyperplasia [25–27]. As reported previously, the joint analysis of locus-specific PCR and long-read DNA sequencing has a 100% diagnostic yield [22]. This method can also determine the precise breakpoint position and cis-trans configuration of multiple variants without the need for parent testing [25]. Owing to the low prevalence of 11β-OHD, LRS in this disease has not yet been investigated before. This study presents the first case report of diagnosing 11β-OHD using T-LRS, thereby broadening the spectrum of pathogenic variants identified in the *CYP11B1* gene. T-LRS has proven to be highly effective for diagnosing 11β-OHD. However, at this stage, LRS is limited by high costs and lower accuracy compared to SRS, making it difficult to use for large-scale studies and routine clinical use [28]. The limitation of this study is the absence of functional validation to confirm the pathogenicity of the delins variant. Nonetheless, based on the ACMG guidelines, it can be designated as a pathogenic SV.

## Conclusions

Routine molecular techniques to gene testing for 11β-OHD are facing challenges. When SRS results were negative, the existence of a chimeric *CYP11B2/CYP11B1* gene and SVs should be explored. T-LRS demonstrates efficacy in identifying these specific variants. Despite its current limitations such as base mismatch rates and cost considerations, T-LRS provides a significant advantage in diagnosing 11β-OHD by addressing the deficiencies of conventional sequencing methods.

### Abbreviations

| | |
|---|---|
| 11β-OHD | 11β-hydroxylase deficiency |
| CAH | Congenital Adrenal Hyperplasia |
| SV | Structural Variants |
| NGS | Next-Generation Sequencing |
| ACTH | Adrenocorticotropin |
| TLRS | Target Long-Read Sequencing |
| CYP11β1 | 11β-hydroxylase |
| DOC | 11-deoxycorticosterone |
| LH | Luteinizing Hormone |
| FSH | Follicle-Stimulating Hormone |
| SRS | Short-Read Sequencing |
| LRS | Long-Read Sequencing |
| CCS | Circular Consensus Sequence |
| BMI | Body Mass Index |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12902-024-01748-5.

Supplementary Material 1

Supplementary Material 2

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**Author contributions**
L.J. and X.Z. contributed to the conception and design of the study. J.L., H.T., X.J., Y.W., Z.Z., M.L., L.D., K.S. contributed to the acquisition and analysis of data. J.L. contributed to drafting the text and preparing the figure. All authors read and approved the final manuscript.

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**Data availability**
The data that support the findings of this study are available in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA007066) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human. Additional data and materials related to this study are available upon request.

## Declarations

**Ethics approval and consent to participate**
The study was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Shandong University Qilu Hospital. Written informed consent was obtained from the patients and their parents, including the permission for details and images related to the patients and their parents to be published.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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