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Long-Read Sequencing of CAH and ADPKD Provides Novel Insights Into the Genetic Diagnosis of Male Infertility

Xiao Li[1] | Chang Liu[2] | Wen Liu[1] | Xianlong Wang[1] | Jiaolong Liu[1] | Feifei Cai[1] | Shaoming Lu[1,3]

[1]Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China | [2]Berry Genomics Corporation, Beijing, China | [3]Center for Reproductive Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China

Correspondence: Shaoming Lu (lsming1110@163.com)

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ABSTRACT

Purpose: This study aimed to evaluate the utility of long-read sequencing (LRS) in identifying variants of congenital adrenal hyperplasia (CAH) and autosomal dominant polycystic kidney disease (ADPKD) in infertile men, which may help further clarify genetic diagnosis and support genetic counseling and reproductive management.

Methods: A total of 722 infertile men underwent next-generation sequencing (NGS) panel testing. LRS was subsequently applied to characterize variants of CAH and ADPKD. Semen parameters, serum sex hormone levels, and assisted reproductive outcomes were further analyzed in males carrying the relevant variants.

Results: Definitive genetic diagnoses were established in 3.3% (24/722) of men by NGS panel. LRS directly determined the trans configurations of two *CYP21A2* variants in a subject with azoospermia. Eighty-seven infertile men with ADPKD-related variants were identified, including 7 subjects carrying pathogenic variants. About 96.6% of subjects had no familial history of PKD, and 85.0% of subjects had abnormal results of semen analysis. Overall, LRS added 33.3% diagnostic yield above for infertile men.

Conclusions: The application of CAH and ADPKD analysis based on LRS extends conventional genetic testing for male infertility by enabling accurate evaluation of complex genomic regions, thereby improving diagnostic clarity and informing reproductive decision-making and clinical management.

1 | Introduction

Infertility is defined as the inability to conceive after at least 12 months of regular, unprotected sexual intercourse, and male factors contribute to about 50% of the cases. Male infertility is a complex and multifactorial condition with highly heterogeneous phenotypic presentations, from complete absence of spermatozoa in the testes to distinct alterations of sperm quality [1]. Genetic abnormalities related to male infertility affect about 15% of men with infertility, including chromosomal abnormalities, such as Klinefelter syndrome (karyotype 47, XXY), Y chromosomal azoospermia factor (AZF) microdeletions, and single gene mutations (e.g., in *CFTR*) [2]. Conventional genetic testing includes a karyotype, Y-chromosome microdeletion, and sequencing of the *CFTR* gene, which help to identify the genetic diagnosis of male infertility. In recent years, next-generation sequencing (NGS) technique has emerged as a powerful tool to achieve more diagnoses and identify new genes for male infertility. Chen et al. performed whole-exome sequencing (WES) of 314 idiopathic non-obstructive azoospermia (NOA) and severe oligospermia (SO) patients, and 3.2% (10/314) of the cohort received diagnosis. Additionally, 20 novel NOA candidate genes were identified [3]. These genetic findings provide insight into the etiology of male infertility and pave the way for further molecular diagnosis. Moreover, genetic testing also plays

Xiao Li and Chang Liu contributed equally to this article.

an important role in guiding clinical decisions, particularly in predicting whether assisted reproductive technology (ART) will be successful [4].

Congenital adrenal hyperplasia (CAH) and autosomal dominant polycystic kidney disease (ADPKD) are two clinically significant hereditary disorders with autosomal recessive and autosomal dominant inheritance patterns, respectively. Although both are primarily recognized for their systemic manifestations, with CAH associated with endocrine dysfunction and ADPKD with renal involvement [5, 6], growing evidence has revealed their potential impact on male fertility. In CAH, excessive adrenal androgen production and the development of testicular adrenal rest tumors can impair the hypothalamic–pituitary–gonadal axis, leading to subfertility or infertility [7]. ADPKD, on the other hand, is frequently associated with reproductive tract abnormalities such as seminal vesicle cysts and ejaculatory duct obstruction, which may result in obstructive azoospermia [8]. Importantly, beyond their reproductive implications, both CAH and ADPKD carry significant genetic transmission risks, making early identification in infertile men crucial not only for etiology-specific diagnosis and management, but also for informed genetic counseling and assisted reproductive planning.

CAH is mainly caused by variants in the *CYP21A2* gene. The corresponding pseudogene *CYP21A1P* is at approximately 30 kb distance from *CYP21A2*. Both the functional *CYP21A2* gene and the nonfunctional *CYP21A1P* pseudogene contain 10 exons and 9 introns, sharing approximately 98% sequence identity in the exons and 96% homology in the introns [9]. Sanger sequencing combined with multiplex ligation-dependent probe amplification (MLPA) has been widely used to identify known and common pathogenic variants. Nevertheless, MLPA may yield false-negative or false-positive results. Moreover, MLPA cannot distinguish subtypes of deletion chimeras. This combined approach is laborious, time-consuming, and unable to determine the cis- or trans- configuration of variants. Because of the limit of short sequencing reads, NGS cannot be correctly aligned with the repetitive regions of the reference genome, making it difficult to accurately detect variants in the highly homologous genes *CYP21A2* and *CYP21A1P*. ADPKD is primarily caused by variants in *PKD1* and *PKD2*, with a prevalence of 85% and 15%, respectively. *PKD1* duplicated segment encompassing exons 1–33 shares 98% sequence similarity to 6 pseudogenes (*PKD1P1* to *PKD1P6*) [10]. The high GC content of exon 1 in both *PKD1* and *PKD2* adds complexity for testing ADPKD based on NGS [11]. Several genetic testing methods have been used for the diagnosis of ADPKD. Although targeted SRS combined with LR-PCR provides a more robust strategy for ADPKD genetic testing, extra input for exon 1 is often needed to achieve balanced sequencing depth due to GC bias. MLPA is limited to detecting large deletions and duplications in *PKD1* and *PKD2*, which increases both turnaround time and diagnostic cost. These conventional methods have limited ability to detect all variant types in *PKD1* and *PKD2*, leading to a diagnostic gap in ADPKD testing, with some patients remaining undiagnosed. Single-molecule GC-unbiased long-read sequencing (LRS) has some advantages in genetic diagnosis for rare diseases, especially for those involving complex genotypes. LRS can sequence the full length of genes dozens of kilobases long in single reads, making it straightforward to read the whole gene and discriminate between highly homologous genes. Therefore, LRS has significant advantages in the comprehensive and precise analysis of these two diseases.

In this study, we conducted an NGS panel for the preliminary analysis of infertile men. Comprehensive analysis of CAH (CACAH) and ADPKD (CAPKD) based on LRS was applied to extend genetic testing for male infertility. The study highlights the advantages of LRS in detecting variants located in complex genomic regions, and contributes to a better understanding of the distribution of these two diseases in infertile males. Moreover, it demonstrates the potential of applying LRS in the genetic diagnosis of male infertility.

2 | Materials and Method

2.1 | Study Subjects

This study enrolled adult male patients with infertility who had visited the Center for Reproductive Medicine, Shandong University, from September 2024 to March 2025 (Figure 1). Patients with chromosomal anomalies, microdeletions in the AZF region, and other potential causes of infertility, including iatrogenic injury, genital tract infection, testicular inflammation, and drug exposure were excluded from this study. In addition, no abnormalities in height, weight, mental status, or external genitalia were found upon physical examination. Ejaculated sperm and peripheral blood samples were collected from all patients for subsequent analysis.

This study adhered to the Declaration of Helsinki and was approved by the institutional review board of Center for Reproductive Medicine at Shandong University. All methods were performed in accordance with approved guidelines. Written informed consent was obtained from all the participants.

2.2 | NGS Panel for Male Infertility

According to previous studies [12–14], a NGS panel consisting of 321 genes related to male infertility was designed (Table S1). The custom Illumina Nextera panel included genomic targets comprising coding exons and 15 bp flanking regions of each gene. DNA samples were processed for library preparation targeted capture and sequencing as previously reported [14]. The 150 bp paired-end reads sequencing was performed on NovaSeq 6000 platform (Illumina, USA). Alignment of the sequencing reads to the human reference genome (GRCh37/hg19) was performed using the Burrows-Wheeler Aligner software (version 0.7.13). Then, sequencing reads were processed using Genome Analysis Toolkit. Finally, ANNOVAR was used to annotate the raw VCF files. The pathogenicity of these variations was evaluated according to the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) [15].

2.3 | Genetic Testing of CAH by LRS

The LRS based approach termed CACAH was conducted as previously described [16]. The 7.0–8.5 kb DNA fragments of *CYP21A2*, *CYP21A1P*, *CYP11B1*, *CYP17A1*, *HSD3B2*, and *StAR* genes were amplified using 6 locus-specific primer pairs

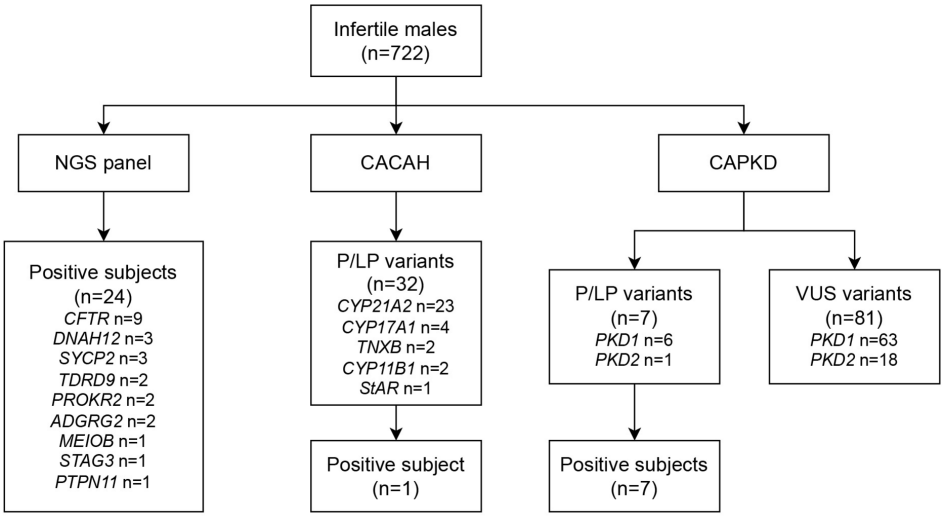

FIGURE 1 | Study flowchart. NGS, next-generation sequencing; CACAH, comprehensive analysis of CAH; CAPKD, comprehensive analysis of ADPKD; P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; CAH, congenital adrenal hyperplasia; ADPKD, autosomal dominant polycystic kidney disease.

through long-range PCR (LR-PCR). For each purified PCR product, a one-step end repair and ligation reaction were done to add a unique barcoded-adaptor. The single-molecule real-time (SMRT) bell library was prepared using the Sequel Binding Kit 2.0 and Internal Control Kit 1.0 (Pacific Biosciences, USA). Primed DNA-polymerase complexes were loaded onto SMRT cells and sequenced with Sequel II Sequencing Kit 2.0 on the Sequel II platform (Pacific Biosciences, USA). The debarcoded and filtered circular consensus sequencing (CCS) reads were then aligned to the human reference genome build GRCh38/hg38 using the SMRT Link analysis software suite (Pacific Biosciences, USA). FreeBayes1.3.4 (Biomatters Inc., San Diego, CA) was used for the identification of single nucleotide variations (SNVs) and small insertions and deletions (InDels). The pathogenic variants were displayed and confirmed in the Integrative Genomics Viewer (IGV) program with BAM files. The classification of variants was performed according to the ACMG guidelines [15].

2.4 | Genetic Testing of ADPKD by LRS

Targeted LRS for *PKD1* and *PKD2* genetic analysis termed CAPKD was performed similarly as previously described [17]. Genomic DNA was subjected to multiplex LR-PCR using KOD FX Neo (Toyobo, Japan). The PacBio SMRT bell libraries were prepared by a 1-step end-repair and ligation reaction to add barcoded adapters, followed by digestion with exonucleases to remove failed ligated DNA. The uniquely barcoded libraries were purified, quantified, and then pooled with equal mass. A SMRT bell sequencing library was prepared using the Sequel II Binding Kit 3.2 (Pacific Biosciences, USA) and sequenced under CCS mode with Sequel IIe platform (Pacific Biosciences, USA) for 30 h. After sequencing, the raw subreads were converted to high-fidelity CCS reads, debarcoded, and aligned to reference genome build GRCh38/hg38 in the SMRT Link analysis software suite (Pacific Biosciences, USA). The aligned CCS reads were then subjected to an in-house developed bioinformatics pipeline to identify SNVs/InDels, deletions, and duplications. The hg38-aligned CCS reads were then filtered to obtain reads with primer sequences used in the panel by blasting to remove misaligned reads. SNVs and InDels of *PKD1/2* were called by FreeBayes 1.3.4 (Biomatters Inc., San Diego, CA). The exact breakpoints of deletions or duplications were determined by pysam and displayed in the IGV. The pathogenicity of variants identified by LRS was classified according to the ACMG guidelines.

2.5 | Semen Parameter Analysis

According to the World Health Organization (WHO) laboratory manual for the examination and processing of human semen (fifth edition) [18], semen samples were collected through masturbation after 2–7 days of sexual abstinence and analyzed after liquefaction at 37°C for 30 min. Routine semen tests for sperm concentration, motility, and morphology were performed following.

2.6 | Serum Sex Hormone Analysis

Serum was isolated from blood samples. Serum levels of follicle-stimulating hormone (FSH) (range: 1.50–12.40 IU/L), luteinizing hormone (LH) (range: 1.70–8.60 IU/L), prolactin (PRL) (range: 4.04–15.20 ng/mL), testosterone (T) (range: 249.00–836.00 pg/mL), oestradiol (E2) (range: 11.3–43.2 pg/mL), and thyroid-stimulating hormone (TSH) (range: 0.75–5.60 uIU/mL) were measured using the electrochemiluminescence method.

3 | Results

3.1 | Genetic Findings of NGS Panel

A total of 722 individuals were subjected to NGS panel tests. Definitive genetic diagnoses were established in 24 cases, accounting for 3.3% (24/722) of all infertile men (Figure 2, Table S2). Sixteen

cases with recessively inherited variants in *CFTR* (9), *DNAH12* (3), *TDRD9* (2), *MEIOB* (1), and *STAG3* (1); 6 cases with dominantly inherited variants in *SYCP2* (3), *PROKR2* (2), and *PTPN11* (1); and 2 cases with X-linked hemizygous variants in *ADGRG2* (2). We collected the results of semen parameters analysis. However, some samples were referred from external hospitals solely for genetic testing related to male infertility, and the unavailability of original semen analysis reports led to missing data. *ADGRG2* and *CFTR* are responsible for obstructive azoospermia (OA) [19]. All 11 samples with pathogenic variants of *ADGRG2* or *CFTR* exhibited azoospermia. Their testicular volumes were within the normal range, and serum FSH levels were normal in all cases except one. *DNAH12* is related to teratozoospermia [20]. The two infertile men carrying *DNAH12* variants both presented with asthenoteratozoospermia and elevated E2 levels. One of them had a testicular volume of less than 12 mL, and his FSH level was below the normal range. *MEIOB*, *STAG3*, *TDRD9*, *PTPN11*, *SYCP2*, and *PROKR2* are reported to contribute to NOA [21]. One infertile male carrying a variant of *PROKR2* showed elevated levels of both FSH and LH. Another sample with a *SYCP2* variant exhibited increased FSH and LH levels, along with a testicular volume of less than 12 mL. A total of 20 infertile men underwent ART. Four achieved clinical pregnancy, four are currently pregnant, and three have already had live births.

3.2 | CACAH Identified CAH-Related Variants in Infertile Men

CACAH identified 31 samples with CAH-related variants from 722 infertile men, including 22 samples with *CYP21A2* variants, 4 samples with *CYP17A1* variants, 2 samples with *CYP11B1* variants, 2 samples with *TNXB* variants, and 1 sample with *StAR* variant (Table S3). A total of 32 variants of CAH were detected in this study (Table 1). The most frequent variation was *CYP21A2*:c.518 T. A (18.8%, 6/32), followed by *CYP21A2*:c.844G>T (12.5%, 4/32). Without the need of genotypic analysis of family members, LRS directly determined the trans configurations in 1 subject with two *CYP21A2* variants, accounting for 0.1% (1/722) of all subjects with male infertility (Figure 3). In sample CT241384, LRS identified c.518 T>A was in one allele, and E6_cluster (c.710 T>A, c.713 T>A, c.719 T>A) was in the other allele. These variants were classified as pathogenic according to ACMG guidelines, supporting the diagnosis of CAH in this patient. This patient had low levels of both FSH and LH but normal levels of T, along with bilaterally reduced testicular volume. These results provide an explanation for the observed oligoasthenozoospermia of this patient, which ultimately contributed to his infertility. Subsequently, he underwent intracytoplasmic sperm injection (ICSI) and his wife achieved clinical pregnancy.

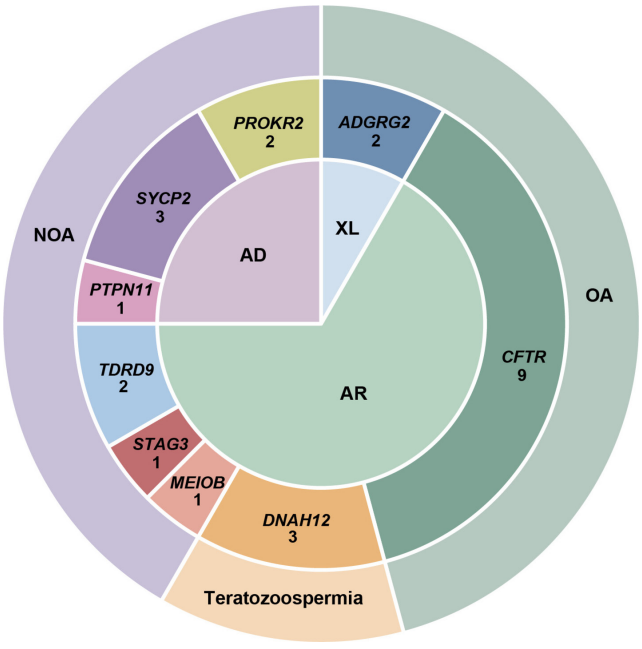

FIGURE 2 | Distribution of genetic findings identified by next-generation sequencing. AD, autosomal dominant; AR, autosomal recessive; XL, X-linked; NOA, non-obstructive azoospermia; OA, obstructive azoospermia.

3.3 | CAPKD Identified ADPKD-Related Variants in Infertile Men

Targeted LRS for *PKD1* and *PKD2* genetic analysis were performed for all 722 infertile patients. A total of 58 ADPKD-related variants were identified in the cohort, including 46 variants in *PKD1* and 12 variants in *PKD2* (Table 2). Allele frequencies in population databases, previously reported classifications in PKDB and ClinVar, and in silico prediction results were summarized in Table 2. According to ACMG criteria, 7 variants were classified as pathogenic, whereas 50 were classified as VUS. Among the identified variants, 14 had been previously reported in PKDB, and 28 were listed in ClinVar. About 87 infertile men were detected to carry the ADPKD variants, including 7 individuals with pathogenic variants accounting for 1.0% (7/722) of all infertile men (Table S4). LRS identified a large deletion in *PKD1* in one sample (Figure 4A). Moreover, the breakpoints of the deletion were directly precisely determined by CAPKD. The breakpoint junctions of the deletion in CT241226 had 1 bp matching sequence, suggesting the involvement of nonhomologous end-joining (Figure 4B). Sample CT241232, CT241390, and CT250317 had a positive family history of ADPKD; 96.6% (84/87) subjects had no familial history of polycystic kidney disease (Table S4). We analyzed the semen parameters, serum sex hormone levels, and assisted reproductive outcomes of samples with pathogenic ADPKD variants (Table S4). Among 7 subjects with pathogenic variants associated with ADPKD, 3 presented with azoospermia, 1 with oligozoospermia, 1 with asthenozoospermia, and 1 with oligoasthenoteratozoospermia. Three samples (CT241226, CT241232, CT241390) carrying pathogenic ADPKD variants presented with azoospermia. Their testicular volumes were within the normal range, and serum FSH levels were also normal. Four individuals opted for preimplantation genetic testing (PGT) as part of their assisted reproductive strategy, and 2 cases achieved clinical pregnancy. One sample underwent ICSI and achieved clinical pregnancy.

Among the 80 infertile males carrying the VUS ADPKD variants, 18 subjects were identified as oligoasthenoteratozoospermia, 17 subjects were identified as azoospermia, 14 were asthenoteratozoospermia, 6 were asthenozoospermia, 4 were oligoasthenozoospermia, 2 were teratozoospermia, and 1 was oligozoospermia (Table S4). Several VUS in *PKD1* were located within functionally relevant regions, including 7 VUS located at the receptor

TABLE 1 | Variants related to CAH identified by CACAH.

Nucleotide change	Protein change	Type	Pathogenicity	ACMG evidence	*n*	Frequency
CYP21A2						
c.518T>A	p.Ile173Asn	Missense	P	PM3_VeryStrong+PS3 + PP1 + PM2_Supporting	6	18.8%
c.844G>T	p.Val282Leu	Missense	P	PM3_VeryStrong+PS3 + PS1 + PM2_Supporting	4	12.5%
c.293-13C>G	—	Intron	P	PM3_VeryStrong+PS3 + PP1_Strong+PM2_Supporting	3	9.4%
TNXA/B-CH-1	—	Deletion	P	—	2	6.3%
CYP21A1P/A2-CH-1	—	Deletion	P	—	1	3.1%
c.92C>T	p.Pro31Leu	Missense	P	PM3_VeryStrong+PS3 + PM2_Supporting	1	3.1%
c.955C>T	p.Gln319Ter	Nonsense	P	PVS1 + PS3 + PM2_Supporting	1	3.1%
c.374G>A	p.Arg125His	Missense	LP	PM5 + PM3 + PM2_Supporting+PP1	1	3.1%
E6_cluster (c.710T>A, c.713T>A, c.719T>A)	p.lle237Asn, p.Val238Glu, p.Met240Lys	Missense	P	PM3_VeryStrong+PS3 + PM2_Supporting	1	3.1%
c.1069C>T	p.Arg357Trp	Missense	P	PM3_VeryStrong+PS3 + PM2_Supporting	1	3.1%
c.[−126C>T, −113G>A]	—	5′ UTR	P	PM3_VeryStrong+PS3 + PM2_Supporting	1	3.1%
c.923dup	p.Leu308PhefsTer6	Frameshift	P	PVS1 + PM3_Strong+PM2_Supporting	1	3.1%
CYP17A1						
c.985_987delinsAA	p.Tyr329LysfsTer90	Frameshift	P	PVS1_Strong+PM3_Strong+PM2_Supporting+PP1	2	6.3%
c.1084C>T	p.Arg362Cys	Missense	P	PS3 + PM3_Strong+PM2_Supporting+PP3 + PP1	1	3.1%
c.286C>T	p.Arg96Trp	Missense	P	PS3 + PP3_Strong+PM3 + PM5 + PM2_Supporting	1	3.1%
TNXB						
c.12174C>G	p.Cys4060Trp	Missense	P	PM3_VeryStrong+PM2_Supporting+PP3 + PP1	1	3.1%
c.11435_11524 + 30del	—	Splice donor	P	PVS1 + PM3_Strong+PM2_Supporting	1	3.1%
CYP11B1						
c.1358G>A	p.Arg453Gln	Missense	P	PS3 + PM3_Strong+PP3	1	3.1%
c.954G>A	p.Thr318=	Splice region	P	PM3_Strong+PP3_Strong+PM2_Supporting	1	3.1%
StAR						
c.707_708delinsCTT	p.Lys236ThrfsTer47	Frameshift	P	PVS1_Strong+PM3_Strong+PM2_Supporting+PP1	1	3.1%

Abbreviations: LP, likely pathogenic; P, pathogenic.

for egg jelly (REJ) domain (Table 2). The *PKD1*:c.7670A>G was identified in two individuals, corresponding to an allele frequency of 0.1390% (2/1444) in our cohort. The allele frequency in gnomAD v4.1.0 is 0.0015% (24/1605326). This yielded an odds ratio of 92.77 (95% CI: 21.91–392.9; $p < 0.001$). This variant is located within the REJ domain of *PKD1* and is classified as VUS in the PKDB database. In silico prediction tools indicated a potential functional impact, with SIFT predicting the variant as disease causing (D) and PolyPhen-2 predicting it as probably damaging (PRD). Among the two individuals carrying *PKD1*:c.7670A>G, one (CT241575) presented with teratozoospermia. A sample (CT241440) with azoospermia in our cohort carried the *PKD2*:c.1343C>T, showing an allele frequency of 0.0690% (1/1444). This variant has not been reported in gnomAD v4.1.0, PKDB, or ClinVar. SIFT and PolyPhen-2 predicted the variant as D and PRD, respectively. Extra-renal manifestations of ADPKD have been known to involve male reproductive organs. Testis cysts were present in Sample CT241610. CT241216 was azoospermia with VUS *PKD1*:c.9889G>A and exhibited renal cysts. CT241116 (*PKD1*:c.9022G>A) and CT241458 (*PKD1*:c.10499 + 10C>T) also carried VUS variants and exhibited liver cysts and epididymis cysts, respectively. A total of 60 infertile men chose different ART approaches to conceive. Two samples (CT241183 and CT241214) carrying *PKD1*:c.9022G>A chose the assisted reproductive methods of ICSI and PGT respectively. One achieved clinical pregnancy, and the other has already had live birth. Three subjects (CT250024, CT250055 and CT250161) carrying *PKD1*: c.11156 + 12C>T chose the assisted reproductive methods of ICSI or invitrofertilization (IVF) and both achieved clinical pregnancy.

4 | Discussion

4.1 | The Importance of Genetic Diagnosis in Male Infertility

In recent years, with the advancement of molecular genetics, genetic testing has played an increasingly prominent role in the diagnosis of male infertility. Primarily, genetic testing enables the precise identification of hereditary causes, thereby enhancing diagnostic accuracy. Genetic testing is particularly valuable for patients who lack overt clinical manifestations or present with inconclusive results from conventional examinations. The results of genetic testing can help us understand the molecular mechanisms of male infertility, thereby facilitating the formulation of corresponding treatment plans. Moreover, the outcomes of genetic testing provide critical guidance for reproductive counseling and the selection of assisted reproductive strategies, ultimately helping to reduce unnecessary treatments and financial burdens. For example, genetic diagnoses can predict the success rate of recovering sperm during testicular sperm extraction (TESE), with success rates reaching up to 50% in men hemizygous for a complete AZFc deletion, while almost zero in cases involving complete deletions of the AZFa, AZFb, or AZFbc regions [22]. Monogenic diagnoses can also predict the success of TESE. Beyond guiding assisted reproductive, genetic diagnosis of male infertility also enables clinicians to evaluate and advise men about the potential health risks beyond infertility. Several studies have identified associations between infertility and an increased risk of other health conditions such as hypertension, diabetes mellitus, autoimmune disorders, and various cancers [23]. Consequently, genetic testing for infertility not only identifies potential risks but also informs preventive healthcare strategies. As an essential component of male infertility assessment, genetic testing should be more widely implemented in clinical practice. The present study further underscores the pivotal role of genetic diagnosis in male infertility.

4.2 | NGS and Genetic Diagnosis of Male Infertility

Genetic factors play a significant role in male infertility. However, a definitive genetic cause can be identified in only about 4% of men within infertile couples [24]. Except for Klinefelter syndrome (karyotype 47, XXY) and microdeletions of the Y-chromosomal AZF region, monogenic causes have also been linked to male infertility. For many years, the diagnosis of monogenic causes of male infertility has remained limited to screening for variants in the *CFTR* gene in patients with azoospermia and suspected congenital bilateral absence of the vas deferens (CBAVD) [25]. Male infertility is a multifactorial pathological condition. The genetic of male infertility is very complex and at least 2000 genes are involved in spermatogenesis [22]. Genetic testing by NGS technologies can be relevant for its diagnostic value in male infertile patients. Due to the reduced costs and increasing accessibility, NGS is becoming a more widely used technique. WES and targeted gene panels allow the simultaneous analysis of large cohorts of infertile patients, thus providing a powerful tool for the detection of variants in infertile men.

NGS provides opportunities for individuals with male infertility, which will already add diagnostic yield. In this study, 3.3% (24/722) individuals received definitive genetic diagnoses through NGS panel for male infertility. Among studies employing exome sequencing in infertile men, the diagnostic yield varies considerably. When analyses are limited to validated disease genes and P/LP variants based on the ACMG guidelines, the yield is approximately 8.5% for cases of azoospermia [26]. However, this figure exceeds 20% in studies that not only consider a broader spectrum of phenotypes but also include VUS identified in candidate genes [12]. Li et al. used WES on 149 infertile men with teratozoospermia and identified several novel gene variants associated with sperm head abnormalities [20]. The study also showed that abnormal sperm-head morphology significantly reduces fertilization success following ICSI. This will help to improve counseling of infertile men prior to invasive testicular biopsy and provide better-personalized treatment. The results of NGS also provided better counseling for potential health risks in the infertile man. A study conducted WES on 521 men with idiopathic spermatogenic failure, identifying a novel gene of severe oligozoospermia. Moreover, a markedly increased cancer risk was observed among genetically infertile men [27]. These results underscore the diagnostic value and clinical implications of NGS analysis in the management of male infertility.

Although NGS methods are frequently used in research laboratories to study the genetics of male infertility, there are no standardized guidelines for clinical genetic testing by NGS in male infertility. Patients with oligozoospermia or azoospermia are

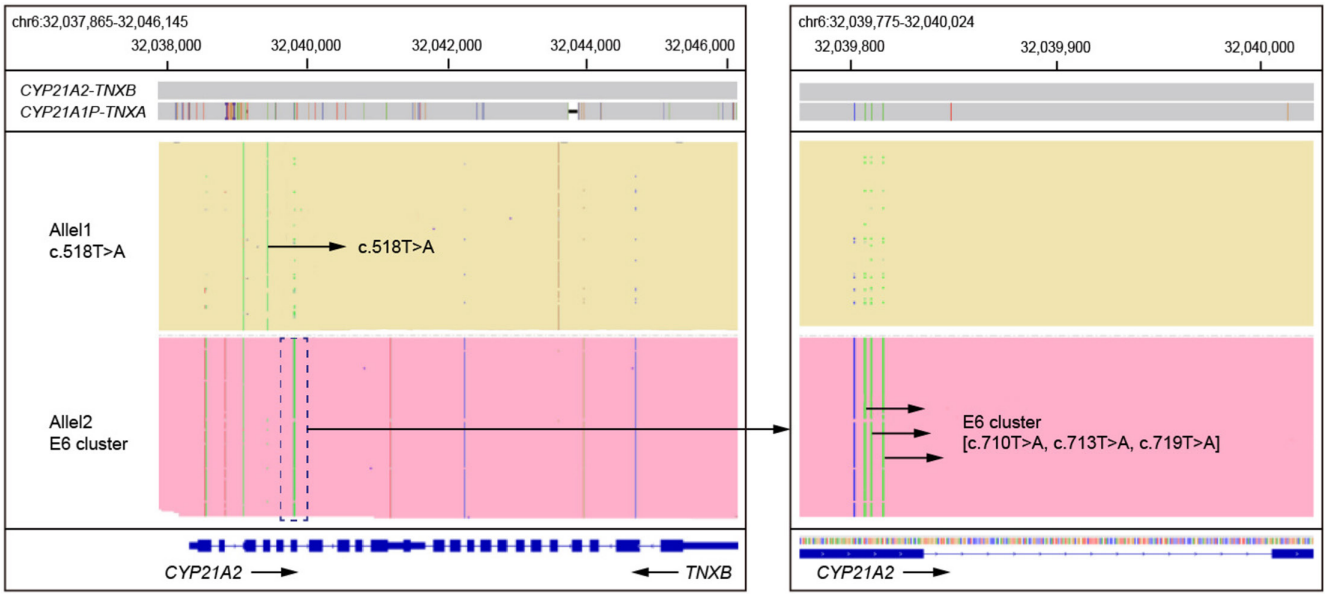

FIGURE 3 | Integrative Genomics Viewer plots showing the variants identified in sample CT241384 by long-read sequencing.

generally offered karyotyping and Y-chromosome microdeletion analysis [28], while CFTR testing is recommended for men with suspected CBAVD [22]. As NGS continues to gain traction in the diagnosis of male infertility, it is necessary to update the genetic testing guidelines for male infertility in the future and clearly define the application of NGS.

4.3 | Utility of Conducting CAH and ADPKD Analysis in the Infertility Men

CAH is a group of autosomal recessive disorders caused by a deficiency of essential enzymes in the adrenal steroidogenesis pathway. About 95% of CAH cases are attributable to 21-hydroxylase deficiency (21-OHD). In 21-OHD affected males, they may develop severe adrenal insufficiency, which can be life-threatening if not promptly treated. Screening for CAH in the male infertile population holds significant clinical value. Studies have demonstrated that CAH may lead to androgen excess through hypersecretion of adrenocorticotropic hormone (ACTH), which in turn disrupts the hypothalamic–pituitary–gonadal axis, suppresses FSH secretion, and impairs spermatogenesis [29]. Additionally, patients with CAH are often found to have testicular adrenal rest tumors (TARTs). They are benign but can exert compressive effects on testicular tissue and further compromise reproductive capacity. This study identified one CACAH-positive subject, but whether he has TARTs remains unknown and requires further follow-up. Given the often subtle or nonspecific clinical presentation of CAH, screening for CAH in men with unexplained oligozoospermia or azoospermia may help to elucidate underlying etiologies and guide hormonal replacement therapy, thereby improving reproductive outcomes. In a previous study, testicular aspiration enabled successful sperm retrieval in a CAH patient presenting with azoospermia, and a successful pregnancy was subsequently achieved via ICSI [30]. One patient diagnosed with CAH also achieved clinical pregnancy after the treatment of ICSI in this study.

ADPKD is the most common inherited kidney disease, which is characterized by the development of renal cysts, hypertension, and extrarenal cysts and results in end-stage renal disease (ESRD). In recent years, it has garnered increasing attention for its potential association with male infertility. Cystic involvement of the epididymis, vas deferens, and seminal vesicles in ADPKD patients can directly compromise the patency of the male reproductive tract, resulting in obstructive azoospermia or abnormal semen parameters [31]. A previous study reported that 80% (37/46) of men with ADPKD variants have abnormal semen, and only some of the male patients with ADPKD carrying definitely pathogenic variants were infertile [32]. In our study, semen analysis results were collected from 80 infertile males with ADPKD-associated variants, including 6 carrying pathogenic ADPKD variants and 74 carrying VUS. Among them, 85.0% (68/80) exhibited abnormal results. All 6 individuals carrying pathogenic variants had abnormal semen, with 3 cases of azoospermia. Of the 74 men with VUS, 24.3% (18/74) of individuals were oligoasthenoteratozoospermia, 23.0% (17/74) were azoospermia, and 16.2% (12/74) of individuals showed normal results. Therefore, the correlation between ADPKD variants and semen quality may require further investigation in a larger cohort. Although ADPKD preferentially manifests with renal cysts, patients often have extra-renal manifestations including liver cysts and seminal vesicle cysts [33]. In this study, two subjects exhibited liver cysts and epididymis cysts, respectively. Given the familial aggregation characteristic of ADPKD, early targeted screening in infertile men with a personal or family history of renal cysts offers critical value for genetic counseling and reproductive planning. Three individuals with CAPKD-positive results had a family history of the disease in our study, while 96.6% (84/87) of subjects had no familial history of polycystic kidney disease and were identified with ADPKD-related variants during infertility evaluations. Apart from infertility, they exhibited no additional manifestations associated with ADPKD. ADPKD is a late-onset disorder, and such findings may offer valuable guidance for intervention and management in the future. Previous studies have reported that most ADPKD patients with genital tract and

TABLE 2 | Variants related to ADPKD identified by CAPKD.

Nucleotide change	Protein change	Type	Region	Predicted location within PKD1 domains	*n*	Allele frequency	Allele frequency in gnomAD	Odds ratio (95% CI)	*p* value	Pathogenicity	PKDB classification	ClinVar classification	SIFT	PolyPhen-2
PKD1														
c.11156+12C>T	—	Intron	Intron 38	Not defined	9	0.6230%	0.0094%	66.54 (33.90–130.61)	<0.001	VUS	LB	—	—	—
c.6878C>T	p.Pro2293Leu	Missense	Exon 15	REJ	4	0.2770%	0.0087%	32.01 (11.83–86.6)	<0.001	VUS	LB	Conflicting	T	B
c.9022G>A	p.Val3008Met	Missense	Exon 25	GAIN-B	4	0.2770%	0.0369%	7.52 (2.81–20.12)	0.002	VUS	LB	B/LB	D	PRD
c.10499+10C>T	—	Intron	Intron 34	Not defined	3	0.2080%	0.0070%	29.53 (9.35–93.29)	<0.001	VUS	—	—	—	—
c.10499+20G>A	—	Intron	Intron 34	Not defined	2	0.1390%	0.0003%	427.23 (82.82–2203.82)	<0.001	VUS	LB	—	—	—
c.6223C>T	p.Arg2075Cys	Missense	Exon 15	PKD domain	2	0.1390%	0.0052%	26.51 (6.52–107.87)	0.003	VUS	—	—	D	PRD
c.7670A>G	p.Asp2557Gly	Missense	Exon 19	REJ	2	0.1390%	0.0015%	92.77 (21.91–392.9)	<0.001	VUS	VUS	—	D	PRD
c.9314G>A	p.Arg3105Gln	Missense	Exon 26	Not defined	2	0.1390%	0.0026%	53.04 (12.82–219.5)	0.001	VUS	—	VUS	D	POD
c.9136C>T	p.Arg3046Cys	Missense	Exon 25	GAIN-B	2	0.1390%	0.0051%	27.25 (6.69–110.97)	0.003	VUS	—	VUS	D	POD
c.9884A>G	p.Asn3295Ser	Missense	Exon 29	Not defined	2	0.1390%	0.0077%	18.11 (4.47–73.34)	0.006	VUS	VUS	VUS	D	PRD
c.*991_*992dup	—	3′ UTR	Exon 46	Not defined	2	0.1390%	—	—	—	VUS	—	—	—	—
c.2854-12T>G	—	Intron	Intron 11	Not defined	2	0.1390%	0.0018%	79.01 (18.81–331.98)	<0.001	VUS	—	VUS	—	—
c.-9G>A	—	5′ UTR	Exon 1	Not defined	1	0.0690%	0.0005%	152 (16.98–1360.78)	0.008	VUS	—	LB	—	—
c.8161+5C>T	—	Intron	Intron 22	Not defined	1	0.0690%	0.0231%	3 (0.42–21.4)	0.284	VUS	—	Conflicting	—	—
c.7288C>T	p.Arg2430Ter	Stop gained	Exon 18	REJ	1	0.0690%	0.0001%	553.74 (50.18–6110.13)	0.003	P	P	P	—	—

(Continues)

TABLE 2 | (Continued)

Nucleotide change	Protein change	Type	Region	Predicted location within PKD1 domains	*n*	Allele frequency	Allele frequency in gnomAD	Odds ratio (95% CI)	*p* value	Pathogenicity	PKDB classification	ClinVar classification	SIFT	PolyPhen-2
c.11713-19C>G	—	Intron	Intron 42	Not defined	1	0.0690%	0.0004%	183.49 (21.42–1571.52)	0.007	VUS	—	—	—	—
c.11603C>T	p.Thr3868Met	Missense	Exon 42	Polycystin domain	1	0.0690%	0.0001%	519.51 (47.08–5732.42)	0.003	VUS	—	—	D	PRD
c.11713-3C>T	—	Splice region	Intron 42	Not defined	1	0.0690%	0.0105%	6.61 (0.92–47.25)	0.141	VUS	LB	LB	—	—
c.7100C>T	p.Ser2367Phe	Missense	Exon 17	REJ	1	0.0690%	0.0120%	5.8 (0.81–41.38)	0.159	VUS	—	Conflicting	D	PRD
c.12004-16G>A	—	Intron	Intron 43	Not defined	1	0.0690%	—	—	—	VUS	—	—	—	—
c.10406-15C>T	—	Intron	Intron 33	Not defined	1	0.0690%	0.0006%	110.73 (14.17–865.59)	0.010	VUS	—	—	—	—
c.12004-5C>G	—	Intron	Intron 43	Not defined	1	0.0690%	—	—	—	VUS	—	—	—	—
c.11185C>G	p.His3729Asp	Missense	Exon 39	Polycystin domain	1	0.0690%	0.0001%	1117.21 (69.85–17870.02)	0.002	VUS	—	—	T	B
c.12138 + 9C>T	—	Intron	Intron 44	Not defined	1	0.0690%	0.0007%	93.07 (12.09–716.24)	0.012	VUS	—	—	—	—
c.11659C>G	p.Pro3887Ala	Missense	Exon 42	Polycystin domain	1	0.0690%	0.0001%	944.67 (59.06–15110.21)	0.002	VUS	—	—	T	B
c.1529G>A	p.Arg510Gln	Missense	Exon 7	C-type lectin	1	0.0690%	0.0001%	1053.44 (65.86–16849.92)	0.002	VUS	—	VUS	D	PRD
c.11712 + 11C>T	—	Intron	Intron 42	Not defined	1	0.0690%	0.0005%	131.63 (15.84–1094.04)	0.009	VUS	—	—	—	—
c.-200C>G	—	5′ UTR	Exon 1	Not defined	1	0.0690%	—	—	—	VUS	—	—	—	—
c.7184A>G	p.Asn2395Ser	Missense	Exon 17	REJ	1	0.0690%	0.0007%	100.73 (12.89–787.38)	0.011	VUS	—	VUS	D	POD
c.10087C>T	p.Gln3363Ter	Stop gained	Exon 31	Not defined	1	0.0690%	—	—	—	P	P	P	—	—

(Continues)

TABLE 2 | (Continued)

Nucleotide change	Protein change	Type	Region	Predicted location within PKD1 domains	*n*	Allele frequency	Allele frequency in gnomAD	Odds ratio (95% CI)	*p* value	Pathogenicity	PKDB classification	ClinVar classification	SIFT	PolyPhen-2
c.-73C>G	—	5′ UTR	Exon 1	Not defined	1	0.0690%	0.0003%	220.34 (22.91–2119.54)	0.006	VUS	—	—	—	—
c.10183C>T	p.Gln3395Ter	Stop gained	Exon 32	Not defined	1	0.0690%	—	—	—	P	P	P	—	—
c.8041C>T	p.Arg2681Cys	Missense	Exon 22	REJ	1	0.0690%	0.0221%	3.13 (0.44–22.3)	0.274	VUS	—	VUS	D	POD
c.5014_5015del	p.Arg1672GlyfsTer98	Frameshift	Exon 15	PKD domain	1	0.0690%	0.0004%	185.97 (22.38–1545.7)	0.006	P	P	P	—	—
c.8360G>A	p.Arg2787His	Missense	Exon 23	REJ	1	0.0690%	0.0061%	11.27 (1.57–80.85)	0.086	VUS	VUS	VUS	D	PRD
c.5923C>T	p.Gln1975Ter	Stop gained	Exon 15	PKD domain	1	0.0690%	—	—	—	P	P	P	—	—
c.11537 + 5G>A	—	Intron	Intron 41	Not defined	1	0.0690%	0.0002%	371.52 (38.62–3573.75)	0.004	VUS	—	—	—	—
c.10652C>T	p.Pro3551Leu	Missense	Exon 36	Not defined	1	0.0690%	0.0015%	45.82 (6.19–338.92)	0.022	VUS	—	VUS	D	PRD
c.9737G>A	p.Arg3246Gln	Missense	Exon 29	Not defined	1	0.0690%	0.0009%	78.69 (10.34–598.83)	0.014	VUS	—	VUS	T	B
c.6704C>T	p.Ser2235Leu	Missense	Exon 15	REJ	1	0.0690%	0.0016%	44.64 (6.05–329.67)	0.023	VUS	—	—	D	PRD
c.9889G>A	p.Val3297Met	Missense	Exon 29	Not defined	1	0.0690%	0.0126%	5.51 (0.77–39.33)	0.167	VUS	—	Conflicting	T	PRD
c.-10T>C	—	5′ UTR	Exon 1	Not defined	1	0.0690%	—	—	—	VUS	—	—	—	—
Del (chr16:1898225–2120800)	—	Deletion		Not defined	1	0.0690%	—	—	—	P	—	—	—	—
c.7066-10C>G	—	Intron	Intron 16	Not defined	1	0.0690%	0.0004%	182.97 (22.02–1520.78)	0.006	VUS	—	VUS	—	—
c.7066-9C>A	—	Intron	Intron 16	Not defined	1	0.0690%	0.0029%	23.88 (3.29–173.24)	0.042	VUS	VUS	—	—	—
PKD2														

(Continues)

TABLE 2 | (Continued)

Nucleotide change	Protein change	Type	Region	Predicted location within PKD1 domains	*n*	Allele frequency	Allele frequency in gnomAD	Odds ratio (95% CI)	*p* value	Pathogenicity	PKDB classification	ClinVar classification	SIFT	PolyPhen-2
c.1724A>G	p.Lys575Arg	Missense	Exon 8	—	5	0.3460%	0.0019%	180.46 (70.07–464.74)	<0.001	VUS	—	VUS	D	B
c.2341G>A	p.Asp781Asn	Missense	Exon 12	—	2	0.1390%	0.0025%	55.74 (13.46–230.86)	0.001	VUS	—	—	D	POD
c.743A>G	p.Tyr248Cys	Missense	Exon 3	—	2	0.1390%	0.0036%	38.59 (9.42–158.14)	0.001	VUS	—	LB	D	PRD
c.1760C>T	p.Ser587Leu	Missense	Exon 8	—	2	0.1390%	0.0024%	58.89 (14.19–244.34)	0.001	VUS	—	VUS	D	B
c.2119-11C>T	—	Intron	Intron 10	—	1	0.0690%	—	—	—	VUS	—	—	—	—
c.595 + 8C>G	—	Intron	Intron 1	—	1	0.0690%	—	—	—	VUS	—	—	—	—
c.1343C>T	p.Thr448Ile	Missense	Exon 6	—	1	0.0690%	—	—	—	VUS	—	—	D	PRD
c.595 + 19C>T	—	Intron	Intron 1	—	1	0.0690%	0.0003%	262.07 (29.27–2346.16)	0.005	VUS	—	—	—	—
c.1271T>C	p.Ile424Thr	Missense	Exon 5	—	1	0.0690%	—	—	—	VUS	—	—	D	PRD
c.2159dup	p.Asn720LysfsTer5	Frameshift	Exon 11	—	1	0.0690%	0.0007%	101.51 (13.1–786.72)	0.011	P	—	P	—	—
c.113_133dup	p.Ala38_Gly44dup	Inframe insertion	Exon 1	—	1	0.0690%	0.0003%	208.92 (23.34–1870.29)	0.006	VUS	—	—	—	—
c.2020-19T>G	—	Intron	Intron 9	—	1	0.0690%	0.0005%	135.78 (16.34–1128.56)	0.009	VUS	—	LB	—	—

Abbreviations: B, benign; D, disease causing; LB, likely benign; LP, likely pathogenic; P, pathogenic; POD, possibly damaging; PRD, probably damaging, REJ, receptor for egg jelly; T, tolerated; VUS, variant of uncertain significance.

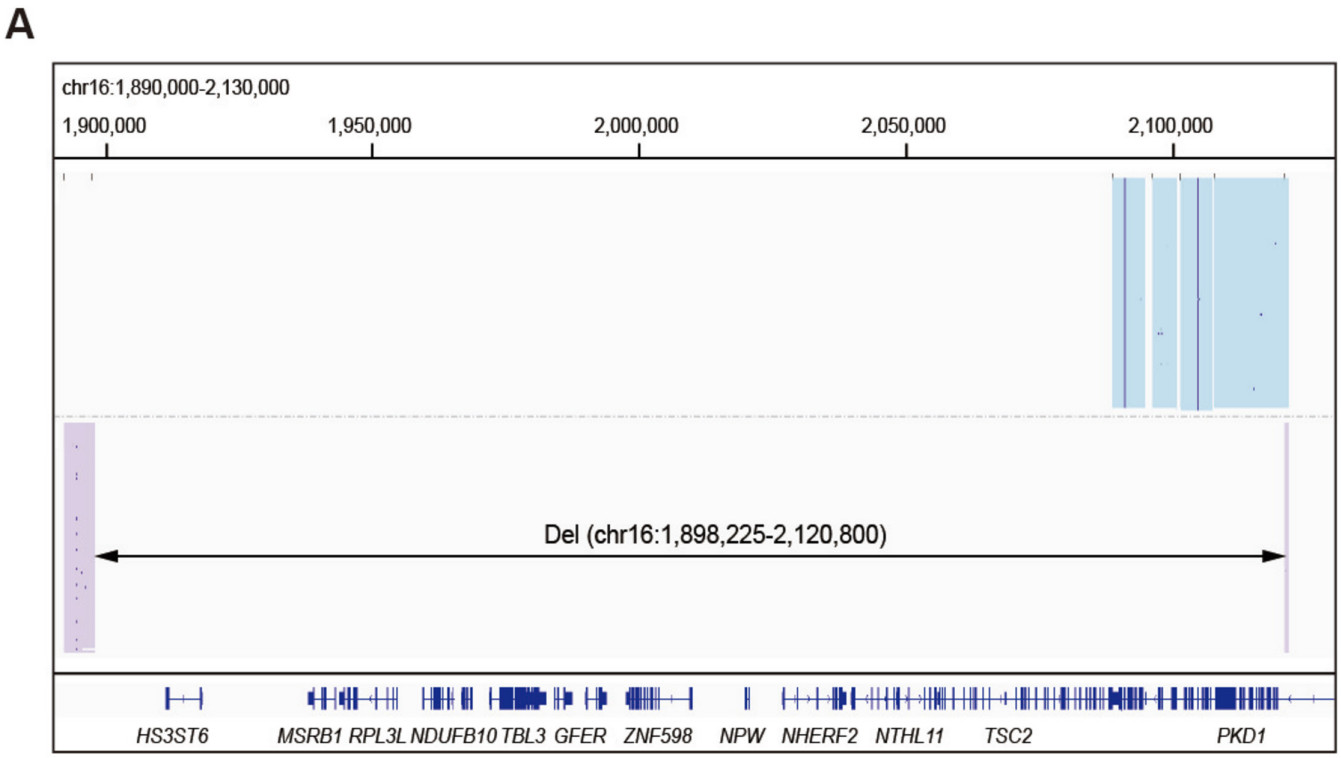

FIGURE 4 | Large deletion identified by long-read sequencing. (A) Integrative Genomics Viewer plots showing the deletion in sample CT241226; (B) Sequence of breakpoint junctions of samples CT241226.

accessory cysts exhibit abnormal semen parameters, which influence natural pregnancy but do not affect ICSI and PGT outcomes [34]. Excluding the ADPKD patients whose treatment cycles were incomplete, 70.8% (17/24) of individuals with semen abnormalities achieved paternity or obtained embryos after the ICSI treatment [32]. In our study, six of the seven patients carrying pathogenic ADPKD variants underwent ART, among whom 3 achieved clinical pregnancy.

4.4 | Application of LRS in Genetics Diagnosis of Male Infertility

Due to the much longer sequencing reads of LRS, it has some advantages in genetic testing of rare diseases which are caused by variants in complex genes with pseudogenes and/or high GC content [35, 36]. LRS demonstrates great potential in the genetic diagnosis of CAH and ADPKD. CACAH has been applied to genetic diagnosis of CAH patients and newborn screening in several clinical studies and has shown advantages over conventional approach in terms of accuracy, cost and turn-around time. Compared with conventional CAH testing using MLPA plus Sanger sequencing, the CACAH assay showed 100% specificity and 100% sensitivity [16]. Furthermore, LRS precisely characterized complex chimeric variants in 17.3% (144/832) of samples that could not be clearly classified previously and clarified the diagnosis and determined the parental origin in 21-OHD patients carrying variants on complex multiple copies of *CYP21A2* [37]. In addition, LRS enables the simultaneous detection of 4 common enzyme-deficient genes in the steroid metabolism pathway (*CYP11B1*, *CYP17A1*, *HSD3B2*, and *StAR*), providing a comprehensive and accurate genotyping approach of CAH [38]. Several studies have demonstrated the effectiveness of LRS-based genetic newborn screening for CAH. In the diagnosis of neonatal CAH, CACAH shows clear advantages over traditional methods and serves as a complementary approach that significantly reduces both false-positive and false-negative rates [39]. Moreover, CACAH enables direct determination of the cis- and trans- configuration of variants without requiring parental genotype analysis, thereby accelerating diagnostic timelines [40]. These strengths facilitate early treatment of patients with CAH and control the disease progression. LRS-based CAPKD generates reads spanning large genomic regions of *PKD1* and *PKD2*, improves detection of complex structural variants and the resolution of repetitive sequences, and provides an accurate and precise approach for genetic analysis of ADPKD [17]. Compared to NGS, CAPKD increased the genetic diagnosis rate from 20.0% (8/40) to 45.0% (18/40) among previously undiagnosed cases. Moreover, CAPKD directly determined the cis- and trans-configurations of 2 or more variants as well as the precise

breakpoints of large deletions and duplications [41]. These findings indicate that LRS provides a novel approach for the research on male infertility-related diseases, such as CAH and ADPKD.

Utilizing CACAH and CAPKD approaches based on LRS, we identified 1 subject with two *CYP21A2* variants and 7 subjects with P/LP ADPKD related variants in this cohort. Compared to NGS-based panel, LRS added the diagnostic yield among infertile men by 33.3% (1.1%/3.3%). LRS can sequence the full length of genes dozens of kilobases long in single reads, making it straightforward to read the whole gene and discriminate between highly homologous genes like *CYP21A1P* and *CYP21A2* [16]. LRS can directly sequence deletions/duplications and large gene conversions, which are not reliably determined by NGS-based methods. In addition, LRS can easily detect the cis- and trans- configuration of 2 or more variants without the need for genotypic analysis of family members. Therefore, in this study, we directly determined the trans configurations in 1 subject with two *CYP21A2* variants. Previous study demonstrated the significant advantages of using LRS for the genetic diagnosis of ADPKD. LRS is capable of spanning large genomic regions and analyzing complex structural variants, which enables a spectrum of variants that were previously undetectable or difficult to characterize by short-read sequencing (SRS) to be identified [17]. LRS-based CAPKD approach for infertile patients with ADPKD is particularly important for their assisted reproductive treatments since accurate genetic diagnosis is required for those who seek PGT for monogenetic diseases (PGT-M) to lower the pregnancy risk [41]. Previous studies sequenced the whole genomes by LRS of over 100 men with various forms of male infertility, demonstrating that LRS can reliably identify structural chromosomal abnormalities, chromosomal aneuploidies, *CFTR* variants, and complete Y-chromosome microdeletions [42]. Moreover, by identifying previously undetectable genomic variants associated with infertility, the results highlighted the enhanced value of LRS in uncovering the genetic mechanisms of male infertility [43].

Although LRS offers advantages for detecting complex structural variants and resolving repetitive regions, its current limitations such as high cost and the need for specialized analytical expertise still restrict widespread clinical adoption. The present findings suggest that the influence of CACAH and CAPKD on clinical management decisions in male infertility remains limited, indicating that LRS-based approaches should be applied cautiously and may not be suitable as routine clinical screening tools. Moreover, the application of screening by LRS requires careful clinical interpretation. International consensus [44] emphasizes that screening for ADPKD in asymptomatic young men without a family history should be approached with great caution. Unselected genetic testing may result in overdiagnosis, psychological burden, and challenges in clinical decision-making. Therefore, CAPKD and CACAH may be considered selectively in individuals with relevant clinical indications, with appropriate genetic counseling as an integral component of the testing process. Overall, our findings primarily underscore the research value of LRS in elucidating the etiological exploration of male infertility and in identifying variants of complex genomic regions, while its clinical application should be considered in a targeted manner rather than as a routine screening approach.

5 | Limitations

This study is not without any limitations. First, all samples were collected from a single center in China, and regional and population-specific characteristics might lead to bias in variants. Second, in addition to the great advantages of genetic analysis for diagnosis, a large number of VUSs were also identified in this study. Some variants were novel and private to a single pedigree, and it was difficult to classify the pathogenicity of these variants. How to interpret the obtained genetic data is also a challenge that we need to address [45]. In the future, we may harness AI-assisted tools to attain precise and efficient interpretation of genetic variants related to male infertility. We employed LRS to analyze only two diseases associated with male infertility in this study, specifically CAH and ADPKD. In the next step, we may include more related diseases to further validate the significance of LRS in the genetic diagnosis of male infertility.

6 | Conclusions

The application of CAH and ADPKD analysis based on LRS extends conventional genetic testing for male infertility by facilitating more precise evaluation of complex genomic regions. It may enhance the clarity of genetic diagnoses in male infertility, particularly in selected patients with relevant clinical indications. This approach contributes to a clearer understanding of the genetic landscape and provides useful information that may support reproductive decision-making and clinical management.

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Ethics Statement

This study had been reviewed and approved by the institutional review board of the Center for Reproductive Medicine at Shandong University.

Consent

All authors give their consent for publication of this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** rmb270038-sup-0001-DataS1.xlsx.