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SHORT REPORT

Whole genome sequencing identifies a homozygous splicing variant in *TDRKH* segregating with non-obstructive azoospermia in an Iranian family

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Abstract

Non-obstructive azoospermia (NOA) resulting from primary spermatogenic failure represents one of the most severe forms of male infertility, largely because therapeutic options are very limited. Beyond their diagnostic value, genetic tests for NOA also hold prognostic potential. Specifically, genetic diagnosis enables the establishment of genotype-testicular phenotype correlations, which, in some cases, provide a negative predictive value for testicular sperm extraction (TESE), thereby preventing unnecessary surgical procedures. In this study, we employed whole-genome sequencing (WGS) to investigate two generations of an Iranian family with NOA and identified a homozygous splicing variant in *TDRKH* (NM_001083965.2: c.562-2A>T). *TDRKH* encodes a conserved mitochondrial membrane-anchored factor essential for piRNA biogenesis in germ cells. In *Tdrkh* knockout mice, de-repression of retrotransposons in germ cells leads to spermatogenic arrest and male infertility. Previously, our team reported *TDRKH* involvement in human NOA cases through the investigation of a North African cohort. This current study marks the second report of *TDRKH*'s role in NOA and human male infertility, underscoring the significance of the piRNA pathway in spermatogenesis. Furthermore, across both studies, we demonstrated that men carrying *TDRKH* variants, similar to knockout mice, exhibit complete spermatogenic arrest, correlating with failed testicular sperm retrieval.

KEYWORDS
azoospermia, male infertility, non-obstructive azoospermia, piRNA pathway, spermatogenesis, TDRKH

1 | INTRODUCTION

Despite the strong genetic basis of non-obstructive azoospermia (NOA), routine screening tests like karyotyping and Y chromosome analysis yield less than 25% for genetic diagnosis.[1] High throughput sequencing (HTS) techniques such as whole exome/genome sequencing (WES/WGS) have revolutionized our ability to detect rare genetic variants and identify novel candidate genes for human diseases, including idiopathic NOA cases. Pathogenic variants in over 40 strong NOA-candidate genes have been identified, underscoring the genetic heterogeneity of NOA.[2–9] Overall, HTS-based testing strategies yield genetic diagnoses in 5.4%–23% of idiopathic NOA cases.[2,10,11]

NOA represents the most severe phenotype of male infertility, with limited therapeutic options available. While testicular sperm extraction (TESE) followed by intracytoplasmic sperm injection (ICSI) has improved treatment, its success rate remains low due to challenges in sperm retrieval and poor gamete quality.[12] Enhancing genetic diagnostic yield and understanding NOA pathophysiology can facilitate genotype–phenotype correlations, aiding in predicting TESE-ICSI outcomes and avoiding unnecessary procedures.[2]

In this study, we conducted whole genome sequencing (WGS) on two generations of an Iranian family affected by idiopathic NOA. We identified a novel rare homozygous variant in *TDRKH* (NM_001083965.2: c.562-2A>T), a gene predominantly expressed in testicular germ cells and involved in the piRNA biogenesis pathway. Our findings corroborate TDRKH's involvement in NOA, as previously reported in a North African cohort.[2] This study further emphasizes the role of the piRNA pathway in spermatogenesis.

2 | MATERIALS AND METHODS

Material and methods are described in online supplementary materials.

3 | RESULTS

3.1 | The clinical report

A 35-year-old man (proband, III:6) from Iran sought treatment at Tehran's Royan Institute (Reproductive Biomedicine Research Center) for primary infertility due to non-obstructive azoospermia (NOA). Further investigation revealed consanguinity in the family, as the proband's grandparents were first cousins, and other family members were also affected by the same reproductive disorder. The proband has three brothers (III:1, III:3, III:6, and III:7) and three sisters. All his brothers and one uncle (II:3) are infertile due to idiopathic NOA (Figure 1A).

On physical examination, the proband appeared normal, except for slightly smaller testicular volumes on both sides (10–15 mL) compared to the reference value (>15 mL). Hormonal investigations revealed FSH levels at 7.8 IU/L (normal range 1.5–12.4 IU/L), LH at 6.3 IU/L (normal range 1.8–8.6 IU/L), and testosterone at 4.7 ng/mL (normal range 2.5–10.6 ng/mL). Sperm analysis showed a complete absence of sperm in the ejaculate, even after centrifugation and examination of the total volume of the pellet. First-line genetic tests showed a normal karyotype (46,XY) and no complete microdeletion of the AZF loci on the Y chromosome. Other affected family members (II:3, III:1, and III:7) also had normal karyotypes and no microdeletions on the Y chromosome.

3.2 | Molecular analyses

WGS revealed a likely pathogenic variant, NM_001083965.2: c.562-2A>T, in *TDRKH* (Tudor and KH Domain Containing), found in the homozygous state in the proband (III:6), his brother (III:1), and his uncle (II:3), confirmed by Sanger sequencing (Figure 1A). Targeted Sanger sequencing further confirmed this variant's homozygosity in the remaining affected brother (III.7), not previously tested by WGS (Figure 1A). Located in *TDRKH* intron 5 within a consensus acceptor splice sequence, this variant was predicted highly deleterious by SPiP (98.4%) and SpliceAI (0.99) (Figure 1B), with no prediction of an adjacent cryptic splice site. The most likely effect is exon 6 skipping (140 bp), causing a frameshift and premature termination codon, potentially yielding a truncated protein. Alternatively, skipping of exons 5 and 6 (462 bp) may occur due to weak splice sites of exon 5 (Figure 2A). This variant is predicted to impact nearly all alternative protein-coding transcripts listed in the Ensembl database. In gnomAD (v4), this variant is found with a minor allele frequency (MAF) of 1.2×10^{-6} and was absent in our control cohort of 507 men. No other convincing deleterious bi-allelic variants in other NOA-candidate genes were found in these subjects.

To assess the detrimental impact of the candidate variant and understand its effect on splicing, we conducted a minigene assay. RT-PCRs were performed on both non-transfected and transfected cells, using minigenes containing either the wild-type or mutated *TDRKH* sequence. In HEK cells transfected with the wild-type minigene, RT-PCR yielded a 692 bp product indicative of normal splicing (Figure 2B). Additionally, we observed a minimal quantity of a shorter fragment (550 bp) lacking exon 5 sequence, suggesting alternative splicing likely due to the inherent weakness of the splice sites flanking this exon. Sanger sequencing confirmed exon 5 skipping in this product. Conversely, RT-PCR from cells transfected with the mutant minigene revealed a small 230 bp amplicon exclusively containing exonic sequences from the vector, as confirmed by Sanger sequencing. This result clearly illustrates the deleterious impact of the candidate variant on *TDRKH* splicing, resulting in the concurrent skipping of exons 5–6.

3.3 | Testicular sperm extraction (TESE) and histopathology

TESE performed on subject III:6 resulted in no sperm retrieval. Histopathological examination of the testicular fragments revealed a predominant post-meiotic maturation arrest at the round spermatid stage

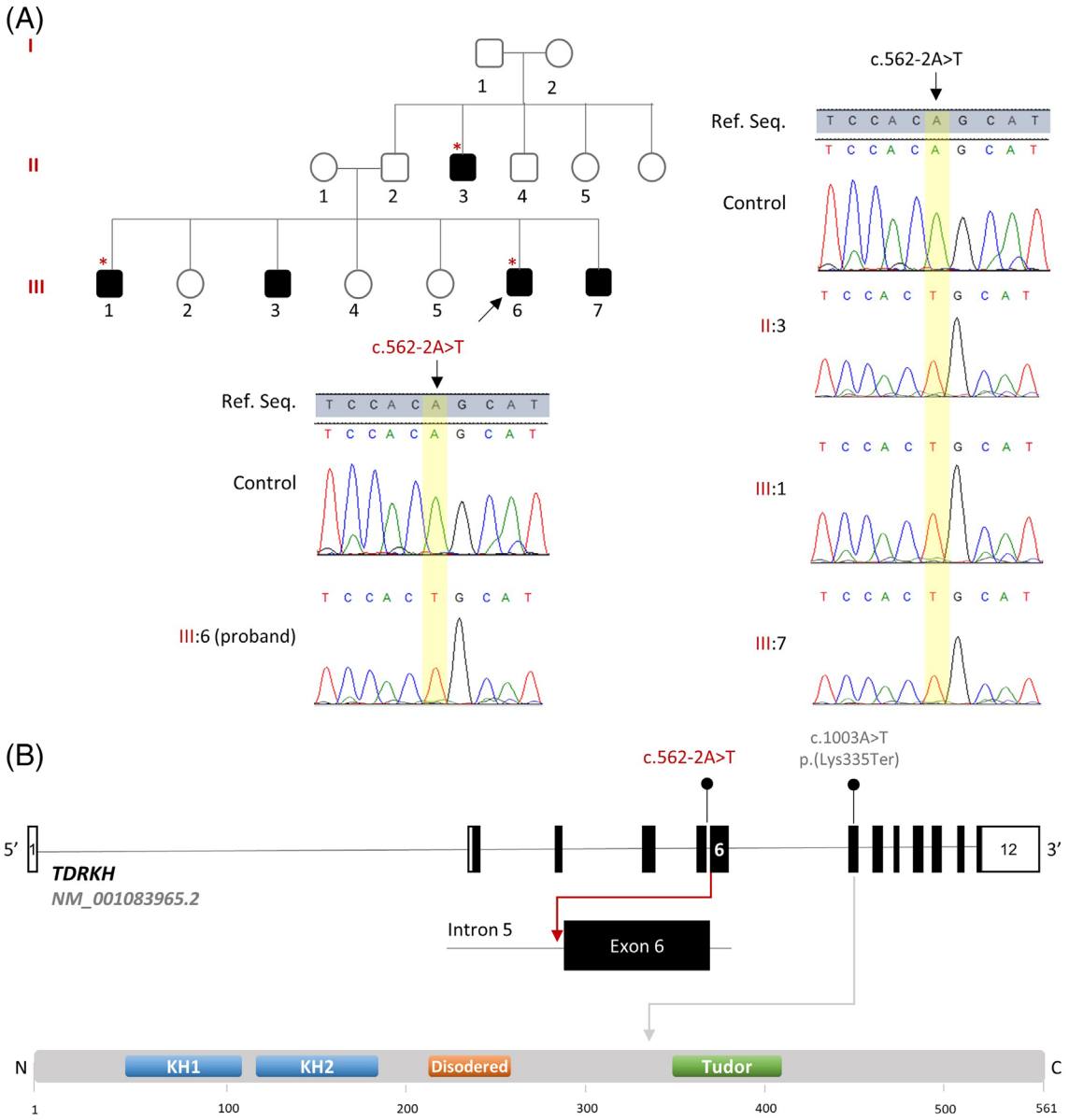

FIGURE 1 Identification of a likely pathogenic *TDRKH* variant in affected family members. (A) Pedigree and genetic analysis of a two-generational family with non-obstructive azoospermia. Red asterisks indicate individuals who underwent whole-genome sequencing (WGS). WGS revealed a homozygous *TDRKH* variant in all tested members (II:3, III:1, and III:6). (B) Location of the *TDRKH* variant (highlighted in red) within intron 5, affecting a splice-acceptor consensus sequence. Previously reported variant is depicted in gray. [Colour figure can be viewed at wileyonlinelibrary.com]

(>95%), with a minor fraction showing maturation arrest at the spermatocyte stage (<5%) (Figure 3). Interstitial fibrosis, tubular sclerosis, inflammation, and germ cell neoplasia were absent, and the Leydig cell count was unremarkable.

4 | DISCUSSION

The discovery of novel monogenic causes of NOA is frequently reported, with the list of candidate genes continually expanding.[2,13,14] Currently, 21 NOA genes have been validated by multiple independent studies.[10] In a previous WES study of 96 North-African men with idiopathic NOA, we identified likely pathogenic variants in 16 genes in 22 individuals (23%).[2] Among these were six genes, notably HENMT1 and TDRKH, which had not previously been linked to male infertility and are crucial for piRNA biogenesis. In this study, using WGS and targeted Sanger sequencing, we identified a novel homozygous likely pathogenic variant in *TDRKH* in an Iranian family with five members affected by idiopathic NOA, present in all four tested affected members, strongly suggesting its role in their infertility. Unfortunately, we

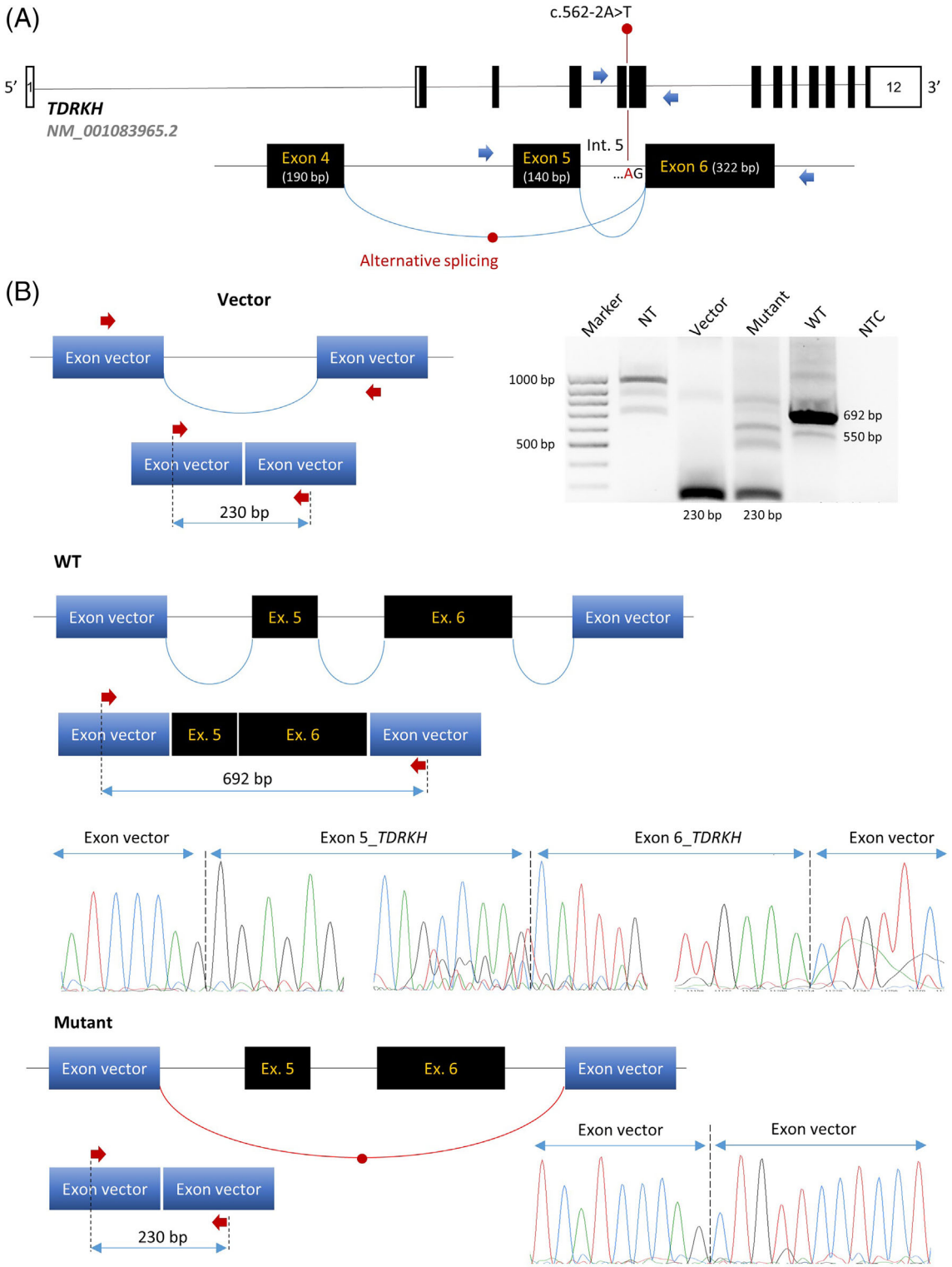

FIGURE 2 Minigene splicing reporter assay. (A) The strategy aimed to amplify the genomic *TDRKH* sequence bordered by introns 4 and 6 in both control samples and patients. The primer set enabled amplification of 330 and 472 bp of introns 4 and 6, respectively, along with the entire sequences of exons 5 (140 bp) and 6 (322 bp). (B) RT-PCR experiments revealed normal splicing in HEK cells transfected with the wild-type *TDRKH* minigene, alongside a minor fragment indicating alternative splicing, confirmed by Sanger sequencing to be exon 5 skipping. Conversely, cells transfected with the mutant minigene showed an aberrant 230 bp amplicon, with Sanger sequencing confirming the concomitant skipping of exons 5–6. [Colour figure can be viewed at wileyonlinelibrary.com]

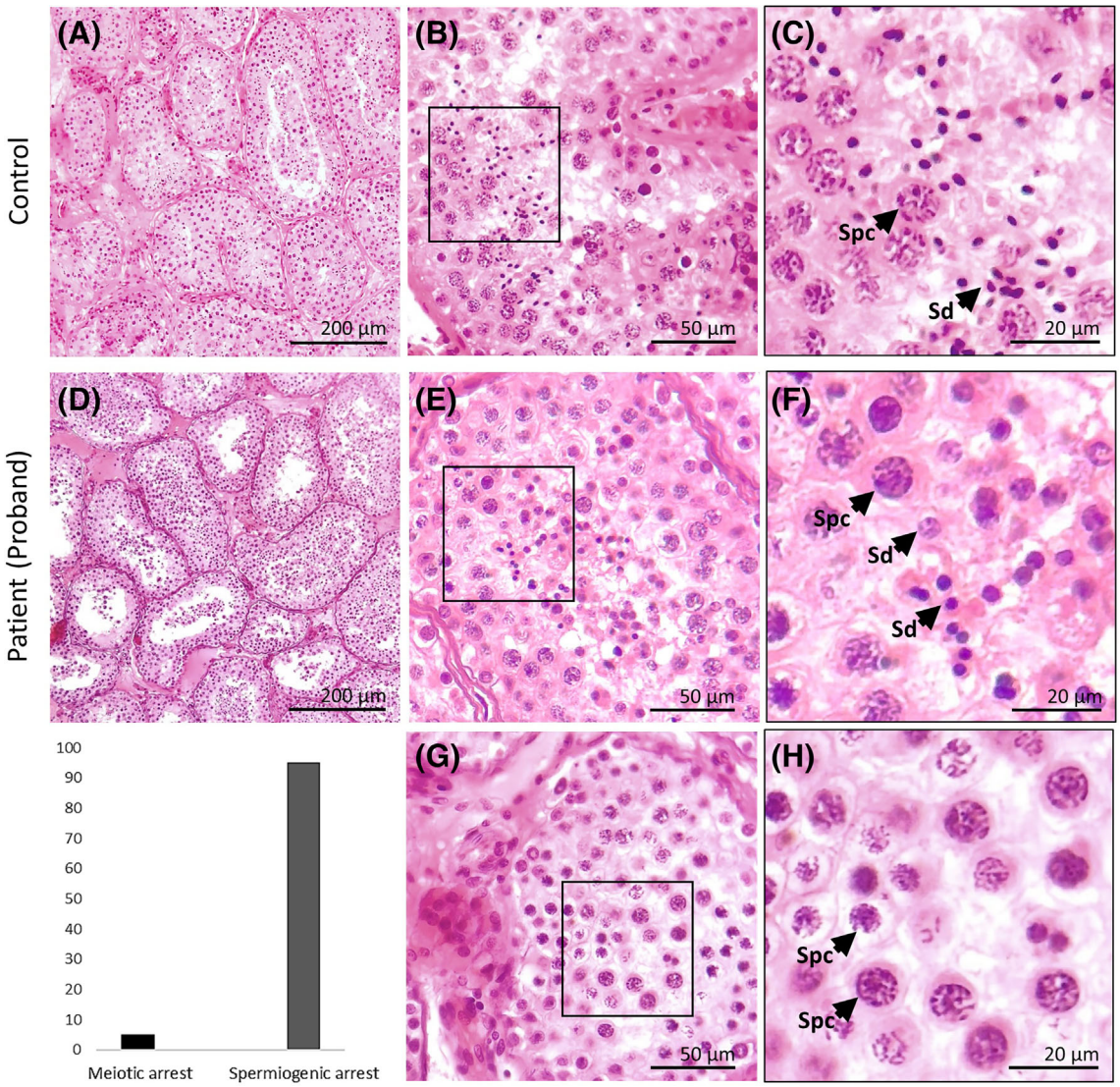

FIGURE 3 Testicular histopathologic findings in the proband. Panels (A–C) show a hematoxylin and eosin stain of a control testicular tissue sample in which seminiferous tubules had complete spermatogenesis. Panels (D–H) show a hematoxylin and eosin stain of a testicular tissue sample from the proband. At higher magnification, we evidenced in the proband a spermiogenic arrest at the round spermatid stage in 95% of analyzed tubules (panel F) and an early meiotic arrest at prophase I in the remaining 5% tubules (panel H). Sd, spermatid; Spc, spermatocyte. [Colour figure can be viewed at wileyonlinelibrary.com]

were unable to access samples from other family members, limiting our ability to conduct further analyses. Moreover, all testicular biopsy fragments were prioritized for retrieving viable spermatozoa for ICSI and histopathological analysis, leaving no fragments available for additional research experiments. To assess the pathogenicity of the candidate variant, we performed a minigene splicing reporter assay, revealing its deleterious impact on *TDRKH* splicing. Specifically, this variant disrupts a consensus splice site, resulting in the concurrent skipping of exons 5–6. This indicates it exerts a highly damaging effect on the gene's splicing process and consequently affects the functional structure of the protein product.

Piwi-interacting RNAs (piRNAs) are a class of small non-coding RNAs, typically 23–31 nucleotides in length, derived from single-stranded precursor transcripts.[15] Initially discovered in the germ cells of *Drosophila melanogaster*, piRNAs were found to regulate transposable element (TE) activity, crucial for maintaining the integrity of the germline genome.[15] TEs, mobile DNA elements, have the potential to excise and re-insert themselves into different genomic loci, posing a threat to genomic stability.[16] Unregulated TE activity in germ cells can lead to defective gametogenesis and infertility. The piRNA-induced silencing complex (pi-RISC) consists of piRNAs and PIWI proteins, with piRNAs possessing sequences complementary to TEs, and PIWI

proteins acting as effectors within the argonaute nuclease family. Together, piRNAs and PIWI proteins serve as a sequence-specific guide, targeting and silencing TE activity to safeguard genomic integrity.[17] piRNA biogenesis is highly conserved across species, starting in the nucleus where single-strand or dual-strand piRNA clusters are transcribed, generating precursor piRNA transcripts (Figure S1). These precursor transcripts then migrate from the nucleus to the cytoplasm. piRNA biogenesis can proceed in two ways depending on the stage: (i) through the Ping-pong cycle, occurring in the nuage, or (ii) via Phasing, which takes place at the mitochondria (Figure S1).

TDRKH, a conserved mitochondrial membrane-anchored factor, drives piRNA processing in pachytenes.[18] Knockout mice lacking Tdrkh exhibit derepression of retrotransposons in developing testicular germ cells, leading to spermatogenic arrest and male infertility.[18,19] The stage of spermatogenic arrest varies among knockout mice. Sax et al.[18] created a global Tdrkh knockout model and observed that mutant males had smaller testes compared to control littermates, with histological analysis revealing homogeneous testicular phenotypes characterized by early meiotic arrest at prophase I. Conversely, Ding et al.[19] generated conditional knockout mice lacking TDRKH expression in testicular germ cells. They found that testicular weight in these mice was twice as high as in global knockout animals, suggesting spermatogenic arrest occurred at a later stage. Testicular sections from conditional knockout animals showed a heterogeneous spermatogenic arrest, with the majority of seminiferous tubules arrested at the round spermatid stage, contrasting with only 20% presenting meiotic arrest at prophase I, consistent with the testicular phenotype observed in our patient, who had negative TESE results and was homozygous for a loss-of-function TDRKH variant.[2]

In conclusion, this study highlights the pivotal role of the piRNA pathway in male germ cell development and spermatogenic failure, while also reaffirming the involvement of TDRKH bi-allelic variants in NOA and male infertility.

AUTHOR CONTRIBUTIONS

Z.-E.K. and A.A-Y. designed the study and supervised all laboratory work. They have full access to all of the data in the study and take responsibility for the integrity of the data and its accuracy. All authors read, corrected, and made a significant contribution to the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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