

ORIGINAL ARTICLE

Novel mutations of *PMFBP1* in a man with acephalic spermatozoa defects

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Abstract

Background: Acephalic spermatozoa (AS) is a serious but rare reproductive genetic disorder that causes infertility in men. To date, only a few genes associated with AS defects have been identified, including the polyamine modulated factor 1 binding protein 1 (*PMFBP1*) gene. Consistent with this, *PMFBP1* localizes to the head–neck connection, which bridges the implantation fossa and basal body. **Methods:** A male patient was diagnosed as having an AS defect. Blood samples from all family members and a sample of the patient's semen were collected to determine the genetic causes of his infertility.

Results: Compound heterozygote mutation in the *PMFBP1* gene, which is associated with AS defects in the present case: two loss-of-function mutations, with one a nonsense mutation c.361C>T p.Gln121Ter, and another a splice donor mutation c.414+1G>T. The current study, together with previous studies, suggests that the nonsense mutation is responsible for a truncated *PMFBP1* protein during its formation; a splice donor mutation c.414+1G>T might lead to new open reading frames, from which the dysfunction of an abnormal *PMFBP1* protein might be predicted. Additionally, the expression of outer dense fiber 1 (ODF1) and ODF2 proteins has been experimentally shown to be regulated by the truncated *PMFBP1* protein.

Conclusion: We herein present a case with AS defects associated with heterozygote mutations of *PMFBP1*, which have been shown to be rare and pathogenic; the association with an AS defect is a monogenic disorder with a recessive inherited pattern in the patient's family.

KEYWORDS

acephalic spermatozoa, headless sperm, *PMFBP1* gene, truncated protein

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1 | INTRODUCTION

A stable sperm head–tail coupling apparatus (HTCA, also known as sperm neck) is required to connect the head and tail of a sperm during the high-speed movement of a spermatozoa competition to fertilize an oocyte (Chemes, 2018; WHO, 2010; Wu et al., 2020). This means that an abnormal structure and/or dysfunction of the HTCA in sperm might cause infertility, although such structure is commonly observed in fertile individuals (the lower reference value for normal sperm is set down at 4%) (WHO, 2010). Case reports and animal model studies highlight similar key features of serious HTCA defects: isolated case of infertility in men associated with the presence of a large number of separated heads and tails of sperm (Chemes, 2018; Chemes & Rawe, 2003; Liu et al., 2020; Nie et al., 2020; Sha, Xu, et al., 2018a; Zhu et al., 2018). A syndrome involving acephalic spermatozoa (AS; OMIM: 617187) is one of the more serious but rare reproductive genetic disorders (Nie et al., 2020). The molecular mechanisms involved in AS have been since the first AS gene (*SUN5*) was identified in 2016. To date, only six AS genes have been reported (Nie et al., 2020; Sha, Xu, et al., 2018a; Zhu et al., 2016, 2018), namely *SUN5*, *BRDT*, *PMFBP1*, *TSGA10*, *SPATA6*, and *HOOK1* (Chen et al., 2018; Li et al., 2017; Liu et al., 2020; Sha, Sha, et al., 2018b; Ye et al., 2020).

The polyamine modulated factor 1 binding protein 1 (*PMFBP1*; GenBank: NM_031293.3) gene has been identified as an AS-associated gene by Zhu et al. (2018) and Sha et al. (2019). The coding protein of *PMFBP1* is a scaffold protein of the centrosome in sperm (Ohuchi et al., 2001), located at the basal body and proximal centriole of the HTCA region (Sha et al., 2019; Wu et al., 2020). It connects upward to *SUN5* protein and downward to the *TSGA10* protein, as described by Nie et al. (2020) and Wu et al. (2020), forming a sandwiched localized structure in the HTCA of sperm. A further study of the interaction between these three proteins demonstrated no direct interaction between each other can be found (Shang et al., 2018; Ye et al., 2020; Yuan et al., 2015; Zhu et al., 2018). This suggested the presence of other proteins might help connect these three proteins and might be involved in the formation of the HTCA of sperm. Furthermore, the incidence of AS defect mutations associated with *PMFBP1* is estimated to account for 11% of all AS cases. As postulated by Zhu et al. (2018), this gene can be used as a genetic marker for the clinical diagnosis of AS defects (Elkhatib et al., 2017; Liu et al., 2020; Nie et al., 2020; Zhu et al., 2018). The reliability of this hypothesis, however, is limited by the small number of relevant published reports. This limitation also makes the management and clinical prognosis of *PMFBP1*-related

AS defects unpredictable: the phenotype of AS sperm does not respond to any pharmacological intervention, and not all AS sperm, through intracytoplasmic sperm injection (ICSI) techniques, can achieve the same clinical results: pregnancy (Nie et al., 2020).

To date, five nonsense and two frameshift mutations of *PMFBP1* have been identified as loss-of-function (LOF) mutations that are associated with AS defects (Nie et al., 2020; Sha et al., 2019; Zhu et al., 2018; see Figure 1c). This means that the LOF of *PMFBP1* might be a major cause of AS sperm formation. However, very recently, a mutant, c.361C > T p.Gln121Ter, was reported to be a nonsense variation in *PMFBP1* transcription, but this has not been demonstrated experimentally (Liu et al., 2020). Therefore, understanding the function and mechanism of *PMFBP1* is essential. In the present study, a novel mutation of *PMFBP1* has been identified through a whole exome sequencing (WES) technique. This study has focused on a genetic contribution to AS defects, namely a discussion of the in-depth changes in the genetic mechanisms of mutant *PMFBP1* in AS sperm formation.

2 | MATERIALS AND METHODS

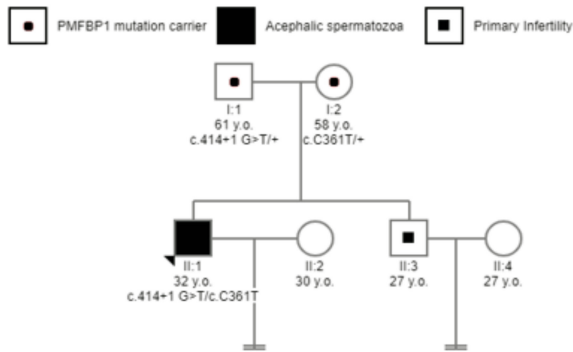
2.1 | Ethical compliance

This study protocol was approved by the Ethics Committee of Guangdong Provincial Fertility Hospital (Former name: Family Planning Special Hospital of Guangdong Province). Informed consent was obtained for collecting blood samples from family members of the patient, as well as for the collection of a sample of the patient's semen for use in this study to determine the genetic causes of his infertility. Four milliliters of peripheral blood and 5 ml of semen (after four days of sexual abstinence) were collected for experiments. The following experiments from this study were conducted in accordance with the relevant principles and rules of the Institutional Ethical Review Committee of Guangdong Provincial Fertility Hospital. Two donated semen samples and blood samples from 100 donors were used as controls for this study.

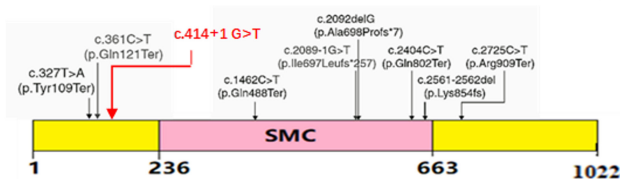
2.2 | Participants

A male patient (II:1) and his wife (II:2) attempted to become pregnant naturally for nearly five years, as shown in the family pedigree (Figure 1). By the time they had both reached 30 years of age, they had visited the reproductive clinic of the Guangdong Provincial Fertility Hospital on a regular

(a) The family history of the patient with acephalic spermatozoa.



(c) localization of PMFBP1 mutation in the protein structure.



(b) Sanger sequencing verification of the *PMFBP1* mutation.

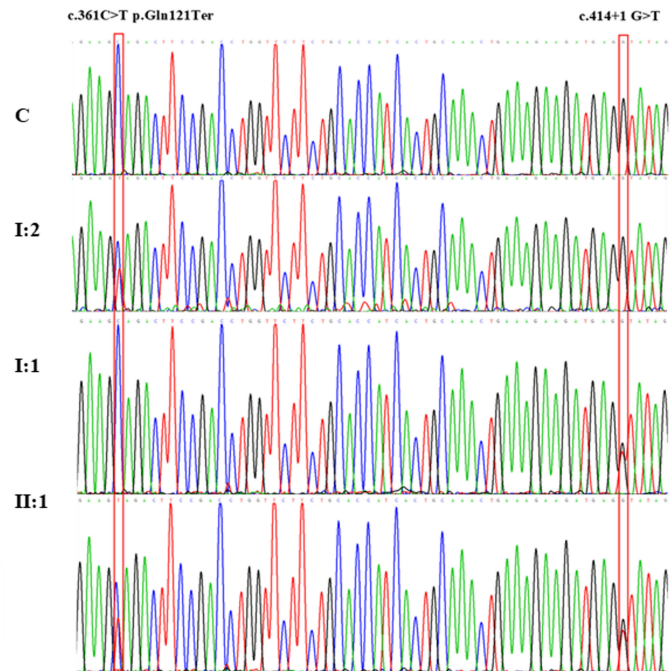


FIGURE 1 The family pedigree and Sanger sequencing analysis of AS mutations. (a) The family history of a AS patient. Patient II:1 (proband, marked with black arrow) was affected by compound heterozygous *PMFBP1* mutations. (b) Sanger sequencing verification of the *PMFBP1* mutation. The results of Sanger sequencing verification in exon 4 and intron 4 of *PMFBP1* for the detection of c.361C>T p.Gln121Ter and c.414+1G>T mutations in the proband's family members. The red box highlights the nucleotide alteration. Two isolated sequences are present from the point of replacement, which indicates that the patient is clearly heterozygous. Human GRCh37 (hg19) assembly was used as a human reference sequence to align the sequence reads of the AS family and an NM_031293.3 transcript was used to annotate all variants. (c) Localization of *PMFBP1* mutation in the protein structure. A novel mutation of *PMFBP1* in the present case is marked in bold red with a red arrow, and black arrows represent reported mutations. Original diagram. AS, Acephalic spermatozoa. *PMFBP1*, Polyamine modulated factor 1 binding protein 1. C, Control sample. SMC, The functional structural maintenance of chromosome region is shown in the pink box from 236 to 663 aa.

basis for the previous three years. The couple was healthy with no history of any significant disorders, excluding infertility. Both had a normal karyotype and microdeletions and microduplications were not found on chromosomes Y and/or X. The hormone levels of both were also normal. Testicular biopsies of the patient were not performed.

2.3 | Sperm analysis and morphological observation

Semen analysis was performed more than once based on the fifth World Health Organization (WHO) criteria (WHO, 2010). Patient II:1's semen volume, semen pH, sperm concentration, and the proportions of motile and acephalic sperm were recorded. Meanwhile, sperm were analyzed by microscopy using a 1000× magnification and oil immersion. In addition, in order to observe sperm morphology, a uranyl acetate and lead citrate staining

protocol (Zhu et al., 2018) for transmission electron microscopy was used to assess the head–neck connect region and midpiece region changes of sperm.

2.4 | Sample preparation

Genomic DNA was extracted from each peripheral blood sample, and total RNA and protein were extracted from the patient's semen and 293FT cell culture (see below), using a QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), a RNeasy Mini Kit (Qiagen), and an acetone (Acetone Optima, Fisher Chemical, Fair Lawn, NJ, USA), respectively, according to the manufacturer's instructions. Extracted samples were collected and stored at -80°C for further experiments. The concentration and purity of genomic DNA and total RNA were determined using a NanoDrop 2000 (Thermo Fisher Scientific™, Waltham, MA, USA) before use. The concentration and purity of proteins were determined by

Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the instruction manual.

2.5 | Whole exome sequencing and Sanger sequencing validation

Sequencing of genomic DNA from the proband and his parents was performed using whole exome sequence (WES) technology to identify possible causes of mutations. Whole exomes of genomic DNA were captured and enriched using a SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) following the protocol of the manufacturer. The WES was determined using a HiSeq X-TEN sequencer (Illumina, San Diego, CA, USA). Human GRCh37 (hg19) Assembly was used as a human reference sequence to align the sequence reads of the using Integrative Genomics Viewer software (<http://software.broadinstitute.org/software/igv/>). ANNOVAR software (http://www.openbioinformatics.org/annovar/annovar_download.html) annotated variations of aligned sequence data based on the NM_031293 transcript. Variations such as missense, splicing, nonsense, and frameshift were retained, while the rest were discarded when the minor allele frequency was higher than 1% in databases, such as 1000 Genome (<https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/>), dbSNP (www.ncbi.nlm.nih.gov/projects/SNP), ExAC Browser (<http://exac.hms.harvard.edu/>), GnomAD Browser (<https://gnomad.broadinstitute.org>), and ESP6500 (<https://evs.gs.washington.edu/EVS/>). Standard Sanger sequencing was used to validate the *PMFBP1* mutation of the AS patient and his family's co-segregation analysis. DNA amplifications were carried out using a PCR technique and a Taq PCR Core Kit (Qiagen) according to the manufacturer's instructions, with primers (BGI Tech Solution, Shenzhen, China) that detected mutations within exons 4 and 5 (see [Figure 1b](#)). The primers for Sanger sequencing are listed as follows:

E4e5 (Forward): 5'AGAAGCAGACTTCCGACCTG3'.

E4e5 (Reverse): 5'TCCCCTGTGTTCTCGTTGTG3'.

2.6 | Transcript analysis

For each reaction, 45 µg of total RNA was successfully reverse transcribed into cDNA using an EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, Biotech, Beijing, China) according to the manufacturer's instructions. Samples were amplified using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA)/StepOnePlus™ Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA) with a Taq PCR Core Kit (Qiagen) according to the manufacturer's instructions. A DL500 DNA Marker and 100bp DNA Ladder (Takara, Dalian, China) were used in agarose gel electrophoresis; and β-actin was used as housekeeping gene in the reverse transcription quantitative PCR (RT-qPCR). The primers (BGI Tech Solution) for PCR are listed as follows:

RT-PCR primers	Sequences
Exon4exon5 (Forward)	5'AGAGAGGCGATCTTGTCCTCC3'
Exon4exon5 (Reverse)	5'CCGCCAGTATCAGTCCATCC3'
Exon5exon6 (Forward)	5' TTGAGAGACCTTTTGCCAGACT3'
Exon5exon6 (Reverse)	5' AGTGAAGATGTTGCAGGGGG3'
Exon6exon7 (Forward)	5' AGCCTTTTCACGCTCCAGAA3'
Exon6exon7 (Reverse)	5'GGCAAAGGTCTCTCAACAGG3'
Exon7exon8 (Forward)	5'CTGGCTGTCTTTCTGCTCT3'
Exon7exon8 (Reverse)	5'TTCCTGTACAGCCACCCACA3'
Exon4exon7 (Forward)	5'ACGGCCTCTAGTTCCACGCGCA3'
Exon4exon7 (Reverse)	5'CCACGACAAGAAGCAAGCACAGGCA3'
ORF4 (Forward)	5' CATTGGGGAACTTGGCAGA3'
ORF4 (Reverse)	5' GCAAGCACAGGCATTAGCAT3'
ORF5 (Forward)	5' ACTGCAGCTTTCACCGAGTTA3'
ORF5 (Reverse)	5' GCAAGCACAGGCATTAGCAT3'
ORF6 (Forward)	5' GACTCCCACTCAACATCTGGT3'
ORF6 (Reverse)	5' GCAAGCACAGGCATTAGCAT3'
RT-qPCR Primers	Sequences
ODF1 (Forward)	5'ATGGCTGCACTGAGTTGTCTC3'
ODF1 (Reverse)	5'GCACCGTGTGCTAAATTCGT3'
ODF2 (Forward)	5' AAGGTGGATGAACTGGAGAGGAAAC3'
ODF2 (Reverse)	5' GGTCCCACATTCCCGTGT3'
β-actin (Forward)	5' TGGCACCCAGCACAATGAA3'
β-actin (Reverse)	5'CTAAGTCATAGTCCGCCTAGAAGCA3'

2.7 | Western blot analysis

Western blot analysis was undertaken according to the protocol of Zhang et al. (2019). Anti-PMFBP1 antibody (rabbit polyclonal, Proteintech, Wuhan, China, 1:1000), anti-outer dense fiber 1 (ODF1) antibody (rabbit polyclonal, Proteintech, 1:1000), anti-ODF2 antibody (rabbit polyclonal, Proteintech, 1:1000), and anti-GAPDH antibody (rabbit polyclonal, Proteintech, 1:10 000) were used as primary antibodies in the western blot. Goat anti-rabbit IgG H&L (HRP; goat polyclonal, Proteintech, 1:10 000) was used as a secondary antibody.

2.8 | Predictions of new open reading frame in the *PMFBP1* mRNA

Open Reading Frame Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) was used to predict the new open reading frame (ORF) from mutated *PMFBP1* mRNA.

2.9 | Transfection of *PMFBP1* protein in human embryonic kidney (HEK) 293FT cells

Cell culture was undertaken according to the protocol of Yin et al. (2020). The HEK-293FT cells (Procell Life Science & Technology, Wuhan, China) were used between four and nine passages and seeded on 12-well plates (1×10^4 cells/well) and incubated 48 h at 37°C in the complete culture medium (KGM12800S, Keygen Biotech, Nanjing, China). Following incubation, the complete medium containing a special ordered exogenous recombinant protein *PMFBP1* (from Met1 to Lys120 of N-terminus, 20 ng/ml, Homo sapiens, Cloud-Clone Corp, Wuhan, China) or random protein fragment (197aa, 20 ng/ml, Homo sapiens, Cloud-Clone Corp) was replaced with the older medium in each three wells. And, the last three wells were cultured in the same medium as older one and used as negative control. After another 24 h incubation, cells were collected to analyze the changes in the expression of ODF1 and ODF2 proteins.

2.10 | Statistical analysis

Image Lab software was used to analyze the relative expression of ODF1 and ODF2 proteins. Minitab17 (<https://www.minitab.com>) and a $-2^{\Delta\Delta CT}$ method were used to analyze the relative quantitation of ODF1 and ODF2 mRNA levels, comparisons of which were done using a two-sample *t*-test (Student's *t*-test) or a one-way ANOVA. A *p*-value <0.05 indicated statistical significance.

2.11 | ICSI for AS patient

Informed consent for intracytoplasmic sperm injection (ICSI) was obtained from the couple (II:1 and II:2). ICSI treatment was performed in the reproductive department of the Guangdong Provincial Fertility Hospital. A long protocol using a gonadotropin-releasing hormone agonist for pituitary downregulation in II:2 has been previously described by Shrestha et al. (2015). Since no single spermatozoa of normal morphology could be found in both semen samples and/or testis of the proband, tailless heads

with a relatively normal morphology and head–neck connected sperm with an abnormal morphology were used for ICSI.

3 | RESULTS

3.1 | Pedigree, WES analysis, and Sanger sequence verification of AS family

Pedigree analysis showed that the patient's parents (I:1 and I:2) were 61 and 58 years old (y.o.), respectively, and had two children: the patient himself (proband) and his brother (27 y.o.; Figure 1a). Both the patient and his brother were married and had no significant medical history in their physical examination except for diagnosed primary infertility. Patient II:1 was diagnosed as having an AS defect. He did not respond to any pharmacological intervention suggesting his infertility could be attributed to the presence of genetic defects. Repeated andrological examinations of patient II:1 showed that he had severe oligospermia and teratozoospermia; most of his semen parameters were abnormal according to sperm, immunological and seminal fluid analyses. The latest semen analysis is shown (see Table 1): the semen volume was 5 ml with a low sperm density of 0.1×10^6 /ml and PH was 7.5. No sperm with a normal morphology with progressive motility was observed. Nearly 99% of sperm were headless. The time for semen liquefaction was more than 60 min. The fructose level was 72.8 μmol.

Whole exome sequencing analysis revealed that the proband was affected by having compound heterozygous c.361C>T p.Gln121Ter and c.414+1G>T mutations (NM_031293.3) in the *PMFBP1* gene (Figure 1b). The patient's parents (I:1 and I:2) were asymptomatic heterozygous carriers, with I:2 carrying the c.361C>T p.Gln121Ter mutation and I:1 carrying the c.414+1G>T mutation, as shown in Figure 1a,b. The identity of this mutation of the proband inherited from each parent was confirmed by the Sanger sequencing. In addition, the *PMFBP1* mutation of the patient was not detected in 100 donated samples without an AS defect phenotype, which revealed that these two variations of *PMFBP1* are rare mutations. These two mutations were present in the study family, suggesting an underlying genetic defect that was heritable. However, no other cases of primary infertility or other genetic symptoms were found in the proband's maternal and/or paternal sides of the family. Thus, this compound heterozygous of proband was inherited from his parents which is consistent with an autosomal recessive pattern. Since the patient's brother (II:3) and his wife were unwilling to be tested, the genetic causes of their primary infertility

TABLE 1 Semen analysis of AS patient based on the fifth World Health Organization criteria (WHO, 2010)

Semen analysis	Volume	PH	Sperm density (10 ⁶ /ml)	Progressive motility (%)	Sperm vitality (%)	Semen liquefaction	Headless sperm (%)	Fructose level	Normal morphology of sperm (%)
II:1	5.0 ml	7.5	0.1	0	0	>60 (min)	98%	72.8 μmol	0

Abbreviation: AS, Acepahalic spermatozoa.

(diagnosed as oligospermia in another hospital two years ago) could not be determined.

3.2 | Ultrastructure observations in AS sperm

Two hundred sperm of the proband were examined, of which a great majority were apparently decapitated and did not show normal progressive motility, as outlined in Figure 2a. Occasionally, isolated sperm heads were observed that contained an intact nucleus with a typical acrosome structure. The size and morphology of these were very similar to that of normal sperm: the ratio of the acrosome to head was one to three (WHO, 2012). Significantly, an implantation fossa and/or basal body, however, were generally absent in most sperm tails. With regard to the tails, it was observed that the proximal centrioles, and incomplete outer dense fibers (ODFs), with/without complete segmented columns surrounded by a cytoplasmic droplet, led to a misrecognition of pinhead-like sperm. Thus, the head of the sperm was sharply separated from the tail at the level of the proximal centriole and plasma membrane of the nucleus. The phenotypes of these headless and tailless spermatozoa were similar to those described by Zhu et al. (2018), Sha et al. (2019) and Nie et al. (2020).

The majority of flagella displayed irregularly structured neck and midpiece regions. In particular, at the midpiece of the headless tail, a normal proximal centriole existed with a disarranged mitochondrial sheath that was observed in nearly half of sperm, with occasional irregular outer dense fibers projecting in the proximal end of headless sperm tails. The axoneme partially presented with a well-formed central pair of microtubules, as shown in Figure 2b,c, a lack of several peripheral microtubular doublets, connected dynein arms, and radial spokes were frequently observed. However, the abnormal axoneme structure can also be observed in normal fertile men. Therefore, whether the defects observed in the axoneme are caused by mutant *PMFBP1* requires further research.

3.3 | Expression analysis of *PMFBP1* gene and protein

The genomic location of *PMFBP1* was determined as 16q22.2; the length of the *PMFBP1* gene is 64,846 bp and is comprised of 27 coding exons (NCBI gene ID: 83449; HGNC:17728). Several cDNA-PCR exon-junction fragments, located between exons 4 and 8 of *PMFBP1*, from the patient were amplified separately (shown in Figure 3a). The absence of both exons 5 and 6 was confirmed from

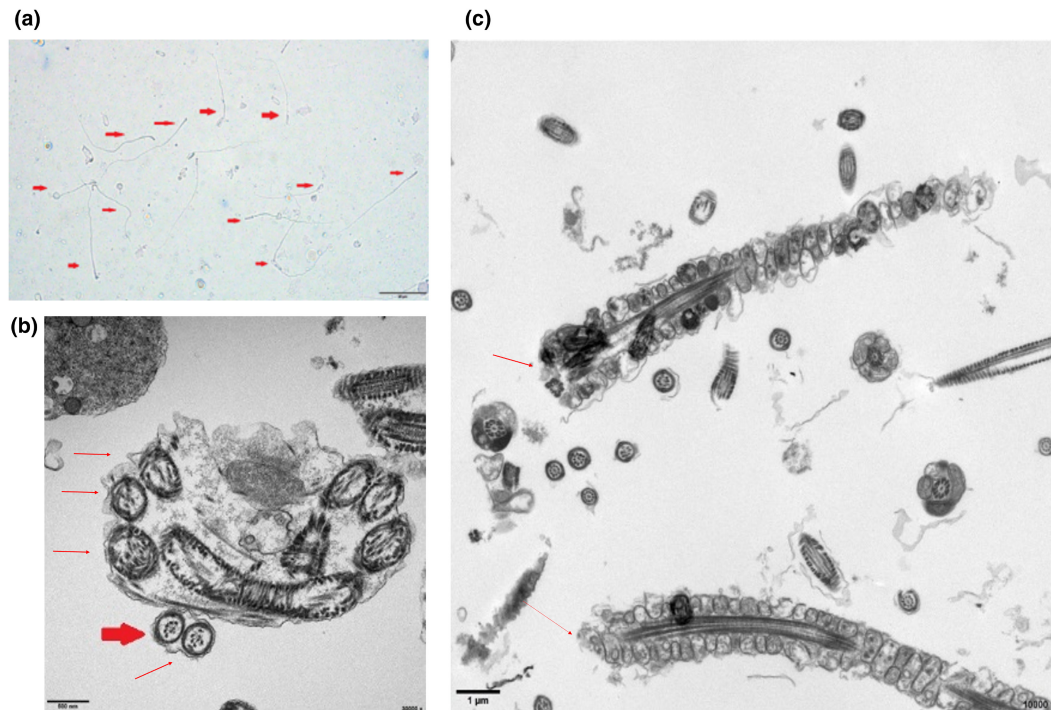


FIGURE 2 Ultrastructural analysis of AS sperm. (a) Light microscopy of stained headless sperm. The red arrows highlight the headless tails of sperm. Scale bar = 20 μ m. (b) Cross section of headless sperm tail. A lack of several peripheral microtubular doublets, connected dynein arms, and radial spokes were frequently observed, marked with a red arrow. Scale bar = 500 nm. (c) The broken point of headless sperm. A sperm head absent from a tail and centrosome can be observed in a headless tail (red arrow). Scale bar = 1 μ m. AS, Acepahlic spermatozoa. Original images.

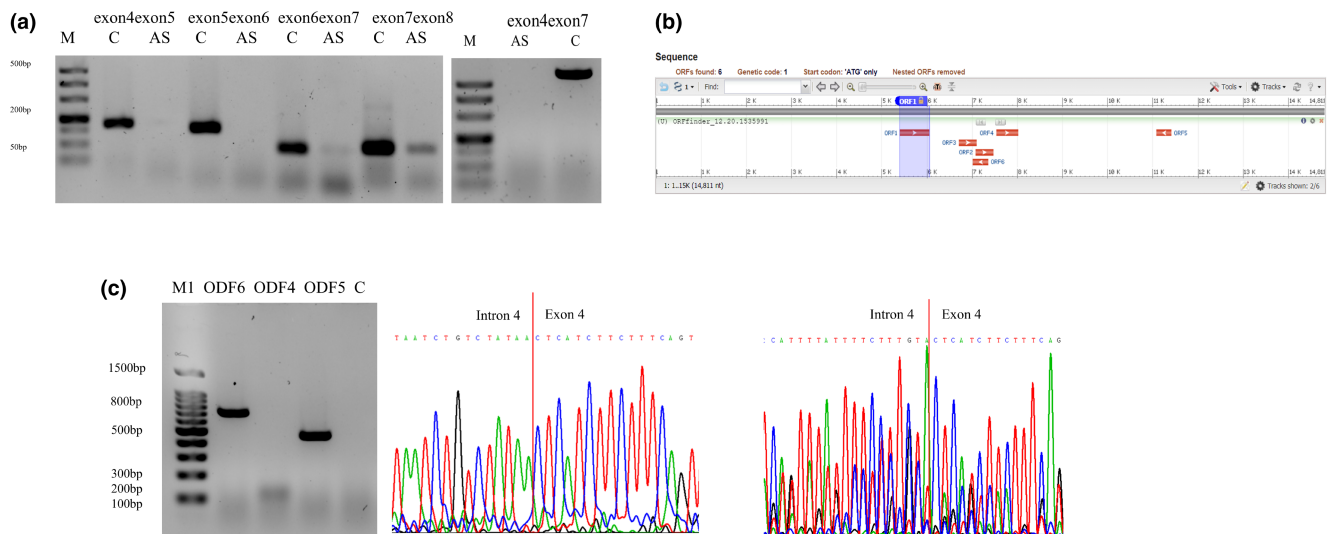


FIGURE 3 cDNA expression analysis. (a) cDNA PCR results confirmed that the exons 5 and 6 were lost due to the splicing variation. (b) New open reading frames (ORF) from intron 4 to intron 6 were predicted through the open reading frame viewer (from NCBI). (c) Using cDNA PCR and cDNA Sanger sequencing to confirm the presence of new ORF and junctions between intron 4 and exon 4. cDNA PCR products are shown for the proband (lane AS), a fertile man (lane C), and DNA markers (lane M or M1) following agarose gel electrophoresis. The bands from cDNA-PCR results are represented by black shapes. AS, Acepahlic spermatozoa; M&M1, DNA markers; ORF, Open reading frame.

gel results, caused by abnormal splicing mutation c.414+1 G>T of *PMFBP1* mRNA. A very low-level cDNA fragment of the junction between exons 7 and 8 indicates

normal splicing has started since exons 7, suggesting that normal C-terminus of *PMFBP1* protein could be generated. The Open Reading Frame (ORF) Finder from NCBI

can be used to predict possible new ORFs from introns 4 to 6, because the normal or shorter length of cDNA fragments between exons 4 and 7 of AS patient was unable to detect (Figure 3a). Only six ORFs (from ORF1 to ORF6) are predicted when the minimal ORF length is set higher than 300 nt (Figure 3b). cDNA-PCR results confirmed the presence of ORF6 and ORF5, and Sanger sequencing also gave the evidence of the existence of junction between intron 4 and exon 4 (Figure 3c), which indicated that the original GU-AG correct splicing pattern between exon 4 and intron 4 has been disrupted, leading to abnormal splicing events, including introns 4, 5, and 6 in the PMFBP1 mRNA. However, partial N-terminus and SMC domain regions of the protein sequence have been changed. These partial changes might lead to the loss of normal function and changes in normal structure of PMFBP1 protein. In addition, c.361C>T p.Gln121Ter is a nonsense mutation predicted to lead to translation termination, inducing the formation of a truncated PMFBP1 protein. The expression of PMFBP1 protein in sperm cells of the proband (Figure 4a) was drastically decreased by 77% ($p < 0.05$), compared to normal control, suggesting that new ORFs from introns 4 and 6 of the aberrant PMFBP1 mRNA might generate similar sized PMFBP1-related proteins.

This protein molecular weight would be similar to that of the normal PMFBP1 protein, resulting in confusing WB results. The variant, c.414+1 G>T, therefore, can be identified as LOF mutation of *PMFBP1*. So, the nonsense mutation, c.361C>T p.Gln121Ter, and the splice donor mutation, c.414+1 G>T, of the *PMFBP1* gene are pathogenic and have negative impacts on the transcription process from the *PMFBP1* gene to mRNA. The normal PMFBP1 functions of these two predicted proteins might be lost due to protein sequence changes in the SMC and N-terminal regions.

3.4 | Transfection experiment analysis

The decreased expression of outer dense fiber 1 (ODF1; HGNC: 8113) and outer dense fiber 2 (ODF2 HGNC: 8114) proteins was observed in WB experiments (see Figure 4a); however, mutations of them were not detected by WES. That meant that either the ODF1 or ODF2 proteins were normally synthesized within the human body but degradation in the sperm cell, or the mutant *PMFBP1* could regulate the expression levels of them. The extensively characterized HEK-293FT cells can be used to

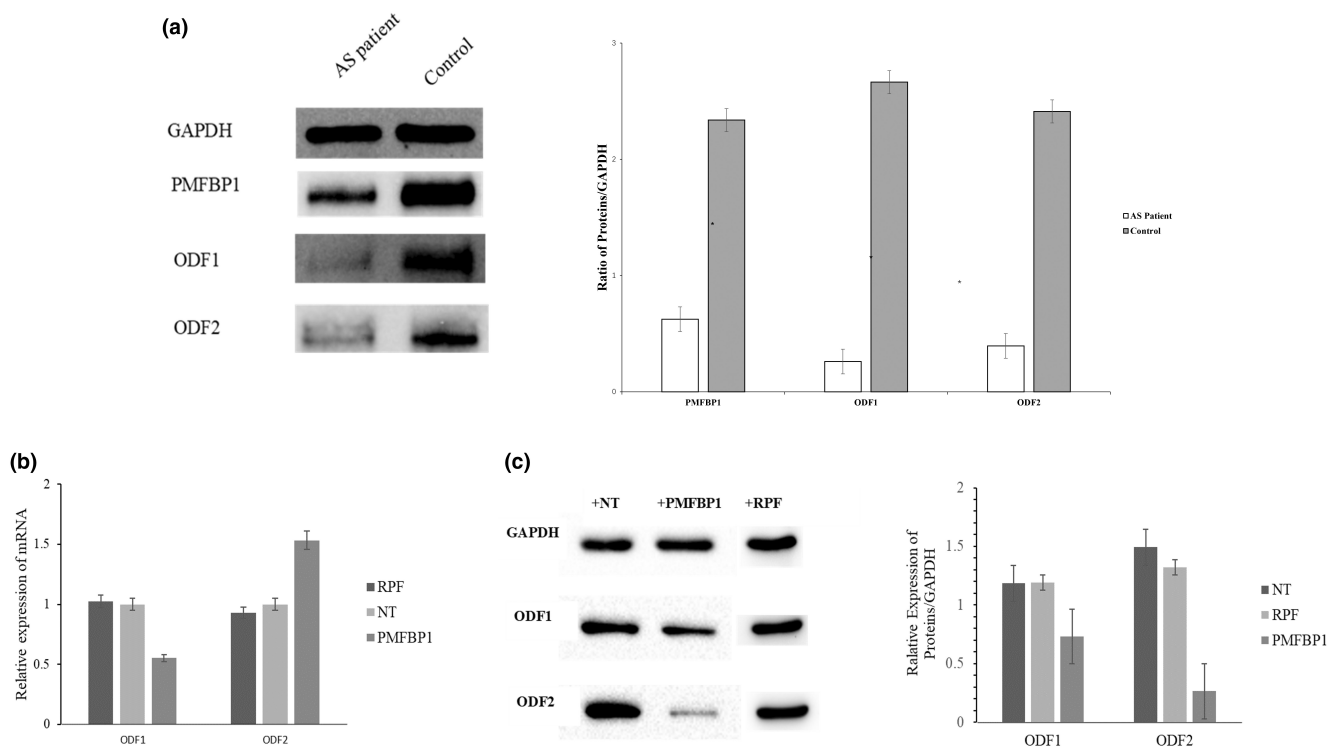


FIGURE 4 The expression of PMFBP1, ODF1, and ODF2 proteins in sperm cells, and in exogenous recombinant protein PMFBP1 transfected 293FT cells. (a) The expression of PMFBP1, ODF1 and ODF2 proteins in sperm cells. (b) The relative ODF1 and ODF2 mRNA expressions were conducted by RT-qPCR in 293FT cells. (c) The relative ODF1 and ODF2 protein expressions were conducted by Western blotting (WB) in 293FT cells. (d): WB results of ODF1 and ODF2 protein expressions. GAPDH was used as a loading control. The order of samples for all gels is specified on the left and/or above the figures. Original image. AS, Acepahlic spermatozoa; ODF, outer dense fibers; PMFBP1, Polyamine modulated factor 1 binding protein 1; NT, non-treated; RPF, random protein fragment; WB, Western blot.

study the mechanism and/or functions of proteins within the reproductive system based on the publications of Yin et al. (2020) and Liu et al. (2010). Consider the instability of abnormal mRNA and degradation rate of the truncated proteins they are produced, an exogenous recombinant PMFBP1 protein was designed to mimic the truncated protein (120aa) from nonsense mutation (c.361C>T p.Gln121Ter), and a random protein (197aa) was used as control. The recombinant PMFBP1 protein transfected into 293FT cell model was created to assess the possible involvement of mutant PMFBP1 in ODF1 and ODF2 proteins' expression regulation. Twenty-four hours post transfections, RT-qPCR results of 293FT cells transfected with PMFBP1 showed a significant increase in *ODF1* mRNA and a significant decrease in *ODF2* mRNA (Figure 4b; $p < 0.05$ for both), revealing that the expression levels of ODF1 and ODF2 are regulated by truncated PMFBP1 protein. In addition, the downregulated ODF1 and ODF2 proteins (Figure 4c,d) in PMFBP1 protein-treated cells were similar to that in the WB results of proband (Figure 4a), confirming the presence of truncated PMFBP1 protein in sperm cells of proband. This means that truncated PMFBP1 might play a key role in the regulation of expression level of ODF1 and ODF2. Interestingly, increased ODF2 mRNA expression and downregulated ODF2 protein might suggest that degraded ODF2 occurred with the presence of truncated PMFBP1 protein. The mechanism of ODF2 protein loss, however, requires further research, due to the disrupted balance between protein synthesis and degradation (Savitski et al., 2018).

3.5 | ICSI outcomes

In order to have children, the patient and his wife underwent one ICSI cycle. Eleven oocytes (diameter > 18mm) were retrieved through hyperstimulation of the ovaries using recombinant follicle-stimulating hormone. Nine oocytes were at the MII stage, one oocyte was at the MI stage, and one was overmature. Five of the MII stage oocytes were used for ICSI. For the ICSI procedure, five oocytes were injected with sperm heads that had a relatively normal morphology or head-tail connected sperm with abnormal morphology; three embryos were then formed. However, syngamy was failed when isolated tail-less sperm heads were used in the ICSI. Two fertilized embryos showed a developmental delay and/or were arrested at the cleavage stage. Only one 3-day-old 8-cell blastocyst was obtained and immediately transferred. However, a clinical pregnancy did not eventuate. Considering the presence of the specific malformation of headless defects that affected the whole sperm population of the proband (patient II:1), normal morphological sperm or head-tail

connected sperm for ICSI could not be found and the couple eventually used artificial insemination with donor sperm.

4 | DISCUSSION

The *PMFBP1* gene is one of the candidate genes for AS disorders which has been confirmed by Zhu et al. (2018); Sha et al. (2019). In the study reported herein, a novel compound heterozygous *PMFBP1* gene has been identified in an AS family. The impacts of a nonsense mutation of c.361C>T p.Gln121 can lead to a dysfunctional truncated protein in the present case; Liu et al. (2020) and Zhu et al. (2018) emphasized that the SMC and C-terminus regions of the PMFBP1 protein are the key elements for the attachment of the basal body to the implantation fossa of sperm. Truncation generally causes complete loss of protein activity (Nie et al., 2020; Wu et al., 2020). In addition, this truncated PMFBP1 protein has been confirmed experimentally that can reduce the expression level of ODF1 and ODF2 proteins. With regard to the splice donor mutation, c.414+1 G>T is a LOF mutation of *PMFBP1*; the aberrant protein sequence of the N-terminus and SMC regions predicted an abnormal secondary structure of the PMFBP1 protein leading to the malfunction of PMFBP1 in spermatogenesis. This led to an AS phenotype. A genetic analysis of the present study, together with those from previous studies, has shown that these two mutations are rare and pathogenic, and that the associated AS defect is a monogenic disorder with a recessive inherited pattern.

The localization of the breakage in the AS patient's sperm, which lacked an implantation fossa and basal body, suggested that the function of the PMFBP1 protein is to attach the sperm tail to the head, ensuring the sperm neck is stable and able to move forward (Nie et al., 2020). An animal study showed that the function was to induce the formation of the implantation fossa and basal body (Zhu et al., 2018), indicating that the PMFBP1 protein is a key element in the formation of the sperm neck. Additionally, the abnormal structure of the sperm tail, including a disorganized mitochondrial sheath, incomplete outer dense fibers, and the lack of several peripheral microtubular doublets, may explain by the reduction of ODF1 and ODF2 proteins. ODF1 and ODF2 proteins are major structural components of sperm tails, as Behnam et al. (2015) emphasized that how ODF1 and ODF2 proteins act as an anchor for axonemal microtubules and the mitochondrial sheath in the sperm tail (Shao et al., 1999). ODF1 is located on the surface of ODFs facing axonemal microtubules, ensuring that the microtubules of the axoneme are in

a highly ordered arrangement (Sha, Xu, et al., 2018a; Shao et al., 1999). The ODF2 protein, in comparison, is present in the cortex facing the mitochondrial sheath, ensuring these are organized in a helix and surrounding the axoneme in the midpiece of sperm (Sha et al., 2019; Shao et al., 1999). Therefore, the downregulated expressions of ODF1 and ODF2 proteins could lead to a disarranged mitochondrial sheath and incomplete ODFs. This might explain why completely immobile sperm has been found in the semen of proband, which is different from other patients.

Normal interactions between SUN5, PMFBP1, and TSGA10 proteins play a key role in the formation of the normal morphology and function of the sperm neck (Liu et al., 2020; Nie et al., 2020; Sha et al., 2019). Thus, a dysfunctional PMFBP1 protein could invalidate normal interaction between SUN5 and TSGA10, disconnect the head and tail of sperm, and lead to AS formation and infertility in men. Zhu et al. (2018), Wu et al. (2020) and Sha, Sha, et al. (2018b) localized SUN5, PMFBP1, and TSGA10 proteins to the sperm neck. These groups predicted that PMFBP1 was a downstream protein of SUN5 and an upstream protein of TSGA10, the relationship of which has not been shown experimentally (Nie et al., 2020; Sha, Sha, et al., 2018b; Zhu et al., 2016). GST pull-down analysis showed that SUN5, PMFBP1, and TSGA10 proteins are not directly bound together (Zhu et al., 2018). Interestingly, SUN5 and TSGA10 proteins are also upstream proteins of ODF1 (Sha, Xu, et al., 2018a) and ODF2 (Sha, Sha, et al., 2018b), respectively. Meanwhile, the N-terminus of PMFBP1 protein could be the key region that interacts with ODF1 and ODF2 protein. Based on this result, therefore, it can be assumed that the functions and interrelationships of SUN5, PMFBP1, and TSGA10 proteins in sperm cells may be through ODF1 and ODF2 proteins, although this requires further research.

Nie et al. (2020) gave an in-depth analysis of the genetic associations of AS defects, of which the ICSI outcomes of AS may be predicted on the basis of subtype. The ICSI procedure, therefore, seems to be the only solution for *PMFBP1* mutated AS defects that at present allows these patients a chance to have offspring (Al Omrani et al., 2018; Moretti et al., 2018; Zhu et al., 2018). All AS candidate genes have been grouped, with the *PMFBP1* gene subdivided into type II, which may contribute to a better clinical outcome (Nie et al., 2020). Several reports have highlighted how a successful clinical pregnancy was achieved using AS sperm; healthy offspring were born in both human and animal studies through an ICSI protocol (Sha, Xu, et al., 2018a; Shang et al., 2018; Zhu et al., 2016, 2018). However, a clinical pregnancy was not always obtained. In the current case, ICSI treatment failed due to

the poor quality of sperm and poor development of the embryo. Nie et al. (2020) and Rawe et al. (2002) explained how the importance of the paternal centrosome in sperm to fertilize the oocyte and help promote the early development of zygotes during an ICSI procedure. Unsuccessful ICSI in the present case may have been caused by dysfunctional centrioles that arrested the development of the embryo or led to the zygote not being formed. Regarding AS defects using an ICSI procedure, a normal head-neck junction sperm is important for a high-quality embryo (Nie et al., 2020). However, the possibility of implantation failure in the patient's wife due to the use of sperm with an abnormal morphology has not been considered during this study.

To conclude, a heterozygote mutation in the *PMFBP1* gene was found to be associated with AS defects in the present case. It was established that the normally expressed PMFBP1 protein is essential for the normal function of expression of ODF1 and ODF2 proteins in sperm. The impacts of mutant *PMFBP1* not only result in an AS disorder, but also decrease the expression of ODF1 and ODF2. Thus, the impacts of a mutant *PMFBP1* gene need to be further explored, and the effects of its final products during spermatogenesis require further research.

ETHICS STATEMENT

The study was reviewed and approved by the Ethics Committee of Guangdong Provincial Fertility Hospital and all patients provided written informed consent.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y. T., X. Z., and W. Q. performed supervision, funding acquisition, and project administration. Y. T. performed

sperm motility, HE and TEM staining, and genotyping. H. N. performed DNA cloning, Western blot, data analysis, designed experiment, and wrote the manuscript. All authors contributed to the article and approved the final manuscript.

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REFERENCES

- Al Omrani, B., Al Eisa, N., Javed, M., Al Ghedan, M., Al Matrafi, H., & Al Sufyan, H. (2018). Associations of sperm DNA fragmentation with lifestyle factors and semen parameters of Saudi men and its impact on ICSI outcome. *Reproductive Biology and Endocrinology*, *16*, 49.
- Behnam, B., Mobahat, M., Fazilaty, H., Wolfe, J., & Omran, H. (2015). TSGA10 is a centrosomal protein, interacts with ODF2 and localizes to basal body. *Journal of Cell Science & Therapy*, *6*(4), 8.
- Chemes, E. H., & Rawe, Y. V. (2003). Sperm pathology: A step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. *Human Reproduction Update*, *9*, 405–428.
- Chemes, H. E. (2018). Phenotypic varieties of sperm pathology: Genetic abnormalities or environmental influences can result in different patterns of abnormal spermatozoa. *Animal Reproduction Science*, *194*, 41–56.
- Chen, H., Zhu, Y., Zhu, Z., Zhi, E., Lu, K., Wang, X., Liu, F., Li, Z., & Xia, W. (2018). Detection of heterozygous mutation in hook microtubule-tethering protein 1 in three patients with decapitated and decaudated spermatozoa syndrome. *Journal of Medical Genetics*, *55*, 150–157.
- Elkhatib, R. A., Paci, M., Longepied, G., Saias-Magnan, J., Courbière, B., Guichaoua, M. R., Lévy, N., Metzler-Guillemain, C., & Mitchell, M. J. (2017). Homozygous deletion of SUN5 in three men with decapitated spermatozoa. *Human Molecular Genetics*, *26*, 3167–3171.
- Li, L., Sha, Y., Wang, X., Li, P., Wang, J., Kee, K., & Wang, B. (2017). Whole-exome sequencing identified a homozygous BRDT mutation in a patient with acephalic spermatozoa. *Oncotarget*, *8*, 19914–19922.
- Liu, G., Wang, N., Zhang, H., Yin, S., Dai, H., Lin, G., & Li, W. (2020). Novel mutations in PMFBP1, TSGA10 and SUN5: Expanding the spectrum of mutations that may cause acephalic spermatozoa. *Clinical Genetics*, *97*, 938–939.
- Liu, L., He, P., Cai, K., Zhang, Y., Li, J., Cao, F., Ding, Z., & Zhang, N. (2010). Lentivirus-mediated expression of MxA in chicken spermatogonial stem cells. *Reproduction in Domestic Animals*, *45*, e131–e137.
- Moretti, E., Gambera, L., Stendardi, A., Belmonte, G., Salvatici, M. C., & Collodel, G. (2018). Characterisation of three systematic sperm tail defects and their influence on ICSI outcome. *Andrologia*, *50*, e13128.
- Nie, H., Tang, Y., & Qin, W. (2020). Beyond Acephalic spermatozoa: The complexity of intracytoplasmic sperm injection outcomes. *BioMed Research International*, *2020*, 1–7.
- Ohuchi, J., Arai, T., Kon, Y., Asano, A., Yamauchi, H., & Watanabe, T. (2001). Characterization of a novel gene, sperm-tail-associated protein (Stap), in mouse post-meiotic testicular germ cells. *Molecular Reproduction and Development*, *59*, 350–358.
- Rawe, Y. V., Terada, Y., Nakamura, S., Chillik, C. F., Olmedo, S. B., & Chemes, H. E. (2002). A pathology of the sperm centriole responsible for defective sperm aster formation, syngamy and cleavage. *Human Reproduction*, *17*, 2344–2349.
- Savitski, M. M., Zinn, N., Faeltsh-Savitski, M., Poeckel, D., Gade, S., Becher, I., Muelbaier, M., Wagner, A. J., Strohmmer, K., Werner, T., Melchert, S., Petretich, M., Rutkowska, A., Vappiani, J., Franken, H., Steidel, M., Sweetman, G. M., Gilan, O., Lam, E. Y. N., ... Bantscheff, M. (2018). Multiplexed proteome dynamics profiling reveals mechanisms controlling protein homeostasis. *Cell*, *173*, 260–274.e225.
- Sha, Y. W., Sha, Y. K., Ji, Z. Y., Mei, L. B., Ding, L., Zhang, Q., Qiu, P. P., Lin, S. B., Wang, X., Li, P., Xu, X., & Li, L. (2018b). TSGA10 is a novel candidate gene associated with acephalic spermatozoa. *Clinical Genetics*, *93*, 776–783.
- Sha, Y. W., Wang, X., Xu, X., Ding, L., Liu, W. S., Li, P., Su, Z. Y., Chen, J., Mei, L. B., Zheng, L. K., Wang, H. L., Kong, S. B., You, M., & Wu, J. F. (2019). Biallelic mutations in PMFBP1 cause acephalic spermatozoa. *Clinical Genetics*, *95*, 277–286.
- Sha, Y. W., Xu, X., Ji, Z. Y., Lin, S. B., Wang, X., Qiu, P. P., Zhou, Y., Mei, L. B., Su, Z. Y., Li, L., & Li, P. (2018a). Genetic contribution of SUN5 mutations to acephalic spermatozoa in Fujian China. *Gene*, *647*, 221–225.
- Shang, Y., Yan, J., Tang, W., Liu, C., Xiao, S., Guo, Y., Yuan, L., Chen, L., Jiang, H., Guo, X., Qiao, J., & Li, W. (2018). Mechanistic insights into acephalic spermatozoa syndrome-associated mutations in the human SUN5 gene. *The Journal of Biological Chemistry*, *293*, 2395–2407.
- Shao, X., Tarnasky, H. A., Lee, J. P., Oko, R., & van der Hoorn, F. A. (1999). Spag4, a novel sperm protein, binds outer dense-fiber protein Odf1 and localizes to microtubules of manchette and axoneme. *Developmental Biology*, *211*, 109–123.
- Shrestha, D., La, X., & Feng, H. L. (2015). Comparison of different stimulation protocols used in in vitro fertilization: A review. *Annals of Translational Medicine*, *3*, 137.
- WHO. (2010). *WHO laboratory manual for the examination and processing of human semen*. Author.
- WHO. (2012). *WHO laboratory manual for the examination and processing of human semen* (Vol. 365, 5th ed.). WHO.
- Wu, B., Gao, H., Liu, C., & Li, W. (2020). The coupling apparatus of the sperm head and taildagger. *Biology of Reproduction*, *102*, 988–998.
- Ye, Y., Wei, X., Sha, Y., Li, N., Yan, X., Cheng, L., Qiao, D., Zhou, W., Wu, R., Liu, Q., & Li, Y. (2020). Loss-of-function mutation in TSGA10 causes acephalic spermatozoa phenotype in human. *Molecular Genetics & Genomic Medicine*, *8*, e1284.
- Yin, Y., Cao, S., Fu, H., Fan, X., Xiong, J., Huang, Q., Liu, Y., Xie, K., Meng, T. G., Liu, Y., Tang, D., Yang, T., Dong, B., Qi, S., Nie, L., Zhang, H., Hu, H., Xu, W., Li, F., ... Li, Z. (2020). A noncanonical role of NOD-like receptor NLRP14 in PGCLC differentiation and spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 22237–22248.
- Yuan, S., Stratton, C. J., Bao, J., Zheng, H., Bhetwal, B. P., Yanagimachi, R., & Yan, W. (2015). Spata6 is required for normal assembly of the sperm connecting piece and tight head-tail conjunction. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, E430–E439.

- Zhang, J., Zhang, X., Zhang, Y., Zeng, W., Zhao, S., & Liu, M. (2019). Normal spermatogenesis in *Fank1* (fibronectin type 3 and ankyrin repeat domains 1) mutant mice. *PeerJ*, 7, e6827.
- Zhu, F., Liu, C., Wang, F., Yang, X., Zhang, J., Wu, H., Zhang, Z., He, X., Zhang, Z., Zhou, P., Wei, Z., Shang, Y., Wang, L., Zhang, R., Ouyang, Y. C., Sun, Q. Y., Cao, Y., & Li, W. (2018). Mutations in *PMFBP1* cause Acephalic spermatozoa syndrome. *American Journal of Human Genetics*, 103, 188–199.
- Zhu, F., Wang, F., Yang, X., Zhang, J., Wu, H., Zhang, Z., Zhang, Z., He, X., Zhou, P., Wei, Z., Gecz, J., & Cao, Y. (2016). Biallelic *SUN5* mutations cause autosomal-recessive Acephalic

spermatozoa syndrome. *American Journal of Human Genetics*, 99, 1405.

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