




A novel homozygous missense mutation of *PMFBP1* causes acephalic spermatozoa syndrome

Mengmeng Lu^{1,2,3,4} · Shuai Kong⁵ · Mingfei Xiang^{1,2,3,4} · Yu Wang^{1,2,3,4} · Jingjing Zhang^{1,2,3,4} · Zongliu Duan^{1,2,3,4} · Xiaomin Zha^{1,2,3,4} · Fengsong Wang⁵ · Yunxia Cao^{1,2,3,4} · Fuxi Zhu^{1,2,3,4} 

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Abstract

Purpose To identify the pathogenic mutation in *PMFBP1* leading to acephalic spermatozoa syndrome.

Methods Sanger sequencing was used to screen for mutations in the known pathogenic genes *SUN5* and *PMFBP1* in a patient with acephalic spermatozoa syndrome. Western blotting and immunofluorescence were used to detect the expression and localization of *PMFBP1* in sperm. At the same time, a *PMFBP1* mutant was constructed, and the expression level of *PMFBP1* protein was further verified by in vitro experiments.

Results We identified a novel homozygous *PMFBP1* missense mutation, c.301A>C (p.T101P), in an infertile male from a consanguineous family. Our results showed that the expression of *PMFBP1* mutant protein was decreased obviously in sperm of the patient.

Conclusion Our results showed that the novel homozygous missense mutation of *PMFBP1* may be a cause of acephalic spermatozoa syndrome, which provided a basis for genetic counseling for the patient.

Keywords Acephalic spermatozoa syndrome · *PMFBP1* · Missense mutation · Sanger sequencing

Introduction

Infertility is a growing reproductive concern worldwide, affecting about 15% of couples. Approximately half of the pathogenic factors are from males, so male infertility is attracting increasing attention [1, 2]. Male infertility is a multifactorial disease with genetic factors accounting for the etiology of about 15% of infertile men [3]. Teratozoospermia, which comes in many forms, is one of the important reasons for male infertility, such as the

rare and severe acephalic spermatozoa syndrome (ASS) [4–10]. Almost no normal sperm are observed during ejaculation in patients with acephalic spermatozoa syndrome, whose sperm morphology in semen includes a large number of headless sperm tails (pinhead sperm), a small number of tailless sperm heads, and sperm with an abnormal head-tail junction. The spermatozoa with abnormal head-tail junctions show a normal-shaped nucleus, an acrosome and midpiece, but the midpiece is not in a linear alignment with the sperm axis [6].

Mengmeng Lu, Shuai Kong, Mingfei Xiang and Yu Wang contributed equally to this work.

✉ Fengsong Wang
fengsongw@ahmu.edu.cn

✉ Yunxia Cao
caoyunxia6@126.com

✉ Fuxi Zhu
fxzhu@ahmu.edu.cn

¹ Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, China

² NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract, Anhui Medical University, Hefei 230032, Anhui, China

³ Key Laboratory of Population Health Across Life Cycle, Ministry of Education of the People's Republic of China, Anhui Medical University, Hefei 230032, Anhui, China

⁴ Anhui Province Key Laboratory of Reproductive Health and Genetics, Anhui Medical University, Hefei 230022, China

⁵ School of Life Science, Anhui Medical University, Hefei 230022, China

Familial aggregation studies suggest that genetic factors play an important role in acephalic spermatozoa syndrome. To date, there have been reported cases of acephalic spermatozoa syndrome related to mutations in *PMFBP1*, *SUN5*, *TSGA10*, *BRDT*, *HOOK1*, and *DNAH6* [11–21]. According to previous studies, mutations in *SUN5* and *PMFBP1* are major causes of ASS in approximately three-quarters of patients [11–15, 18]. However, these mutations do not account for all cases of ASS, which requires more studies to elucidate its pathogenesis. Despite the presence of abnormal spermatozoa, there have been cases of successful progeny from patients with ASS attributed to the use of intracytoplasmic sperm injection [11, 13, 15].

Our previous work identified multiple mutations in *SUN5* and *PMFBP1* as important causes of ASS [11–15, 18]. Hence, in this study, we aimed to screen novel mutations of these genes in a patient with acephalic spermatozoa syndrome from a consanguineous family, where a novel homozygous missense mutation (NM_031293.2, c.301A>C; p.T101P) in *PMFBP1* was identified. Both western blotting and immunofluorescence analysis showed the expression level of *PMFBP1* was consequently decreased. Our findings thus further confirmed that *PMFBP1* may be one of the main pathogenic genes of acephalic spermatozoa syndrome.

Materials and methods

Patient information

A 30-year-old infertile man from family IV-3, whose parents were cousins, was recruited to the Reproductive Center of the First Affiliated Hospital of Anhui Medical University in Aug. 2018. The patient was diagnosed with ASS by performing semen analysis at least twice and Papanicolaou staining. In addition, the patient's bilateral testicular size, spermatic vein, and prostate were examined by color ultrasound.

The study was approved by the Ethics Committee of Anhui Medical University, and written consent was obtained from the participant.

Semen analysis

According to the Guidelines of the World Health Organization, the patient is instructed to undergo a routine semen examination at the same reproductive center at least twice. The following parameters were observed: semen volume, pH, sperm concentration, sperm motility, and proportion of headless sperm. Sperm morphology was evaluated by Papanicolaou staining.

Sanger sequencing of *PMFBP1* and *SUN5*

The blood samples of patients were collected and preserved in anticoagulant tubes containing EDTA and

stored in a $-80\text{ }^{\circ}\text{C}$ refrigerator. Genomic DNA was extracted from the peripheral blood of the proband using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's protocol. The coding regions of *PMFBP1* and *SUN5* genes were amplified as described in previous studies [11, 13]. The PCR products were detected using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Finally, Sanger sequencing was performed to verify whether *PMFBP1* and *SUN5* contained any mutations, which was predicted using *in silico* tools.

Plasmid construction

The full-length cDNA of *PMFBP1* with different restriction sites of *EcoRI* or *BamHI* at both ends (General Biosystems (Anhui) Co., Ltd., Anhui, China) was synthesized by Tongyong Biotechnology Company. The recombinant plasmid was inserted into pCMV-3×FLAG (Sigma) vectors by subcloning. *PMFBP1* with c.301A>C mutation plasmid was constructed using the Mut Express II Fast Mutagenesis kit (Vazyme Biotech Co., Ltd., Nanjing, China).

Transfection

HEK293T cells (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle growth medium (DMEM, Invitrogen) containing 10% (vol/vol) fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 . Subsequently, FLAG-*PMFBP1* (wild-type/WT) and *PMFBP1* mutant (p.T101P) expression plasmids were transfected into the cells respectively. The final concentration of each plasmid after transfection was $0.01\text{ }\mu\text{g}/\mu\text{l}$. After 5 h, the medium was changed with fresh medium containing MG132 (a ubiquitination protease inhibitor), and the cells were harvested after 24 h.

Western blotting

The fresh semen was washed three times with PBS by centrifugation (6000 rpm, 1 min). The supernatant was discarded, and the precipitation was dissolved in 1 mM loading buffer at $100\text{ }^{\circ}\text{C}$ for 10 min. The proteins were separated using SDS-PAGE and were electroblotted onto a nitrocellulose membrane at 100 V for 1.5 h at $4\text{ }^{\circ}\text{C}$. The membrane was then blocked with 5% BSA solution for 1 h at room temperature before it was incubated overnight at $4\text{ }^{\circ}\text{C}$ with primary antibodies (anti-*PMFBP1*, 1:1000 dilution; or anti- α -tubulin, 1:1000 dilution; Sigma). The secondary antibodies (goat anti-

rabbit IgG or goat anti-mouse IgG, 1:5000 dilution; ProteinTech Group, Hubei, China) were used to detect antigen content.

Immunofluorescence analysis

Immunofluorescence analysis of sperm was performed as previously described [10, 12]. HeLa cells were cultured in DMEM containing 10% FBS, and the corresponding plasmids were transfected into HeLa cells on coverslips in 24-well plates with Lipofectamine 2000 (Invitrogen). After 24 h, the transfected cells were fixed with 2% formaldehyde in PBS for 5 min followed by staining the nuclei of HeLa cells with DAPI (4', 6-diamidino-2-phenylindole). The images were collected using a confocal laser scanning microscope.

Results

Clinical findings

The proband presented with primary infertility over 3 years. The patient was diagnosed with ASS based on the routine semen analysis, which showed complete teratozoospermia with severe oligoasthenozoospermia (Table 1). The karyotype was 46; XY and Y chromosome microdeletions analysis showed no obvious abnormalities. Color ultrasound showed normal size of bilateral testis, and no obvious varicose veins and abnormalities in the prostate. Meanwhile, drugs, environment, poisons, diet, and other possible causes leading to infertility were excluded. The patient had an older sister and brother (Fig. 1a, IV-1, IV-2) who got married and had sons, respectively. The pedigree analysis showed an autosomal recessive inheritance pattern (Fig. 1a).

Identification of the *PMFBP1* mutation c.301A>C (p.T101P) (NM_031293.2)

To determine the pathogenic factor of ASS in the patient, peripheral blood DNA was extracted. According to previous reports, *PMFBP1* and *SUN5* are the main pathogenic genes of ASS. Therefore, we performed Sanger sequencing on the

SUN5 and *PMFBP1* coding regions of the proband. No mutation was found in *SUN5*, and a novel homozygous missense mutation in *PMFBP1*, c.301A>C (p.T101P), was identified in the patient (Fig. 1b). Here, we summarized the known data on *PMFBP1* mutations that caused acephalic spermatozoa syndrome (Fig. 1c). Moreover, the amino acid mutation Thr-101/T101 was highly conserved in different species (Fig. 1d).

Effect of the novel *PMFBP1* mutation predicted using in silico tools

SIFT, Polyphen2, and Mutation Taster were used to predict the effect of c.301A>C mutation on the function of *PMFBP1*, which was predicted to be a disease-causing mutation. The mutation was not found in SNPs, genome aggregation database (gnomAD) and exome aggregation consortium database (ExAC).

Expression and localization of *PMFBP1* mutant

To evaluate the impact of missense mutations on *PMFBP1*, *PMFBP1* expression level and localization in sperm of the patient were analyzed by western blot and immunofluorescence. Our results showed that there was a specific 117-kDa band in sperm samples from control and the patient, but the intensity of band in the patient's sperm decreased significantly (Fig. 2a). In vitro experiments also confirmed the effect of this mutation on *PMFBP1* (Fig. 2b and c). The proteasome inhibitor MG132 treatment could restore the expression level of *PMFBP1* mutant (Fig. 2d).

The localization of *PMFBP1* was determined by immunofluorescence (Fig. 3), we used 4', 6-diamidino-2-phenylindole (DAPI), *PMFBP1* mouse antibody, and CD46 antibody to stain control and patient's sperm. The results suggested that nuclear membrane, nucleus, and acrosome were unaffected regardless of mutation (Fig. 3a). In both the two samples, *PMFBP1* was detected at the head-tail junction of spermatozoa. However, in the spermatozoa of patient, the fluorescence intensity of *PMFBP1* was obviously decreased (Fig. 3b). Furthermore, the immunofluorescence analysis was also performed using HeLa cells, and data showed that the p.T101P mutation did not change the localization of *PMFBP1* in the cytoplasm (Fig. 3c).

Table 1 Semen parameters of the patient with ASS

Volume (ml)	pH	Concentration ($\times 10^6$ / ml)	Motility B+C (%)*	Abnormal spermatozoa
3.1	7.4	5.6	8.4	100% Abnormal head-tail junction 0.3% Decaudated 0.7% Acephalic 99%

* Motility A (%)=0

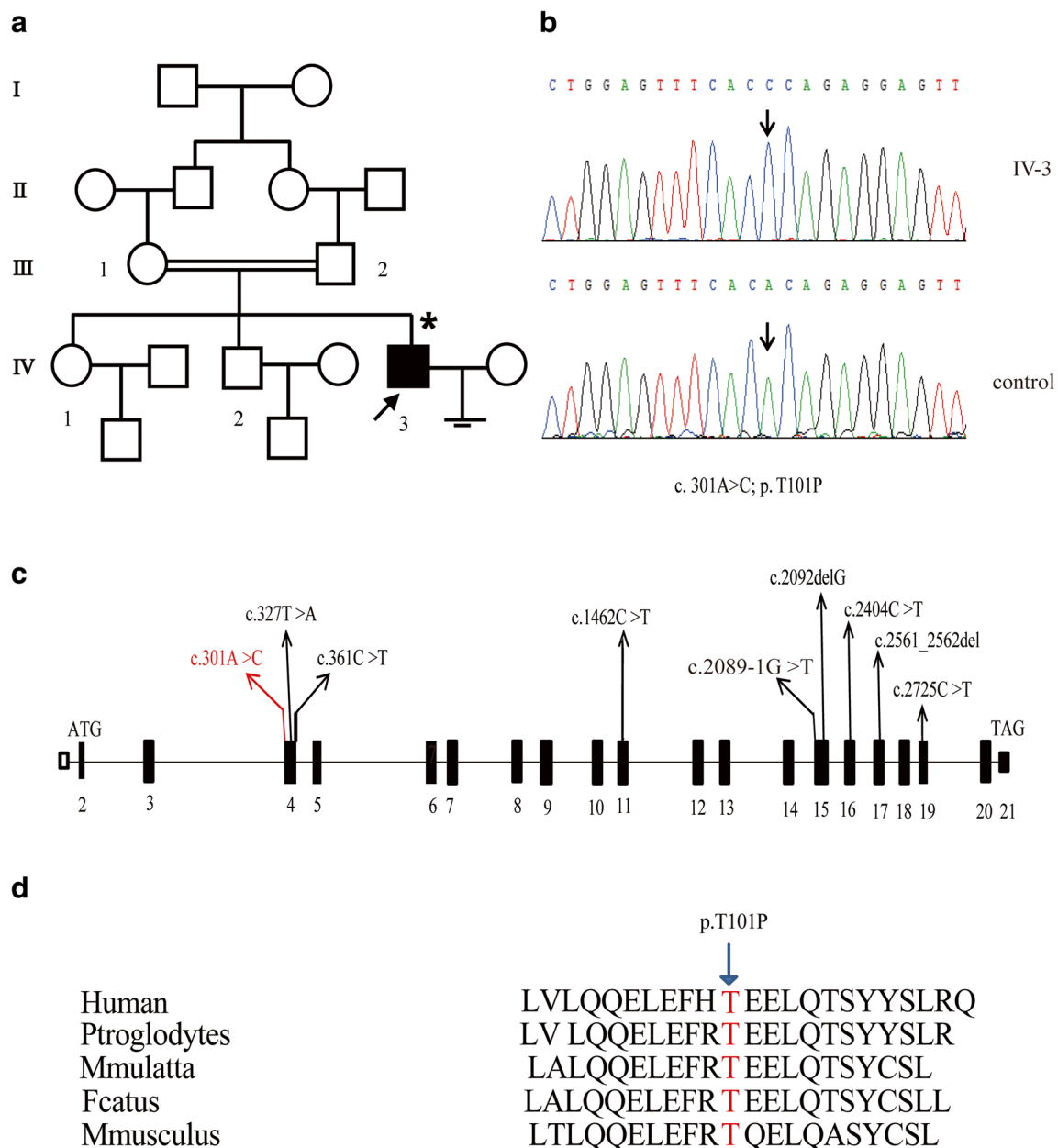


Fig. 1 **a, b** Pedigree of a family with inherited *PMFBP1* mutation. The individual representing a filled black box was Sanger sequenced. The proband (IV-3) has a homozygous mutation in *PMFBP1*. **c** The black boxes represent the coding region and the white box indicates noncoding exon 1, while introns are black bars; the novel missense mutation in this

study and other reported mutations in *PMFBP1* are represented by red wire and black wires, respectively. **d** Cross-species comparisons support that *PMFBP1* is highly conserved and the detected *PMFBP1* mutation in IV-3 is located in a highly conserved region

Discussion

PMFBP1 is a testis-specific protein located at the head-tail junction of sperm, and forms a “sandwich” structure with *SUN5* and *SPATA6* proteins. *SUN5* is a nuclear membrane protein and *SPATA6* is a structural protein, so it is speculated that *PMFBP1* may be a scaffold protein connecting the coupling device and the sperm nuclear membrane [11, 12, 22, 23]. In a study of mutations in *PMFBP1* leading to acephalic spermatozoa, an

independent team found that *SUN5* and *TSGA10* were upstream and downstream of *PMFBP1* respectively. According to previous studies, there is no significant difference in semen parameters when compared to those of patients with acephalic spermatozoa caused by *PMFBP1*, *SUN5*, *BRDT*, and *TSGA10* [11–19]. However, another team reported that two patients with ASS, caused by mutations in *PMFBP1*, not only had sperm with rounded or amorphous heads upon ejaculation but also had lower sperm concentrations, indicating

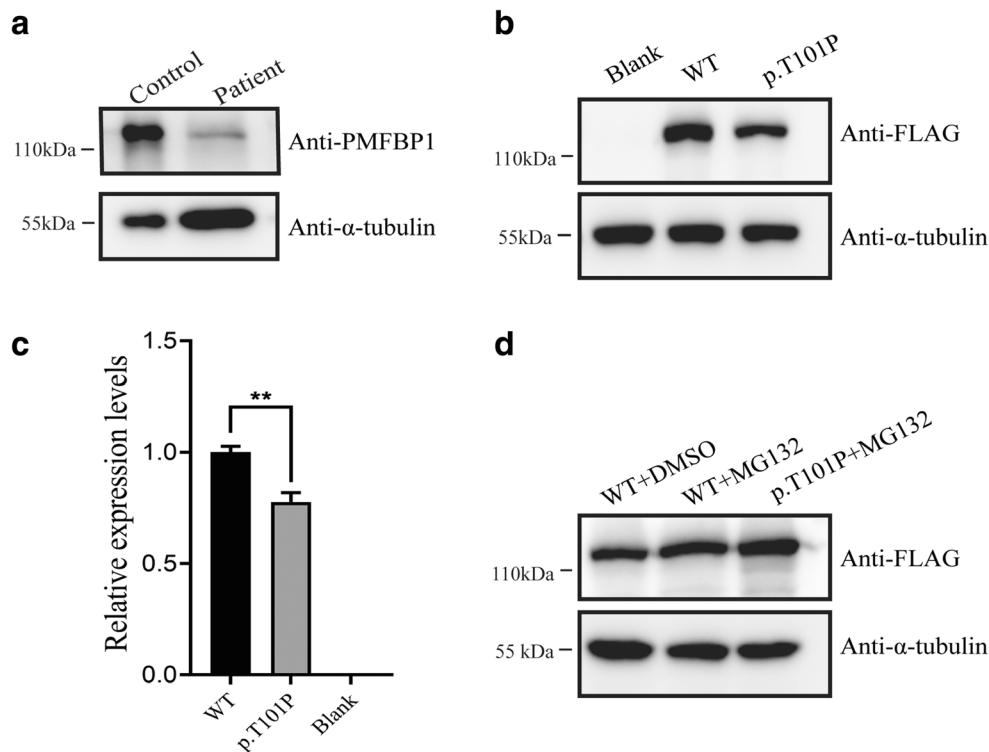


Fig. 2 Protein expression of PMFBP1 in sperm and HEK293T cells. **a** Western blotting was performed to test the expression level of PMFBP1 in the normal and patient sperm. **b** The influence of the c.301A>C mutation on *PMFBP1* protein expression levels in HEK293T cells transfected with wild-type and mutant plasmids of *PMFBP1* was tested with Western blotting. **c** The relative expression levels of wild-type and

mutant-PMFBP1 (p.T101P) proteins in **b** represented by bar charts. Information is indicated as the mean and SEM. $**P < 0.05$. **d** MG132 was added to the culture medium for 12 h before the cells were collected. The expression levels of wide-type and mutant-PMFBP1 (p.T101P) were measured by western blotting. DMSO treatment alone was used as the control

that the phenotype of acephalic spermatozoa caused by *PMFBP1* mutation is more severe [12].

It has been established that *PMFBP1* and *SUN5* mutations are mainly responsible for ASS. Previous reports on *PMFBP1* include three compound heterozygous mutations, three homozygous nonsense mutations, and one homozygous frameshift mutation [11, 12, 18]. Because ASS is severe and rare, there are no clear epidemiological statistics on the incidence of this disease. The identification of these mutations in humans represents a major step forward in the study of the disease. Even so, some cases cannot be explained by known mutations. Therefore, more studies are needed to elucidate the pathogenesis of ASS.

In our study, we identified a novel homozygous missense mutation c.301A>C (p.T101P) in exon 4 of *PMFBP1* in a patient with ASS. In the present study, the mutant PMFBP1 could be detected in the patient's sperm but the expression level drops significantly, which was consistent with the in vitro experimental data. To further verify whether the mutant PMFBP1 was degraded by the ubiquitination pathway, the cells transfected with *PMFBP1* mutant plasmids were treated or not with MG132. Western blotting analysis showed

that MG132 could improve the expression level of *PMFBP1* mutant, which suggested that MG132 may block the degradation of the mutant *PMFBP1*. Furthermore, our hypothesis verified that the degradation of the mutant *PMFBP1* was mediated by the ubiquitination pathway [24–26]. In this study, *PMFBP1* was expressed in the sperm of the patient, but the expression level of the mutant protein was significantly lower than that of the normal control. The mutation did not change the location of *PMFBP1*, but the immunofluorescence signal was attenuated. These results also verified that the novel missense mutation of *PMFBP1* identified in this study may be a pathogenic mutation leading to ASS.

In conclusion, we identified a novel homozygous missense *PMFBP1* mutation that could cause ASS. Altogether, these results suggested that *PMFBP1* mutations appeared to be a “hotspot” of causative factors underlying patients suffering from ASS. Therefore, identification and characterization of more novel mutations of *PMFBP1* would undoubtedly provide more approaches for diagnosis, genetic counseling, and selection of assisted reproductive treatment for patients with the disease.

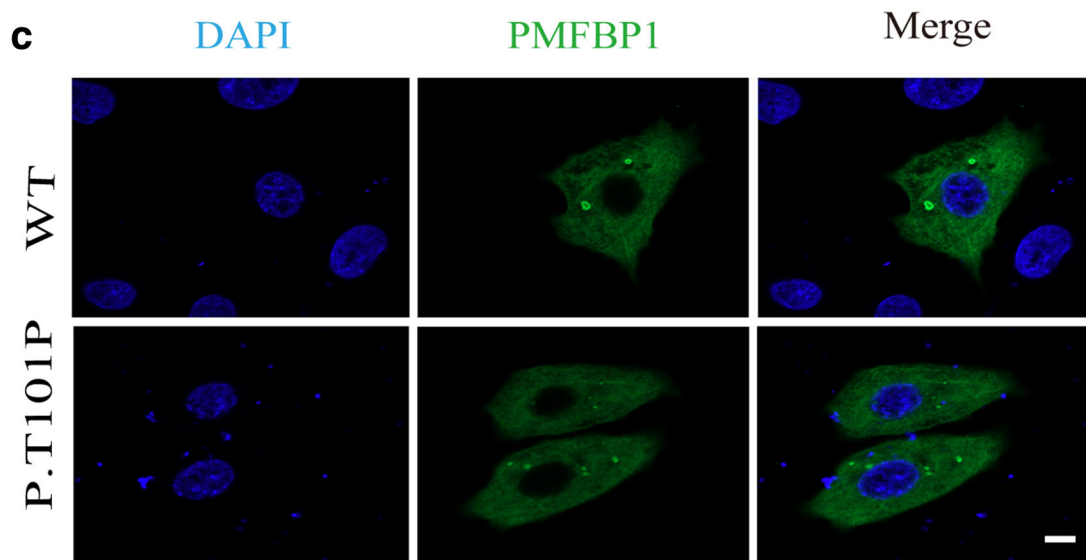
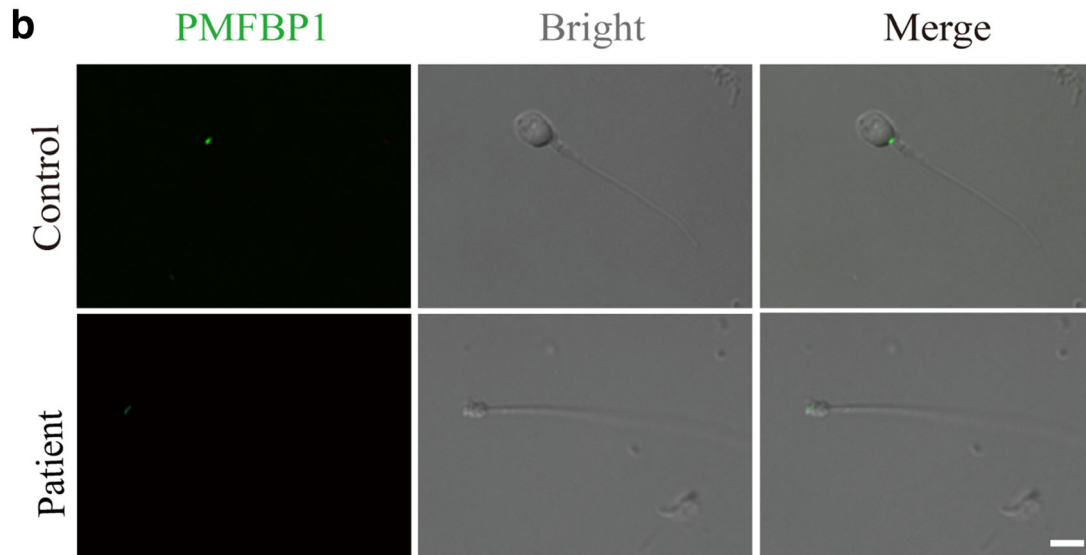
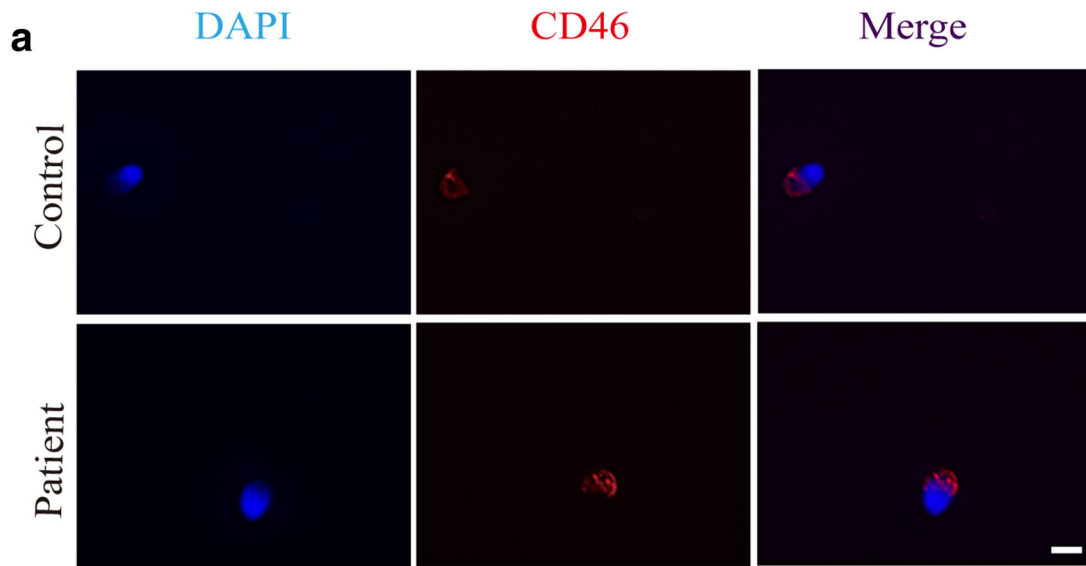


Fig. 3 Immunofluorescence analysis and acrosomal staining in sperm of patient and control. **a** The acrosome and nucleus of the sperm are stained with CD46 antibody (red) and DAPI (blue), respectively. **b** The localization and expression level of PMFBP1 in sperm from the patient and control were demonstrated by immunofluorescence staining. **c** Representative images of HeLa cells transfected with FLAG-*PMFBP1* and mutant plasmids and stained with anti-FLAG antibody. Bar: 10 μ m

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Compliance with ethical standards

This study was approved by the ethics committee of Anhui Medical University. Written informed consent was obtained from each participant.

Competing interests The authors declare that they have no conflict of interest.

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