Molecular Genetics & Genomic Medicine

WILEY

Molecular Genetics & Genomic Medicine
Open Access

CLINICAL REPORT OPEN ACCESS

Long-Read Sequencing Identifying the Genetic Complexity of Congenital Adrenal Hyperplasia in the Pedigree

Ximin Chen[1] | Jing Zhao[2] | Danhua Li[3] | Na Xi[1] | Danying Yi[1] | Mengjia Yan[1] | Yan Yin[1] | Xueyan Wang[1]

[1]Department of Medical Genetics and Prenatal Diagnosis, Sichuan Provincial Women's and Children's Hospital, the Affiliated Women's and Children's Hospital of Chengdu Medical College, Chengdu, Sichuan, China | [2]Department of Ultrasound, Sichuan Provincial Women's and Children's Hospital, the Affiliated Women's and Children's Hospital of Chengdu Medical College, Chengdu, Sichuan, China | [3]Department of Research and Development, Berry Genomics Corporation, Beijing, China

Correspondence: Xueyan Wang (wanxueyang@163.com)

Received: 17 April 2024 | **Revised:** 17 October 2024 | **Accepted:** 22 October 2024

Funding: This study was supported by the Medical Research Project of the Chengdu Health Commission (202321).

Keywords: compound heterozygote | congenital adrenal hyperplasia | long-read sequencing | prenatal diagnosis

ABSTRACT

Background: High sequence homology between *CYP21A2* and *CYP21A1P* poses challenges to genetic diagnosis of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD). Traditional genetic testing is unable to provide an accurate diagnosis due to the genetic complexity of CAH.

Methods: Deletions, duplications, and recombination breakpoints were precisely identified by long-read sequencing (LRS).

Results: This study presented a pregnant woman, a 21-OHD carrier detected by MLPA, and her husband, a normal subject also detected by MLPA. The fetus was suspected of having 21-OHD based on clinical presentations such as enlarged adrenal glands, atypical external genitalia and karyotyping of 46, XX. LRS further identified the fetus as having the most severe salt-wasting (SW) form of 21-OHD with a compound heterozygote genotype. One allele was *TNXA/TNXB* CH-2, while the other allele was *CYP21A1P/CYP21A2* CH-8. LRS precisely determined the genotypes of the fetus's father and grandmother with duplications, which misdiagnosed by MLPA. The multidisciplinary team recommended immediate glucocorticoid and mineralocorticoid treatment for the child after birth to prevent life-threatening adrenal crisis.

Conclusions: LRS provides precise diagnosis for family members with *CYP21A2* deletion or duplication, improving disease management and preventing potential adrenal crises. When used in pre-pregnancy genetic testing, LRS can indicate high genetic risk and guide the appropriate therapy during pregnancy and immediately after birth.

1 | Introduction

Congenital adrenal hyperplasia (CAH, OMIM #201910) is a group of autosomal recessive inherited disorders resulting from genetic variants that affect adrenal steroidogenesis. The most prevalent type of CAH is 21-hydroxylase deficiency (21-OHD) due to the pathogenic variants in *CYP21A2* (a gene encoding 21-hydroxylase enzyme) (Auer et al. 2023; El-Maouche, Arlt, and Merke 2017). 21-OHD accounts for approximately 95% of patients with CAH. According to the clinical presentation, 21-OHD can be classified into classic and nonclassic (NC) forms (Auer et al. 2023). The nonclassic form is more common than the classic one (Speiser et al. 2018; Miller 2018). Classic form is further divided into life-threatening salt wasting (SW) and simple virilizing (SV). The classic form is the most common cause of atypical genitalia in females, and the severe classic SW

Ximin Chen and Jing Zhao contributed equally to this work.

form is characterized by life-threatening adrenal crisis (Speiser et al. 2018; Kim et al. 2015). The nonclassical form presents with variable symptoms of mild androgen excess during puberty such as hirsutism, acne, menstrual irregularities, infertility, or it may be asymptomatic (New et al. 2013; Macut et al. 2019; Nakhleh et al. 2022).

The functional *CYP21A2* gene and its pseudogene *CYP21A1P* are tandemly located in the major histocompatibility complex class III region on chromosome 6p21.33, forming a genetic repeat structure along with several neighboring genes (known as RCCX module). The genes in the RCCX module include serine/threonine kinase 19 (*STK19*, formerly *RP*), complement 4 (*C4*), steroid 21-hydroxylase (*CYP21*), and tenascin-X (*TNX*) genes (Doleschall et al. 2017). The bimodular RCCX unit is composed of two DNA segments including *STK19B-C4B-CYP21A2-TNXB* and *STK19-C4A-CYP21A1P-TNXA*. Because of the high sequence homology between the *CYP21A2* and the pseudogene *CYP21A1P*, microconversions, large deletions and duplications occur during meiosis (Carrozza et al. 2021). The microconversions are responsible for deleterious variants transferred from *CYP21A1P* to *CYP21A2* (Concolino and Costella 2018). Large deletions and duplications, resulting from misalignment and unequal crossing over during meiosis, cause the *CYP21A1P/CYP21A2* chimeras with a 30 kb deletion including the *CYP21A2* gene and the 3′ portion of the *TNXB* gene and duplicated *CYP21A2*, which might complicate the determination of carrier status (Ezquieta et al. 2006; Kleinle et al. 2009). It is noteworthy that nine haplotypes with *CYP21A1P/CYP21A2* chimeric genes were identified and defined as CH-1 to CH-9, which are related to SW of 21-OHD, except for CH-4 and CH-9, which carry milder phenotypes (Chen et al. 2012). The three different haplotypic *TNXA/TNXB* (CH-1 to CH-3) chimeras with a contiguous gene deletion of *CYP21A2* and *TNXB* have been reported (Chen et al. 2012; Kim et al. 2023). patients with 21-OHD who have deletions of both the *CYP21A2* and *TNXB* genes are more likely to have Ehlers–Danlos syndrome (EDS), characterized by joint hypermobility, skin hyperextensibility, and tissue fragility due to Tenascin-X deficiency. Up to 10% of patients with SW form of 21-OHD exhibit clinical features of EDS due to the haploinsufficiency of TNX and heterozygosity of the *TNXB* deletion (Merke et al. 2013; Chen et al. 2009).

The correlation between genotype and phenotype increases with the severity of 21-OHD (Nandagopal et al. 2011). Accurate and efficient identification of the genotype in 21-OHD is important for the prenatal diagnosis and newborn screening. Due to the challenges in detecting *CYP21A2* deletions and duplications caused by genetic recombination between the active *CYP21A2* gene and its inactive pseudogene *CYP21A1P*, it is necessary to introduce more precise genetic testing strategies for 21-OHD diagnosis. In this study, a pregnant woman, whose female fetus was suspected to have 21-OHD due to clinical features, sought genetic counseling. The fetus, which showed enlarged adrenal glands and atypical external genitalia on ultrasound imaging, was determined to have 21-OHD. It was noted that the woman and her husband were identified as a carrier and a normal subject using MLPA analysis. Long-read sequencing (LRS) identified the fetus was a SW form of 21-OHD and corrected the genotype of the father. Based on LRS and the junction sites detected, the fetus's father actually carried a duplication. The proband has a *CYP21A1/CYP21A2* CH-8 chimeric gene on one allele inherited from her father and a *TNXA/TNXB* CH-2 chimeric gene on the second allele inherited from her mother.

2 | Materials and Methods

2.1 | Ethical Compliance

This study was approved by Ethics Committee of Sichuan Provincial Hospital for Women and Children. Written informed consent was obtained from all the family members.

2.2 | Subject Presentation

A consanguineous Chinese family was enrolled. The amniotic fluid from the pregnant woman and peripheral blood from other family members were sent to Berry Genomics Corporation for genetic testing. Family members include fetus' mother, father, and paternal grandparents.

2.3 | Genetic Testing by LRS

The specific primers were designed to amplify the *CYP21A2*, *CYP11B1*, *CYP17A1*, *HSD3B2*, and *StAR* genes as previously published (Liu et al. 2022; Li et al. 2023). The PCR products were purified using 1 × Ampure PB beads (PacBio) and then quantified using the Qubit dsDNA BR test kit. The library was constructed using the Sequel Binding Kit (PacBio) and sequenced with the Sequel II Sequencing Kit in circular consensus sequencing (CCS) mode.

2.4 | Data Analysis

After high-throughput sequencing, the CCS were aligned to the reference sequence of the human genome GRCh38 to determine the exact position of each base sequence obtained by sequencing on the chromosome. The GenBank reference sequence of *CYP21A2* and *TNXB* are NG_007941.3 and NG_008337.2. The SNPs/INDELs was detected by FreeBayes (1.3.4). For the analysis of 30 kb deletion chimeras and *CYP21A2* duplications, the *CYP21A2* reads, *CYP21A1P/CYP21A2* and *CYP21A2* duplications reads were aligned with the reference build *CYP21A2-TNXB*. The pathogenicity information of the mutation site was obtained to assist interpreters in data interpretation.

3 | Results

3.1 | Clinical Findings

A 34-year-old pregnant woman with a gestational age of 23 weeks presented to the hospital for prenatal diagnosis. It was noted that both the woman and her husband previously undergone genetic testing using MLPA. The results revealed that the woman was a carrier of 21-OHD, while her husband was identified as a normal subject.

A colour Doppler ultrasound was performed at 23 weeks plus 5 days of gestation (Figure 1). The ultrasound results showed the fetus had an enlarged left adrenal gland, diminished corticomedullary demarcations and a normal right adrenal gland. The karyotyping was performed and showed that the fetus was 46,XX. Based on these clinical features, the fetus was suspected to have 21-OHD. Genetic testing using LRS was performed on amniotic fluid collected via amniocentesis.

During a follow-up examination four weeks later, both the enlarged left and right adrenal gland of fetus were observed by ultrasound imaging at 27 weeks plus 5 days of gestation (Figure 2A,B). Additional, clitoral enlargement and thickened labia majora were also determined by the ultrasound images of external genitalia (Figure 2C). The combination of these phenotypic characteristics and the genotype of the fetus detected by LRS indicated that the fetus carried the most severe SW form of 21-OHD.

3.2 | LRS Identified *CYP21A2* Deletion and Duplication in the Pedigree

Based on the LRS, the genotypes of the family members, including the proband, father, mother, and paternal grandparents, were determined and displayed by IGV plots and schematic diagrams (Figure 3). The results showed that the proband (a female fetus) carried a compound heterozygote of a *CYP21A1P/CYP21A2* CH-8 chimeric gene on one allele and a *TNXA/TNXB* CH-2 chimeric gene on another allele. The junction site of the *CYP21A1P/CYP21A2* CH-8 chimeric gene (chr6: 32040535–32040797) was located downstream of the common mutation R356W (c.1069C > T) in exon 8. The break interval of the *TNXA/TNXB* CH-2 chimeric gene (chr6:32042509–32043712) was located upstream of the variant c.12174C > G (p.Cys4058Trp) in exon 40 derived from *TNXA* pseudogene (Figure 3).

The comprehensive genotypic analysis of this family lineage suggested that the proband's *CYP21A1P/CYP21A2* CH-8 was inherited from her father and grandfather. In addition, *TNXA/TNXB* CH-2 was inherited from her mother. Of note, LRS clarified the confusion caused by a misdiagnosis of father's carrier status by MLPA. The proband's father actually had a duplicated *CYP21A2* gene on one allele inherited from his mother and a monodular unit with *CYP21A1P/CYP21A2* CH-8 chimeras inherited from his father. The break interval for this allele was located at chr6:32045151–32045850. All the family members except the proband at least had one allele without pathogenic variants. The fetus's father, mother, and grandfather were carriers for 21-OHD (Figure 3). Pedigree diagram of this investigated family is showed in Figure 4.

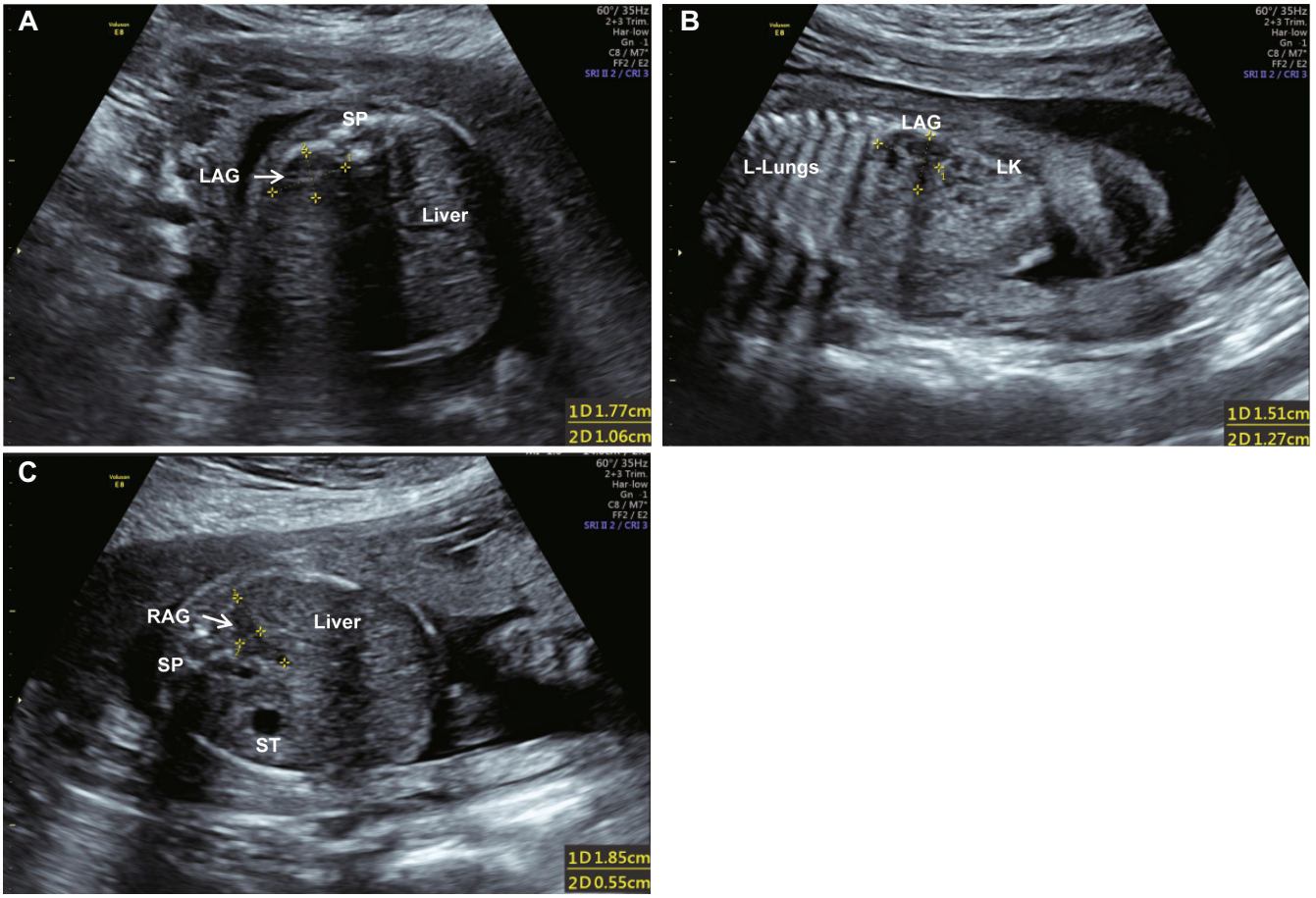

FIGURE 1 | Color Doppler ultrasound Images at 23 weeks plus 5 days of pregnancy. (A) Transverse ultrasound sections of the abdomen showing enlarged left adrenal gland. (B) Long-axis view of the left kidney. (C) Transverse ultrasound sections of the abdomen revealing normal right adrenal gland. LAG: Left adrenal gland. RAG: Right adrenal gland. SP: spine. LK: left kidney. ST: stomach.

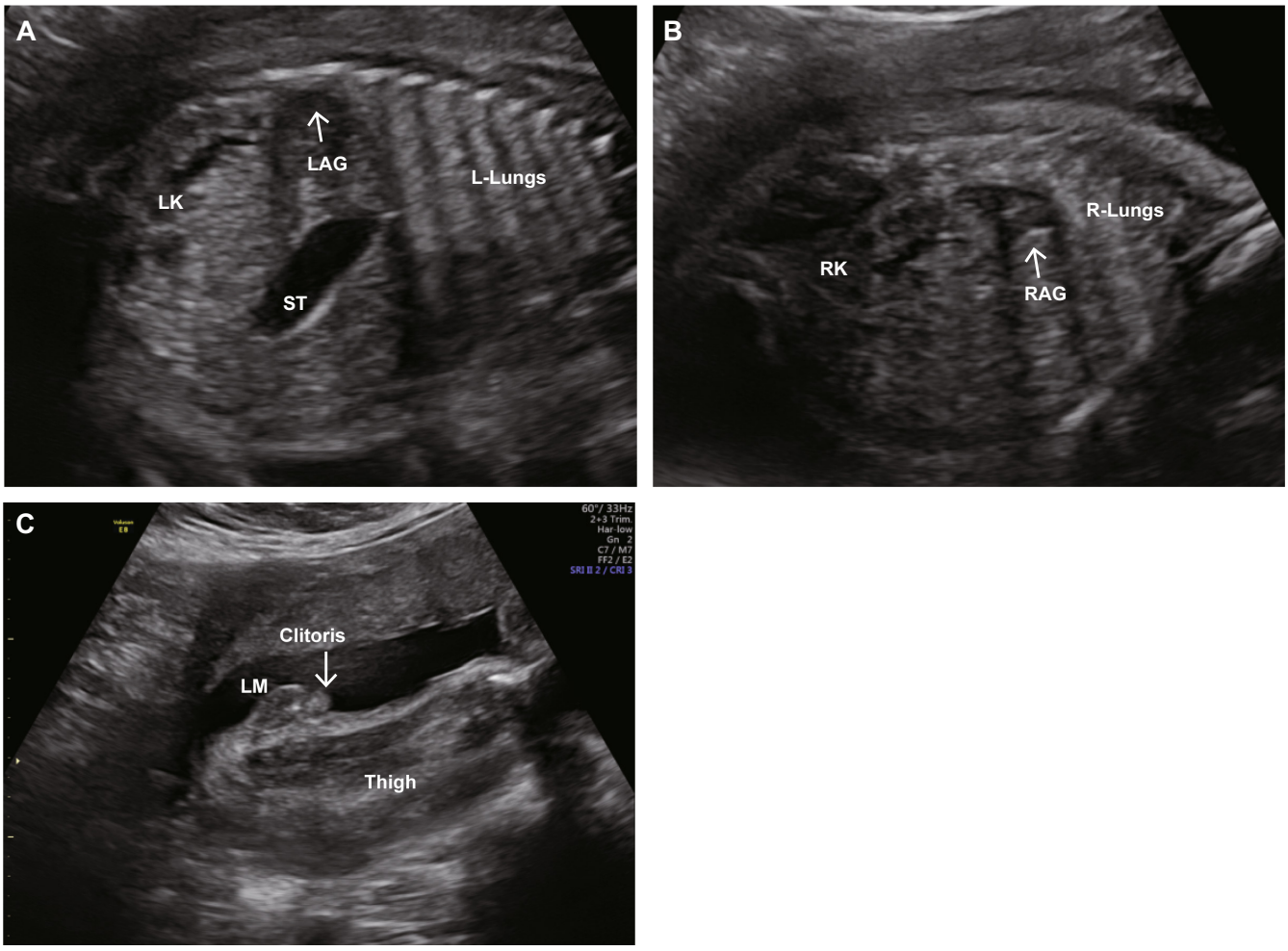

FIGURE 2 | Color Doppler ultrasound Images at 27 weeks plus 5 days of pregnancy. Long-axis view of the left idney (A) and right kidney (B) showing enlarged adrenal gland. (C) The clitoral enlargement and thickened labia majora visualized by the ultrasound imaging of external genitalia. LAG: left adrenal gland. RAG: right adrenal gland. LK:left kidney. RK: right kidney. ST: stomach. LM: labia majora.

Based on the comprehensive analysis of clinical and molecular results, the fetus was diagnosed as the life-threatening SW type of 21-OHD. LRS accurately identified the genotypes of her family members, especially the father with a duplicated *CYP21A2*, which was misdiagnosed by MLPA. As previously reported, dexamethasone therapy administered before 6–7 weeks of postconception, which is the critical window of sexual differentiation, can suppress fetal androgen production and has been shown to effectively prevent or reduce prenatal virilization (Nowotny et al. 2022). Unfortunately, the fetus in this study had already developed virilization of the external genitalia at the time of diagnosis. After consultation by a multidisciplinary team involving pediatric endocrinologists, urologists, and geneticists, it was recommended that the child would receive glucocorticoid and mineralocorticoid treatment immediately after birth to prevent life-threatening adrenal crises. The decision for genital surgery in child should be made by parents or patient herself when she is grown up with the guidance of a multidisciplinary team. This case holds significant importance for couples falling into the similar genetic condition. Accurate pre-pregnancy genetic testing could indicate a higher risk for a genetic disorder and ensure the appropriate therapy during gestational period and immediately after birth. The family was recommended to pursue adequate genetic counseling for family planning.

4 | Discussion

As more than 95% of CAH is caused by 21-OHD due to the *CYP21A2* variants, *CYP21A2* genotyping is essential for clinical genetic counselling, prenatal diagnosis, newborn screening and early treatment of 21-OHD patients. The *CYP21A2* gene, which has 10 exons and encodes 21-hydroxylase enzyme, is located on chromosome 6p21.33 and forms the RCCX module with the closely linked *CYP21A1P* pseudogene and their neighboring genes (Auer et al. 2023). The chimeric *CYP21A1P/CYP21A2* genes, with a frequency of about 30% (Chen et al. 2012; Vrzalová et al. 2011), result from intergenic recombination and are classified into 9 haplotypes (CH-1 to CH-9) with CH-1, CH-2, CH-3, CH-5, CH-6, CH-7 and CH-8 belonging to the classic group due to the SW type of 21-OHD and CH-4 and CH-9 belonging to the attenuated group due to the SV or NC type of 21-OHD. The frequency of classic *CYP21A1P/CYP21A2* CH-8 chimeras is 5.45% (Chen et al. 2012). The junction region was located downstream of the mutation c.1069C > T (R356W) (Concolino

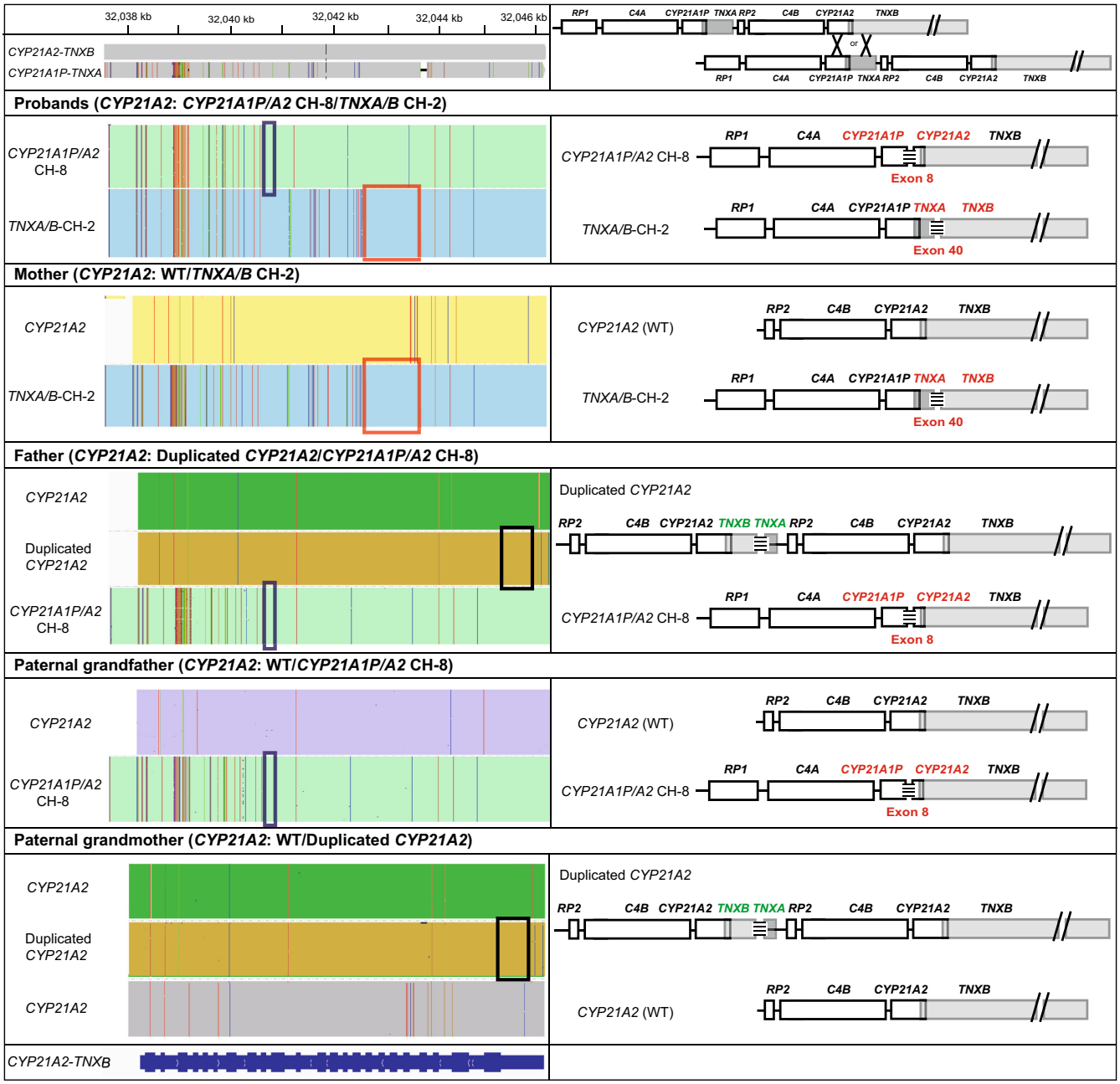

FIGURE 3 | The genotypes of family members identified by LRS and depicted by IGV plots and schematic diagram. *CYP21A2*: *CYP21A2* without pathogenic variants. *CYP21A1P/A2* CH-8: *CYP21A1P/CYP21A2* CH-8; *TNXA/B* CH-2: *TNXA/TNXB* CH-2; a duplicated *CYP21A2*: The fusion gene of functional gene TNXB and pseudogene TNXA. WT: Wild type. Blue box: The break interval of *CYP21A1P/CYP21A2* CH-8; Red box: The break interval of *TNXA/TNXB* CH-2; Black box: The break interval of a duplicated *CYP21A2*. Dashed line: Chimeras resulted from meiotic unequal crossover.

and Costella 2018; Cantürk et al. 2011). In the past, the location could not be narrowed down further. In this study, the proband with the most severe SW form of 21-OHD harbored this CH-8 haplotype, inherited from her father and grandfather (Figure 3). The exact junction region was determined using LRS through the alignment of haplotypic sequence to reference chromosome.

The chimeric *TNXA/TNXB* genes are characterized by a contiguous gene deletion of *CYP21A2* that extends into the *TNXB* gene. Three different haplotypic *TNXA/TNXB* (CH-1 to CH-3) genes have been identified based on the junction site location

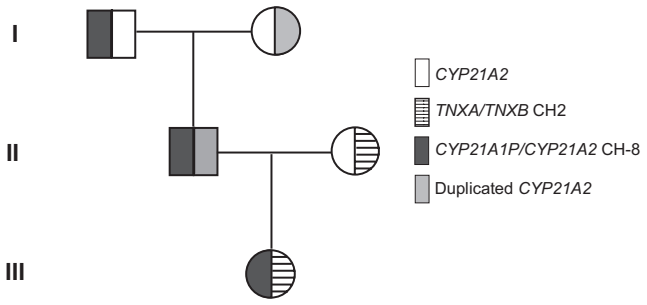

FIGURE 4 | Pedigree chart of the investigated family.

with *TNXA/TNXB* CH-2 determined by the pseudogene variant c.12174C > G in exon 40 (Chen et al. 2012; Kim et al. 2023). The variants in *TNXA/TNXB* CH-2 alter the TNX protein structure, which may cause a more severe phenotype than *TNXA/TNXB* CH-1 with altered protein expression (Merke et al. 2013). The proband in this study carried a monoallelic *TNXA/TNXB* CH-2 inherited from her mother. The exact junction region was also determined using LRS though the alignment to reference sequence (Figure 3).

Patients with biallelic *TNXA/TNXB* chimeras have severe Ehlers–Danlos syndrome (EDS), which is characterized as joint hypermobility, skin hyperextensibility, and tissue fragility due to Tenascin-X deficiency. Patients with monoallelic *TNXA/TNXB* chimeras are more likely to develop EDS (Merke et al. 2013; Chen et al. 2009). Although clinical evaluation for EDS phenotypes was not applicable to this study, it should be routinely performed in CAH patients with *TNXA/TNXB* chimeras.

As previously reported, a large number of variants and their related phenotypes have been identified in 21-OHD. This demonstrates a good correlation between genotype and phenotype, which indicated the significance of genetic testing. Three genetic strategies have been commonly used to diagnose 21-OHD, including Sanger sequencing, an MLPA analysis and next-generation sequencing. Sanger sequencing is labor-intensive and not applicable for large-scale use. A MLPA analysis, which is dependent on the limited probes, cannot detect the variants outside the target probes. Moreover, the duplications in this case can not been identified by MLPA, resulting in misdiagnosis of father's carrier status. MLPA was mainly used to identify exon-level variation based on the copy number of specific sections. When an individual carried a duplicated *CYP21A2* on one allele and 30-kb deletion on another allele, like in this case, a duplicated *CYP21A2* could mask a 30-kb deletion. Next-generation sequencing in 21-OHD diagnosis faces challenges when aligning short reads, such as 150 bp, to the reference genome due to high homology (98%) between *CYP21A2* and its *CYP21A1P* pseudogene (Higashi et al. 1986; White, New, and Dupont 1986). LRS can generate highly accurate (99.8%) long reads, and it can match or exceed the ability of short-read sequencing to detect variants. Additionally, the variants can be phased into haplotypes, further improving variant detection (Wenger et al. 2019). In the diagnosis of 21-OHD, LRS has advantages over the other methods in terms of high throughput, accurate diagnosis, and direct detection of genetic complexity without parental genotyping (Liu et al. 2022; Wenger et al. 2019; Pervez et al. 2022). The chimeric *CYP21A1P/CYP21A2* and *TNXA/TNXB* genes in the family members were diagnosed using LRS. In addition, the duplicated *CYP21A2* was also accurately identified by LRS, suggesting that LRS can improve the diagnostic accuracy of 21-OHD at the molecular level for prenatal diagnosis, newborn screening, and the clinical management. Based on the clinical examination and genetic testing results, the fetus with SW form of 21-OHD should receive the appropriate therapy early during the gestational period and immediately after birth. The parents were advised to pursue adequate genetic counseling for family planning.

5 | Conclusions

In conclusion, a 34-year-old pregnant woman, a 21-OHD carrier detected by MLPA, and her husband, a normal subject detected by MLPA, was reported in this study. The prenatal ultrasound examination determined the enlarged adrenal gland and atypical external genitalia in the fetus, suggesting the female fetus was a suspected 21-OHD patient. Further genetic testing using LRS determined the genotypes of the fetus and her family, which supported the SW form of 21-OHD diagnosis of the fetus and provided a precise diagnosis of the proband's father, who was found to have a duplicated *CYP21A2* that has been misdiagnosed by MLPA. Pedigree analysis showed that the proband had a *CYP21A1P/CYP21A2* CH-8 chimeric gene in one allele, which was inherited from her father and grandfather, and a *TNXA/TNXB* CH-2 chimeric gene in the second allele, which was inherited from her mother. Both the proband's father and grandmother carried a duplicated *CYP21A2* gene. In this study, large deletions and duplications caused by unequal crossover were determined by LRS. The application of LRS for rapid and accurate molecular diagnostics can assist clinicians in prenatal diagnosis and genetic counseling.

Author Contributions

Ximin Chen and Jing Zhao have contributed equally to the article. They drafted the manuscript and revised it. Na Xi is responsible for the clinical studies. Danhua Li, Danying Yi, Mengjia Yan, and Yan Yin are responsible for experimental studies. Xueyan Wang ensured the integrity of the entire study and reviewed the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

The authors have nothing to report.

Ethics Statement

This study was approved by Ethics Committee of Sichuan Provincial Hospital for Women and Children. The family provided their written informed consent to participate in this study.

Consent

The written informed consent to publish this information was obtained from study participants.

Conflicts of Interest

D.L are employees of Berry Genomics Corporation. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

All data generated during this study are included in this article.

References

Auer, M. K., A. Nordenström, S. Lajic, and N. Reisch. 2023. "Congenital Adrenal Hyperplasia." *Lancet* 401, no. 10372: 227–244.

Cantürk, C., U. Baade, R. Salazar, N. Storm, R. Pörtner, and W. Höppner. 2011. "Sequence Analysis of CYP21A1P in a German Population to

Aid in the Molecular Biological Diagnosis of Congenital Adrenal Hyperplasia." *Clinical Chemistry* 57, no. 3: 511–517.

Carrozza, C., L. Foca, E. De Paolis, and P. Concolino. 2021. "Genes and Pseudogenes: Complexity of the RCCX Locus and Disease." *Frontiers in Endocrinology (Lausanne)* 12: 709758.

Chen, W., M. S. Kim, S. Shanbhag, et al. 2009. "The Phenotypic Spectrum of Contiguous Deletion of CYP21A2 and Tenascin XB: Quadricuspid Aortic Valve and Other Midline Defects." *American Journal of Medical Genetics. Part A* 149a, no. 12: 2803–2808.

Chen, W., Z. Xu, A. Sullivan, et al. 2012. "Junction Site Analysis of Chimeric CYP21A1P/CYP21A2 Genes in 21-Hydroxylase Deficiency." *Clinical Chemistry* 58, no. 2: 421–430.

Concolino, P., and A. Costella. 2018. "Congenital Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency: A Comprehensive Focus on 233 Pathogenic Variants of CYP21A2 Gene." *Molecular Diagnosis & Therapy* 22, no. 3: 261–280.

Doleschall, M., A. Luczay, K. Koncz, et al. 2017. "A Unique Haplotype of RCCX Copy Number Variation: From the Clinics of Congenital Adrenal Hyperplasia to Evolutionary Genetics." *European Journal of Human Genetics* 25, no. 6: 702–710.

El-Maouche, D., W. Arlt, and D. P. Merke. 2017. "Congenital Adrenal Hyperplasia." *Lancet* 390, no. 10108: 2194–2210.

Ezquieta, B., M. Beneyto, R. Muñoz-Pacheco, et al. 2006. "Gene Duplications in 21-Hydroxylase Deficiency: The Importance of Accurate Molecular Diagnosis in Carrier Detection and Prenatal Diagnosis." *Prenatal Diagnosis* 26, no. 12: 1172–1178.

Higashi, Y., H. Yoshioka, M. Yamane, O. Gotoh, and Y. Fujii-Kuriyama. 1986. "Complete Nucleotide Sequence of Two Steroid 21-Hydroxylase Genes Tandemly Arranged in Human Chromosome: A Pseudogene and a Genuine Gene." *Proceedings of the National Academy of Sciences of the United States of America* 83, no. 9: 2841–2845.

Kim, J. H., G. H. Kim, H. W. Yoo, and J. H. Choi. 2023. "Molecular Basis and Genetic Testing Strategies for Diagnosing 21-Hydroxylase Deficiency, Including CAH-X Syndrome." *Annals of Pediatric Endocrinology & Metabolism* 28, no. 2: 77–86.

Kim, M. S., A. Ryabets-Lienhard, A. Dao-Tran, et al. 2015. "Increased Abdominal Adiposity in Adolescents and Young Adults With Classical Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency." *Journal of Clinical Endocrinology and Metabolism* 100, no. 8: E1153–E1159.

Kleinle, S., R. Lang, G. F. Fischer, et al. 2009. "Duplications of the Functional CYP21A2 Gene Are Primarily Restricted to Q318X Alleles: Evidence for a Founder Effect." *Journal of Clinical Endocrinology and Metabolism* 94, no. 10: 3954–3958.

Li, H., X. Zhu, Y. Yang, et al. 2023. "Long-Read Sequencing: An Effective Method for Genetic Analysis of CYP21A2 Variation in Congenital Adrenal Hyperplasia." *Clinica Chimica Acta* 547: 117419.

Liu, Y., M. Chen, J. Liu, et al. 2022. "Comprehensive Analysis of Congenital Adrenal Hyperplasia Using Long-Read Sequencing." *Clinical Chemistry* 68, no. 7: 927–939.

Macut, D., V. Zdravković, J. Bjekić-Macut, G. Mastorakos, and D. Pignatelli. 2019. "Metabolic Perspectives for Non-Classical Congenital Adrenal Hyperplasia With Relation to the Classical Form of the Disease." *Frontiers in Endocrinology (Lausanne)* 10: 681.

Merke, D. P., W. Chen, R. Morissette, et al. 2013. "Tenascin-X Haploinsufficiency Associated With Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia." *Journal of Clinical Endocrinology and Metabolism* 98, no. 2: E379–E387.

Miller, W. L. 2018. "Mechanisms in Endocrinology: Rare Defects in Adrenal Steroidogenesis." *European Journal of Endocrinology* 179, no. 3: R125–R141.

Nakhleh, A., L. Saiegh, N. Shehadeh, et al. 2022. "Screening for Non-Classic Congenital Adrenal Hyperplasia in Women: New Insights Using Different Immunoassays." *Front Endocrinol (Lausanne)* 13: 1048663.

Nandagopal, R., N. Sinaii, N. A. Avila, et al. 2011. "Phenotypic Profiling of Parents With Cryptic Nonclassic Congenital Adrenal Hyperplasia: Findings in 145 Unrelated Families." *European Journal of Endocrinology* 164, no. 6: 977–984.

New, M. I., M. Abraham, B. Gonzalez, et al. 2013. "Genotype-Phenotype Correlation in 1,507 Families With Congenital Adrenal Hyperplasia Owing to 21-Hydroxylase Deficiency." *Proceedings of the National Academy of Sciences of the United States of America* 110, no. 7: 2611–2616.

Nowotny, H., U. Neumann, V. Tardy-Guidollet, et al. 2022. "Prenatal Dexamethasone Treatment for Classic 21-Hydroxylase Deficiency in Europe." *European Journal of Endocrinology* 186, no. 5: K17–K24.

Pervez, M. T., M. J. U. Hasnain, S. H. Abbas, M. F. Moustafa, N. Aslam, and S. S. M. Shah. 2022. "A Comprehensive Review of Performance of Next-Generation Sequencing Platforms." *BioMed Research International* 2022: 3457806.

Speiser, P. W., W. Arlt, R. J. Auchus, et al. 2018. "Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline." *Journal of Clinical Endocrinology and Metabolism* 103, no. 11: 4043–4088.

Vrzalová, Z., Z. Hrubá, E. S. Hrabincová, et al. 2011. "Chimeric CYP21A1P/CYP21A2 Genes Identified in Czech Patients With Congenital Adrenal Hyperplasia." *European Journal of Medical Genetics* 54, no. 2: 112–117.

Wenger, A. M., P. Peluso, W. J. Rowell, et al. 2019. "Accurate Circular Consensus Long-Read Sequencing Improves Variant Detection and Assembly of a Human Genome." *Nature Biotechnology* 37, no. 10: 1155–1162.

White, P. C., M. I. New, and B. Dupont. 1986. "Structure of Human Steroid 21-Hydroxylase Genes." *Proceedings of the National Academy of Sciences of the United States of America* 83, no. 14: 5111–5115.