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GENETICS

A novel variant in *CFAP69* causes asthenoteratozoospermia with treatable ART outcomes and a literature review

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Abstract

Purpose Multiple morphological abnormalities of the sperm flagella (MMAF) are a severe form of sperm defect causing male infertility. Previous studies identified the variants in the *CFAP69* gene as a MMAF-associated factor, but few cases have been reported. This study was performed to identify additional variants in *CFAP69* and describe the semen characteristics and outcomes of assisted reproductive technology (ART) in *CFAP69*-affected couples.

Methods Genetic testing with next-generation sequencing (NGS) panel of 22 MMAF-associated genes and Sanger sequencing was performed in a cohort of 35 infertile males with MMAF to identify pathogenic variants. Morphological, ultrastructural, and immunostaining analyses were performed to investigate the characteristics of probands' spermatozoa. ART with intracytoplasmic sperm injection (ICSI) was carried out for the affected couples to get their own progenies.

Results We identified a novel frameshift variant in *CFAP69* (c.2061dup, p. Pro688Thrfs*5) from a MMAF-affected infertile male with low sperm motility and malformed morphology of sperm. Furthermore, transmission electron microscopy and immunofluorescence staining revealed that the variant induced the aberrant ultrastructure and reduction of CFAP69 expression in the proband's spermatozoa. Moreover, the partner of the proband birthed a healthy girl through ICSI.

Conclusions This study expanded the variant spectrum of *CFAP69* and described the good outcome of ART treatment with ICSI, which is beneficial to the molecular diagnosis, genetic counseling, and treatment of infertile males with MMAF in the future.

Keywords *CFAP69* · Asthenoteratozoospermia · MMAF · Assisted reproductive technology

Introduction

Infertility is a reproductive disorder caused by a variety of factors and has become a global medical and social problem that affects about 10–15% of couples of childbearing age in the world [1]. Male factors in infertility are mainly reflected as the low sperm count, absence of spermatozoa, reduced sperm motility, and abnormal sperm morphology, which account for approximately 50% of infertile couples [2]. Based on the results of routine semen analysis, infertile males are diagnosed as having azoospermia, oligospermia, asthenospermia, and teratospermia, or a combination of these factors, such as oligoasthenospermia, asthenoteratozoospermia, and oligoasthenoteratozoospermia [3, 4]. Interestingly, multiple morphological abnormalities of the sperm flagella (MMAF) is an idiopathic asthenoteratozoospermia with severely impaired motility and morphology, characterized by compound abnormalities of the sperm flagella, including absent, short, coiled, bent, and irregular caliber [5, 6].

Genetic variants are the main causative factors of MMAF, and the currently identified more than 20 genes are responsible for approximately 60–75% of MMAF-affected

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cases [6–8]. Among them, the cilia- and flagella-associated gene *CFAP69* (alternatively termed *C7ORF63*, MIM 617949) is mapped to chromosome 7q21.13 and contains 23 exons, while the encoded cilia- and flagella-associated protein 69 (CFAP69) contains 941 amino acids [9]. CFAP69 is an evolutionarily conserved protein that regulates olfactory transduction kinetics and is also required for flagella assembly and stability in sperm cells [10]. The experimental evidence from *C. reinhardtii* suggested that FAP69 (the orthologue of CFAP69 in *C. reinhardtii*) is involved in the C1b projection of central pair microtubules in flagella [11, 12]. Dong et al. reported that CFAP69 is highly expressed in the human testis and mainly localized to the midpiece of the human sperm flagellum [13].

Animal model studies in mice demonstrated that the male *Cfap69*-knockout mice were sterile and showed profound flagellum morphology defects [13]. Biallelic variants of *CFAP69* have been related to approximately 2.5 to 5.7% of studied human MMAF cohorts [13, 14]. However, only four variants in *CFAP69* were identified in four MMAF subjects, and no following outcomes of assisted reproductive technology (ART) with intracytoplasmic sperm injection (ICSI) were described [13, 14]. Furthermore, it should be noted that the genotype–phenotype correlation in the absence of clinical cases is difficult to characterize. Whether there are other variants in *CFAP69* related to MMAF and the ICSI outcomes of *CFAP69*-affected patients needs to be further explored in larger studies.

In this study, we recruited 35 infertile Chinese males suffering from MMAF at the Center for Reproductive Medicine, Women and Children's Hospital of Chongqing Medical University (Chongqing, China). Genetic testing with next-generation sequencing (NGS) panel of 22 MMAF-associated genes found a homozygous loss-of-function variant in *CFAP69* in a non-consanguineous family. The semen characteristics of *CFAP69*-affected proband, including sperm concentration, sperm morphology, and sperm motility, were described and compared to the previously reported cases. Moreover, the *CFAP69*-affected couples have got a healthy girl following ART with ICSI. These findings extend the variant spectrum of *CFAP69* and can hopefully promote the genetic counseling of infertile males with MMAF in the future.

Materials and methods

Subjects and ethical approval

Thirty-five Chinese males who were diagnosed with primary infertility with MMAF were recruited from the Center for Reproductive Medicine, Women and Children's Hospital of Chongqing Medical University (Chongqing, China) from August 2019 to October 2022. All participants were healthy, with no previous history of exposure to any toxic substances, testicular injury, or obstruction. The chromosomal karyotypes were normal (46, XY), and no deletion was found in the Y chromosome. Written informed consent was obtained from every participant before the collection of peripheral blood and semen samples. This study was approved by the Clinical Application and Ethics Committee of Human Assisted Reproductive Technology of the Chongqing Health Center for Women and Children (2022-RGI-05).

Next-generation sequencing (NGS), sanger sequencing, and variant analysis

As previously reported [15], the genome DNA was extracted from peripheral blood samples with a QIAamp® DNA Blood Midi Kit (69504, QIAGEN, Germany), and subsequently, the NGS of 22 MMAF-associated genes was performed according to the flowchart of the variant-filtration pipeline as shown in Fig. S1. All the identified variants in the *CFAP69*-affected male were listed in Table S1. The identified variant in *CFAP69* was validated through Sanger sequencing with the following primers: forward-5′-CCACAGAGTGGGGAAGAAAT-3′ and reverse-5′-AGCAAAACAGACTCTTGCAAAT-3′. The PCR products were sequenced with an ABI 3500 (Thermo Fisher, USA) and analyzed with Chromas 2.6.5 (Technelysium Pvt. Ltd., USA). The gnomAD database (http://gnomadsg.org/), Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php), and VarSome (https://varsome.com/) were referred for novelty and frequency analysis [16]. The guidelines of the American College of Medical Genetics and Genomics (ACMG) were used to annotate the pathogenicity of variants [17]. Name Checker (https://mutalyzer.nl/name-checker) was used in normatively naming the mutant CFAP69 protein. Clustal Omega (http://www.clustal.org/omega/) and ESPript 3.0 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) were referred to analyze the amino acid conservation between different species. Illustrator for Biological Sequences (IBS, http://ibs.biocuckoo.org/online.php) was used in modeling the diagram images of the CFAP69 protein.

Semen analysis

The semen analysis was conducted according to the guidelines of the World Health Organization (WHO) published in 2010 and 2021 [18, 19]. Briefly, within 1 h of ejaculation into a sterile container after 2–7 days of abstinence, the semen parameters, including semen volume, sperm concentration, semen pH, normal morphology, and sperm motility, were evaluated via a computer-aided sperm analysis system (Jiangsu Rich Life Science Instrument Co., Ltd., Nanjing, China).

Asthenozoospermia is indicated as the reduced sperm motility (progressive motility < 32%), while teratospermia means abnormal sperm morphology (normal sperm < 4%). The co-existence of these two symptoms was collectively defined as asthenoteratozoospermia. Semen analysis was conducted four times for the *CFAP69*-affected individual in our clinic (Table S2).

Sperm morphology analysis

The modified Papanicolaou staining (Cariad Medical Technology Co., Ltd., Zhuhai, China) was used to assess sperm morphology. Shortly, the sperm were smeared onto a slide, air-dried at room temperature, and then fixed for 3 min with 95% ethanol. Then, the slides were submerged in hematoxylin for 3 min, acidic ethanol for 5 s, and eosin and bright green for 3 min, respectively. Washing was performed between each step, and the prepared slides were placed onto absorbent paper to thoroughly dry. More than 200 stained sperm were assessed for morphology analysis with a light microscope according to the WHO guidelines. The morphology of sperm flagella was divided into normal, absent, short, coiled, bent, and irregular caliber. The observation and evaluation were conducted twice. Reference values (5th centiles and their 95% confidence intervals) according to the WHO (2010) manual criteria and the distribution range of normal morphology of sperm flagella observed in 926 fertile individuals [20]. The statistical approach is according to the previous classification systems [21].

Immunostaining of human sperm

Following smearing onto the slides, the sperm were fixed with 4% paraformaldehyde for 6 min, permeabilized with 0.1% Triton X-100 (X100, Sigma) for 30 min, and then blocked with 3% BSA for 2 h. After that, the slides were incubated with antibodies of CFAP69 (1:100, bs-15278R-A647, Bioss), TUBULIN (1:500, F2168, Sigma), and DNAI1 (1:100, ab171964, Abcam), DNALI1 (1:300, HPA028305, Sigma) overnight at 4 °C. The goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (Alexa Fluor™ 555, 1:500, A-21428, Invitrogen) and goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody (Alexa Fluor™ 488, 1:1000, A-11001, Invitrogen) were incubated for 2 h at room temperature for the secondary amplification. Finally, all slides were incubated with 4′-6-diamidino-2-phenylindole (DAPI, P0131, Beyotime) for 2 h to label the nuclei and observed under a laser scanning confocal microscope (TCS SP8, Leica, Germany).

Electronic microscopy evaluation

Scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM) were used to investigate the human sperm flagellar ultrastructure as previously described [15]. The sperm collected from normal subjects and the *CFAP69*-affected male were fixed with 2.5% phosphate-buffered glutaraldehyde at 4 °C overnight. For SEM, the prepared samples were sputter coated by an ionic sprayer meter (ACE200; Leica, Germany) and imaged by the SEM (Nova NanoSEM 450, FEI, USA) at an accelerating voltage of 5 kV. As for TEM, the samples were dehydrated with graded alcohol and embedded in Epon 812 (SPI, USA). Ultrathin (70 nm) sections were stained with lead citrate and uranyl acetate and then observed with the TEM (TECNAI-10, Philips, Netherlands) at an accelerating voltage of 80 kV.

Ovarian stimulation and assisted reproductive technology

The wife of the *CFAP69*-affected male was 36 years old, with a height of 153 cm, a weight of 44.6 kg, a BMI of 19.05 kg/m^2, and an AMH of 1.2 ng/mL. The ovarian stimulation was conducted based on the ovarian reserve of the female partner, as previously reported [22]. E2 pretreatment (Estradiol Valerate, 2 mg/day, Bayer Vital GmbH) was carried out from the middle luteal phase of the previous menstrual cycle to the second day of the next menstruation. On the third day of menstruation, the hormonal level of the female was FSH 2.89 mIU/mL, LH 1.6 mIU/mL, E2 145 pg/mL, and P 0.3 ng/mL, and two sinus follicles were seen in both ovaries. After 10 days of stimulation with Gonal-F (300 IU/day, Merck Serono S.p.A., Italy), the hormonal level of the female was FSH 19.54 mIU/mL, LH 3.61 mIU/mL, E2 2239 pg/mL, and P 0.8 ng/mL on the trigger day. Ten follicles were punctured from both ovaries, and the retrieved oocytes were fertilized through intracytoplasmic sperm injection (ICSI) and cultured in a time-lapse monitoring system (Embryoscope Plus, Vitrolife, Sweden) to minimize the embryos' exposure to sub-optimal conditions as previously reported [23]. G1 medium (Vitrolife, Sweden) was used from day 1 to day 3 and G2 medium (Vitrolife, Sweden) for blastocyst formation was used from day 3 to day 5. The embryos were evaluated according to the ESHRE consensus [24] and the previously reported criteria [25]. Routine prenatal examination and follow-up were conducted until the birth of the fetus.

Statistical analysis

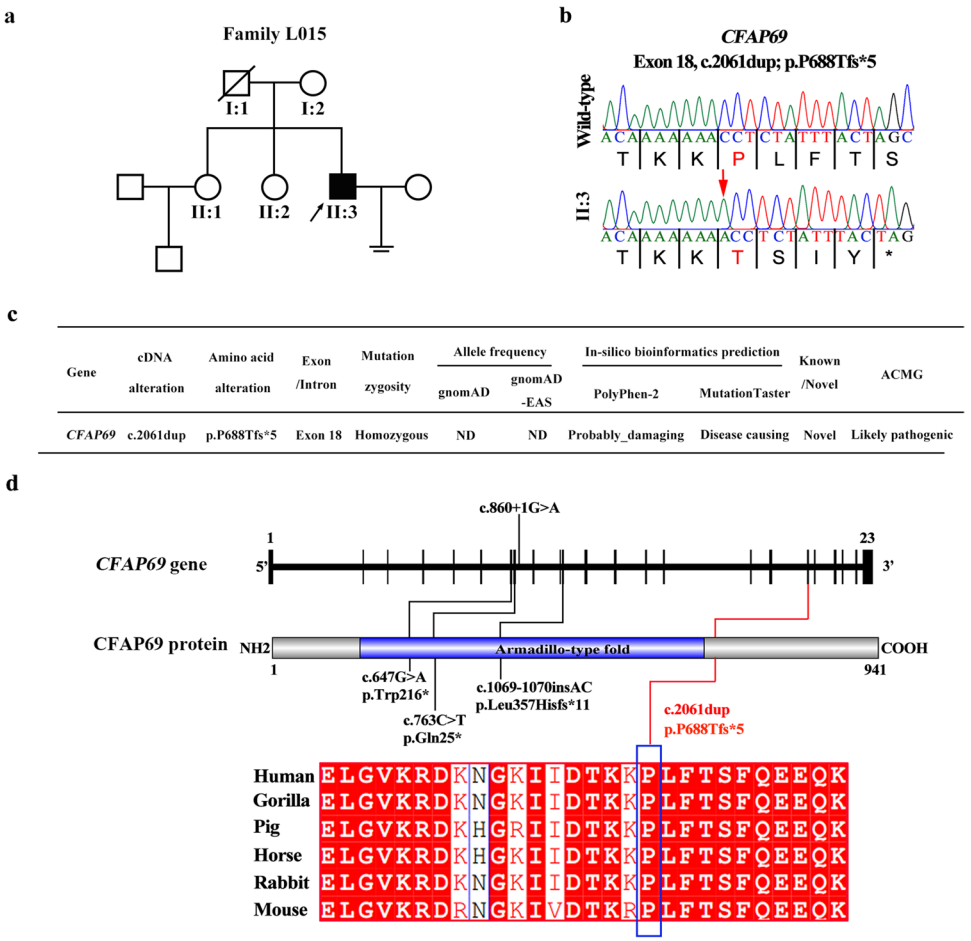
Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, USA). Statistical comparison of mutant individual to the control group was performed via Student's *t*-test; *p* values lower than 0.05 were regarded as significant.

Results

Identification of a loss-of-function variant in *CFAP69* from males with MMAF

Among the cohort of 35 MMAF-affected males analyzed by NGS in this study, a homozygous loss-of-function variant in *CFAP69* was identified in a non-consanguineous family L015 (Fig. 1a). The proband L015-II-3 was 36 years old and suffered from primary infertility for 9 years due to severe asthenospermia (progressive motility < 5%). Pedigree analysis of the family manifested an autosomal recessive inheritance pattern, which is consistent with the previous studies [13, 14]. The variant in *CFAP69* (ENST00000389297, c.2061dup) was located in exon 18 and induced the frameshift and premature termination of the CFAP69 protein (p.Pro688Thrfs*5) (Fig. 1b). This variant was absent from the available human population databases, such as the gnomAD and ExAC, and in silico analysis with PolyPhen-2 and Mutation Taster deduced the deleterious effect of this variant on the function of CFAP69 (Fig. 1c). Moreover, this loss-of-function variant is novel and has not been previously reported in patients with MMAF.

To date, only four variants in *CFAP69* were related to four MMAF patients in the literatures (including c.763C>T, p.Gln255*; c.860+1G>A; c.647G>A, p.Trp216*; and c.1069_1070insAC, p.Leu357Hisfs*11) [13, 14]. We reviewed the previously reported variants and the identified variant in *CFAP69* in this study and analyzed them with a protein diagram (Fig. 1d and Table 1). These five variants are mainly of the loss-of-function subtype, including frameshift (2), nonsense (2), and splice-site loss (1), and are localized along the full length of the CFAP69 protein with



Gene	cDNA alteration	Amino acid alteration	Exon /Intron	Mutation zygosity	Allele frequency		In-silico bioinformatics prediction		Known /Novel	ACMG
					gnomAD	gnomAD -EAS	PolyPhen-2	MutationTaster		
CFAP69	c.2061dup	p.P688Tfs*5	Exon 18	Homozygous	ND	ND	Probably_damaging	Disease causing	Novel	Likely pathogenic

Fig. 1 Identification of a *CFAP69* variant in the man with MMAF. **a** Pedigree of the family affected by the homozygous *CFAP69* variant. The *CFAP69*-affected proband is indicated by a black square and an arrow. The "=" sign indicates infertility. **b** Sanger sequencing of the wild-type and variant allele of *CFAP69* demonstrated that the duplication of adenine at 2061 induced the transition of Pro (CCT) to Thr (ACC). The red arrow shows the variant position. **c** Detailed description of the genetic variant of *CFAP69* identified in the infertile males with MMAF. The transcript used in this study was ENST00000389297. ND—not found. **d** Overview of the *CFAP69* variants. The red font represents the novel variant identified in this study, and the black font represents the previously reported variants. The blue square stands for an armadillo-type fold as predicted by the InterPro server. The altered amino acid (Pro688) is highly conserved among different species

Table 1 Variants of *CFAP69* identified in infertile males with MMAF

	Gene	Transcript	cDNA change	Protein change	Effect	Number of patients	Origin	Allelic status	References
1	*CFAP69*	ENST00000389297	c.763C>T	p.Gln255*	Nonsense	1/78	Iranian	Homozygous	Dong et al. 2018 [13]
2	*CFAP69*	ENST00000389297	c.860+1G>A		Splice-site loss	1/78	Tunisian	Homozygous	Dong et al. 2018 [13]
3	*CFAP69*	ENST00000389297	c.647G>A	p.Trp216*	Nonsense	1/35	Han Chinese	Homozygous	He et al. 2019 [14]
4	*CFAP69*	ENST00000389297	c.1069_1070insAC	p.Leu357Hisfs*11	Frameshift	1/35	Han Chinese	Homozygous	He et al. 2019 [14]
5	*CFAP69*	ENST00000389297	c.2061dup	p.Pro688Thrfs*5	Frameshift	1/35	Han Chinese	Homozygous	This study

Table 2 Semen characteristics and sperm morphology of the *CFAP69*-affected males identified in this study and the previous literatures

Individual	This study		Dong et al. 2018 [13]		He et al. 2019 [14]		Reference Value[#]
	Control	L015 proband	CFAP69_1	CFAP69_2	A001 proband	A006 proband	
Age (years)	29	36	42	51	N/A	N/A	-
cDNA change	-	c.2061dup	c.860+1G>A	c.763C>T	c.1069_1070insAC	c.647G>A	-
Protein change	-	p.Pro688Thrfs*5		p.Gln255*	p.Leu357Hisfs*11	p.Trp216*	-
Allelic status	-	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	-
Semen parameters							
Semen volume (mL)	3.4 ± 0.4	2.5 ± 0.3	5	4	3.0	4.2	1.5 (1.4–1.7)
Sperm concentration (10^6/mL)	27.7 ± 6.7	29.8 ± 7.4	4	6	110.7	24.2	15 (12–16)
Total sperm count (10^6/ejaculate)	95.3 ± 25.7	74.0 ± 24.7	20	24	332.1	101.6	39 (33–46)
Sperm motility (%)	68.3 ± 9.5	5.3 ± 3.8***	1	10	4.5	0.7	40 (38–42)
Progressive motility (%)	63.0 ± 12.8	1.0 ± 1.2***	N/A	N/A	0.8	0.4	32 (31–34)
Immobility (%)	31.7 ± 9.5	94.8 ± 3.8***	99	90	95.5	99.3	-
Normal sperm morphology (%)	61.3 ± 7.1	3.0 ± 1.0**	1	12	3	5.0	4 (3–4)
Morphology of sperm flagella							
Normal flagella (%)	81.0 ± 0.6	30.1 ± 0.8***	18	56	14	2.0	-
Absent flagella (%)	2.9 ± 0.1	10.6 ± 0.2***	1	12	13	10.0	5 (4–6)
Short flagella (%)	1.8 ± 0.2	22.0 ± 0.7***	79	13	28	47.5	1 (0–2)
Coiled flagella (%)	6.4 ± 0.8	20.5 ± 0.5***	1	7	35	32.5	17 (15–19)
Bent flagella (%)	5.1 ± 1.3	6.1 ± 1.1^{ns}	0	5	7.5	5.5	13 (11–15)
Irregular-caliber (%)	2.8 ± 0.2	9.9 ± 0.3***	1	7	2.5	2.5	2 (1–3)
Morphology of sperm head							
Tapered head (%)	2.5 ± 1.8	13.2 ± 0.7***	20	1	18.0	7.5	3 (2–4)
Thin head (%)	3.2 ± 1.2	5.2 ± 0.2^{ns}	14	37	N/A	N/A	14 (12–16)
Abnormal acrosomal region (%)	9.1 ± 1.8	37.5 ± 0.5***	64	70	29.5	28.5	60 (57–63)

For the L015 proband, four independent experiments were performed. Data are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns—not significant; Student's *t*-test. For the previous reported four cases, the data were obtained from the literatures [13, 14]. N/A—not available. [#]Reference values (5th centiles and their 95% confidence intervals) were according to the WHO (2010) manual criteria and the distribution range of normal morphology of sperm flagella observed in 926 fertile individuals [20]. The statistical approach is according to the previous classification systems [21]

highly evolutionary conservation. These findings demonstrated that the loss-of-function variants in *CFAP69* were one of the key genetic factors for MMAF.

Semen analysis of the *CFAP69*-affected male

All the physical characteristics and semen parameters of the *CFAP69*-affected proband are shown in Table 2 and Table S2. According to the confidence intervals published by the World Health Organization in 2010 and 2021 [18, 19], the semen volume, sperm concentration, and total sperm count of the L015-II-3 proband were all normal. However, sperm motility, especially progressive motility, was extremely low (sperm motility: 1–9%; progressive motility: 0–3%). These characteristics are all consistent with the previously reported *CFAP69*-affected probands [13, 14], except for those two subjects described by Dong et al. who had oligozoospermia along with asthenozoospermia [13].

The morphology of the sperm was assessed with Papanicolaou staining and SEM. Sperm morphology analysis with Papanicolaou staining reflected the severe abnormalities of the sperm flagella of the proband, including absent (10.6%), short (21.5%), coiled (20%), bent (5.9%), and irregular caliber (10.1%), that are typical characteristics of MMAF (Fig. 2a, b). The results of SEM also showed similar morphological abnormalities in the flagella of the *CFAP69*-affected individual (Fig. 2c). In addition, a high rate of head malformations, in particular tapered heads and an abnormal acrosomal region, were also observed, which is consistent with another four cases reported (Table 2). The statistical data demonstrated the high heterogeneity in sperm morphology between different probands harboring biallelic variants in *CFAP69* and that the probands had various proportions of spermatozoa with normal flagella (Fig. 2d).

CFAP69 variant is associated with spermatozoa axonemal malformations

To investigate the effect of the *CFAP69* variant on the ultrastructure of sperm flagella, we performed immunostaining with antibodies of CFAP69, TUBULIN, DNAI1, and DNALI1 for the spermatozoa from control subjects and the *CFAP69*-affected male. The results showed that CFAP69 was co-localized along the spermatozoa

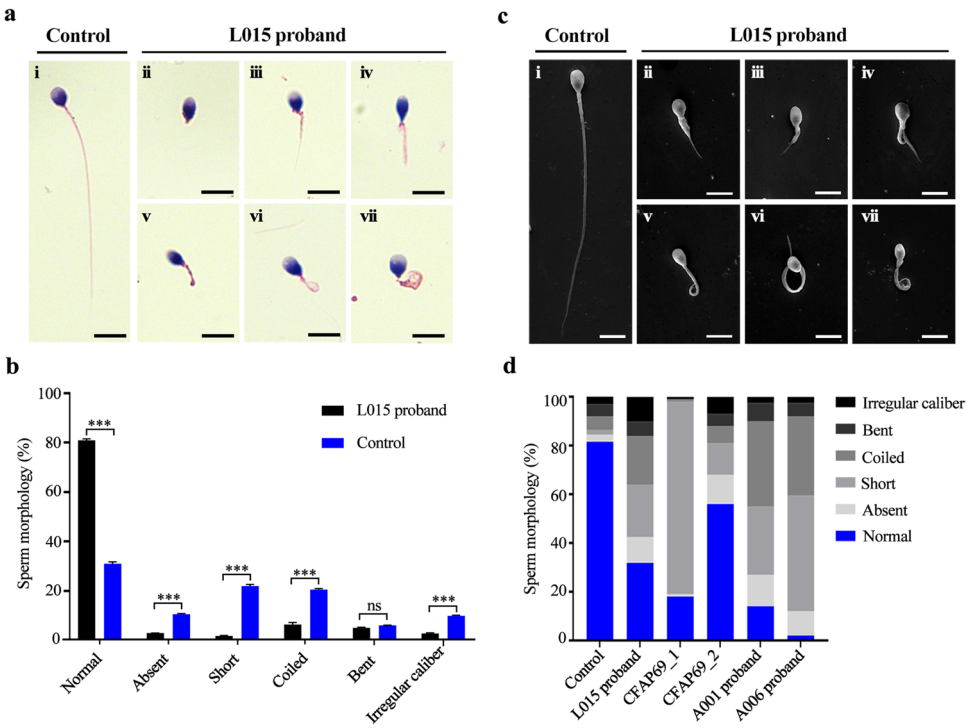


Fig. 2 Morphology of normal and *CFAP69*-affected spermatozoa. **a** The Papanicolaou staining of the normal control and *CFAP69*-affected spermatozoa. (i) Normal morphology of the sperm from a healthy control male. (ii–vii) Most sperm obtained from the L015 proband displayed typical MMAF phenotypes, including absent, short, coiled, bent, and irregular-caliber flagella. Scale bars, 5 μm. **b** Frequencies of flagellar with different morphologies from the fertile control and proband. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ns—not significant; Student's *t*-test. **c** SEM analysis of the sperm obtained from control subjects and the L015 proband. (i) Normal morphology of the sperm from a healthy control male. (ii–vii) Malformed flagella, such as short, absent, bent, coiled, and irregular flagella, were often observed in the L015 proband. Scale bars, 5 μm. **d** Quantification of different categories of flagellar morphologies, including normal, absent, short, coiled, bent, and irregular-caliber, in the five *CFAP69*-affected probands

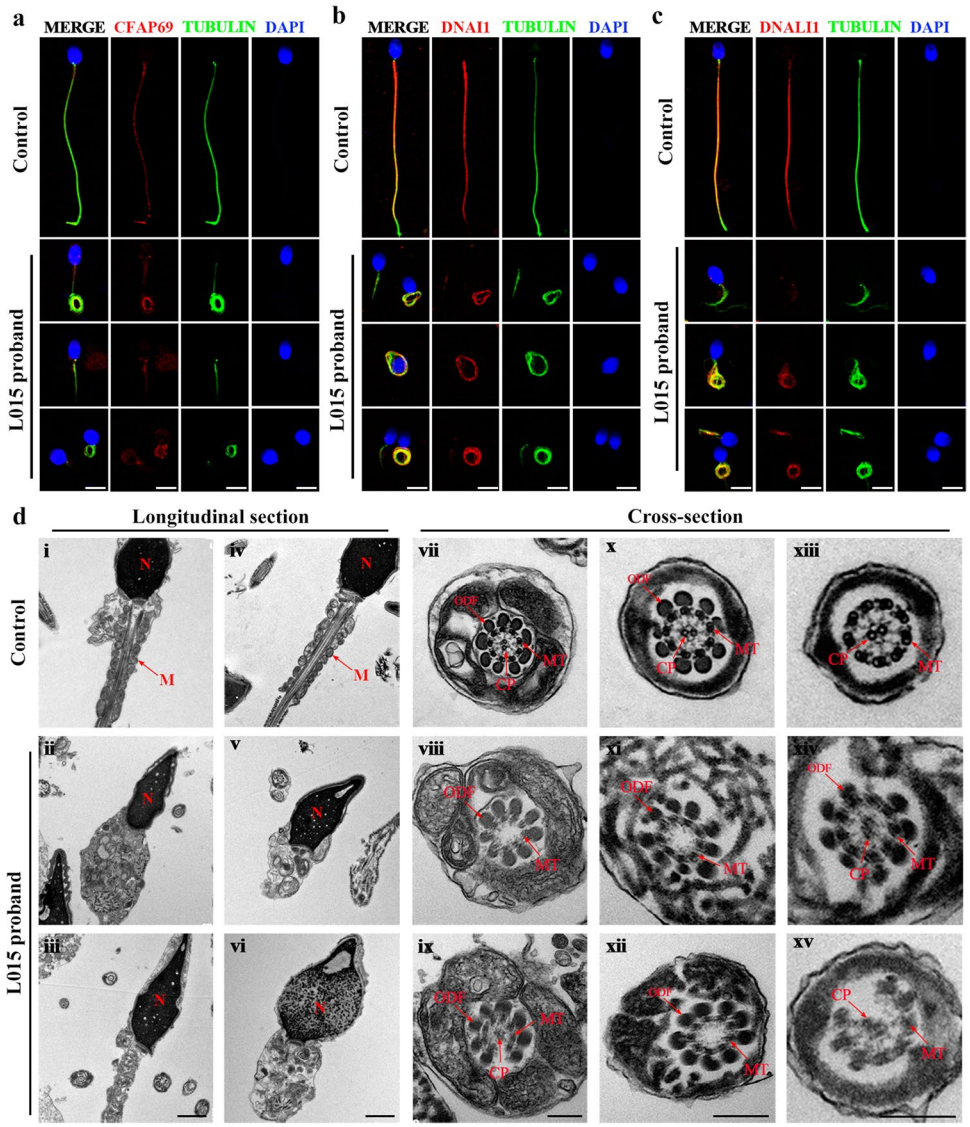


Fig. 3 Localization of CFAP69 and sperm ultrastructure in normal and *CFAP69*-affected spermatozoa. **a–c** Immunostaining in human spermatozoa from control subjects and L015 proband. Sperm cells from a fertile control individual and the L015 proband were stained with anti-CFAP69 (red), anti-DNAI1 (red), anti-DNALI1 (red), and anti-TUBULIN (green) antibodies. The nucleus was counterstained with DAPI. Scale bars, 4 μm. **d** Transmission electron micrographs of testicular sperm from the control subject (i, iv, vii, x, xiii) and L015 proband (ii, iii, v, vi, viii, ix, xi, xii, xiv, xv). (i–vi) Longitudinal sections of the sperm of control subjects and L015 proband. N, nucleus; M, mitochondria. Scale bar, 1 μm. (vii–xv) Cross-sections of the sperm flagella in the control subjects and L015 proband. ODF, outer dense fibers; MT, microtubule doublets; CP, central pair. Scale bars, 200 nm

flagella with microtubule protein TUBULIN, especially in the midpiece in normal samples (Fig. 3a). For the abnormally morphological sperm flagella obtained from the L015 proband, the expression of CFAP69 was downregulated and even absent from the flagella, while the reduced expression of TUBULIN also showed the abnormal morphologies of sperm flagella. DNAI1 and DNALI1, which function as structural components of the outer dynein arm and inner dynein arm in the ciliary axoneme respectively, are localized along the smooth flagella in normal samples (Fig. 3b, c). The CFAP69 deficiency in the L015 proband also reduced the expression of these two pivotal proteins, implying the probable destruction of ultrastructure in sperm flagella.

Herein, TEM was conducted to explore the sperm flagellar ultrastructure in the *CFAP69*-affected male. The normal sperm had a symmetrical midpiece with a smooth axoneme surrounded by regularly arranged mitochondria (Fig. 3d). Structurally, the axoneme usually consists of a "9+2" microtubule structure containing nine peripheral microtubule doublets (MT) paired with nine outer dense fibers (ODF) and the central pair (CP) in the midpiece and principal piece, while the ODF is absent from the endpiece. In contrast, *CFAP69*-affected sperm displayed as a large cytoplasmic bag in the longitudinal section and presented with disorganized axonemal and peri-axonemal components, such as the absence of CP and the disorder of MT and ODF arrangement. These findings demonstrate that CFAP69 plays an important role in the assembly and maintenance of sperm flagella.

Clinical outcome of the *CFAP69*-affected couple

The *CFAP69*-affected couples have accepted ART treatment with ICSI in our clinic (Fig. 4a). Briefly, the female partner of the L015 proband was consecutively treated with an antagonist for ovarian stimulation, and 10 mature oocytes at

a

Cycle No.	No. of oocytes retrieved	No. of MII Oocytes (%)	Insemination method	No. of 2 PN (%)	No. of transferable embryos (%)	No. of Blastocysts (%)	No. of embryos transferred (FET)	Clinical Outcome
1	10	10/10 (100)	ICSI	8/10 (80)	6/8 (75)	3/6 (50)	1	Delivery

b

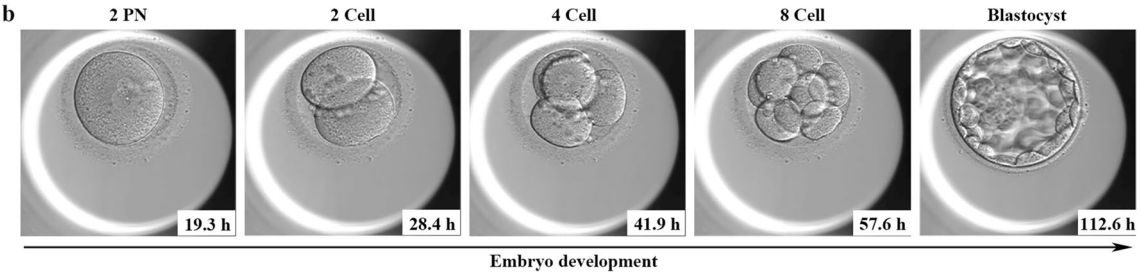


Fig. 4 The outcome of ART with ICSI for the *CFAP69*-affected couple. **a** Statistical data of the oocyte collection, early embryonic development, and the clinical outcome from the *CFAP69*-affected couple. **b** Images of in vitro early embryonic development from the *CFAP69*-affected couple. ICSI, intracytoplasmic sperm injection; MII, metaphase II; PN, pronucleus; FET, frozen-thawed embryo transfer

metaphase II stage were retrieved through the laparoscopic ovarian puncture method. Following ICSI, eight oocytes got fertilized, six zygotes developed into transferable embryos, and three blastocysts formed at day 5 (Fig. 4b). The rate of fertilization was 80%, and the rate of blastocyst formation was 50%, respectively. After the frozen-thawed embryo transfer, the female partner of L015 proband got pregnant and gave birth to a full-term healthy girl.

Discussion

In the present study, we described an additional family with MMAF harboring a biallelic variant in *CFAP69*. The genetic screening with a panel of 22 MMAF-associated genes identified the homozygous state of c.2061dup in *CFAP69* in the L015 proband. Pedigree analysis, Sanger sequencing, and in silico analysis further confirmed the pathogenicity of the variant in a recessive pattern. The results of Papanicolaou staining, SEM, immunofluorescence, and TEM showed abnormalities of sperm flagella and severe disorganization of the axoneme in *CFAP69*-affected male compared to normal control subject. We also found that the patient with the *CFAP69* variant who received ICSI had a good prognosis of fertility.

Over the review, there are some similarities in all five MMAF patients with *CFAP69* variants: (1) patients present typical MMAF manifestation, such as immotility and five major types of flagellar malformation. And according to the statistics of abnormal morphology of sperm flagella in patients, the most common abnormal types were short (21.5%, 79%, 13%, 28%, and 47.5%, respectively) and coiled flagella (20%, 1%, 7%, 35%, and 32.5%, respectively). (2) TEM results showed abnormal spermatozoa with an atypical "9+0" microtubule structure, a normal arrangement of peripheral microtubules, and a lack of the central pair of microtubules. (3) Despite the main defects in sperm flagella, we observed that the L015 proband, like the previously reported cases [13, 14], had obvious head morphological abnormalities. Small acrosome or tapered heads were the main abnormal types. Based on the above results, CFAP69 could also be important for sperm head shaping during spermiogenesis. But how CFAP69 is involved in sperm head shaping and whether CFAP69 is required for a process common to head and flagellum development or in distinct processes is not clear. More patients and animal models with variants in MMAF-associated genes should be investigated for their association with head shaping and flagella development.

Interestingly, the sperm concentrations were discrepant between cases. Dong et al. [13] reported a very low sperm concentration in *CFAP69*-affected males, whereas the sperm concentration is normal in the cases He et al. [14] reported and the L015 proband in our study. It suggested that phenotypic variance was present between cases. It is well known that many factors can affect sperm concentration, such as endocrine factors, environmental factors, and infection. We suspect that these factors may be responsible for the phenomenon. This reminds us that we cannot use oligospermia as one of the reference criteria for *CFAP69*-related MMAF.

In view of the abnormal morphology and low motility of sperm in patients with MMAF, the ICSI technique is now widely used for assisted reproduction for MMAF patients. However, none of the previous literatures reported the reproductive outcomes of MMAF patients with *CFAP69* variants. In our study, the *CFAP69*-affected couple achieved a positive pregnancy outcome following ICSI, which is hopeful to predict the ICSI outcomes for MMAF patients with *CFAP69* variants in the future. This also suggests that

it is worthwhile for researchers and clinicians to apply ICSI for *CFAP69*-affected patients. Notably, the genetic screening of the wives of male patients carrying *CFAP69* variants should also be considered before the couple asks for ICSI to reduce the risk of hereditary diseases in offspring. A limitation of this study is the low number of variants identified in *CFAP69* and the small number of *CFAP69*-mutant cases available. Additional *CFAP69* variants and cases are needed to better characterize the genetic etiology of the MMAF phenotype and improve the management of MMAF patients with *CFAP69* variants.

Conclusion

In summary, our study identified a novel pathogenic variant in *CFAP69* related to MMAF. Our results indicate a good ICSI prognosis for the patient carrying the *CFAP69* variant. This study extended the mutant spectrum of the *CFAP69* gene and could facilitate researchers and clinicians to understand the genetic etiology of MMAF better, thus improving the counseling of infertile males with MMAF in the future.

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Author contribution Tingting Lin, Guoning Huang, and Xiangrong Tang conceived and designed the study and drafted the manuscript. Jing Ma, Shunhua Long, Xiangrong Tang, and Ling Wan carried out the genetic studies and the immunoassays. Haibing Yu provided the clinical samples. Xinglin Wang conducted the ART cycle. Tingting Lin, Guoning Huang, and Jigao Yang critically commented on and edited the manuscript. All authors read and approved the final version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval This study was approved by the Clinical Application and Ethics Committee of Human Assisted Reproductive Technology of Chongqing Health Center for Women and Children (2022-RGI-05).

Consent to participate Obtained.

Competing interests The authors declare no competing interests.

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