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GENETICS

Patient with multiple morphological abnormalities of sperm flagella caused by a novel ARMC2 mutation has a favorable pregnancy outcome from intracytoplasmic sperm injection

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Abstract

Purpose To investigate the potential genetic cause in a primary infertility patient with multiple morphological abnormalities of sperm flagella (MMAF).

Methods The patient's sperm was observed by light and electron microscopy. Whole-exome sequencing (WES) was carried out to identify candidate genes. Then, the mutation found by WES was verified by Sanger sequencing. The proteins interacting with ARMC2 were revealed by co-immunoprecipitation (co-IP) and mass spectrometry. Intracytoplasmic sperm injection (ICSI) was carried out to achieve successful pregnancy.

Results Typical MMAF phenotype (absent, short, coiled, bent irregular flagella) was shown in the patient's sperm. A novel homozygous mutation in *ARMC2* (c.1264C > T) was identified. The proteins interacting with ARMC2 we found were CEP78, PGAM5, RHOA, FXR1, and SKIV2L2. The ICSI therapy was successful, and boy-girl twins were given birth.

Conclusion We found a novel mutation in *ARMC2* which led to MMAF and male infertility. This is the first report of ICSI outcome of patient harboring *ARMC2* mutation. The interacting proteins indicated that ARMC2 might be involved in multiple processes of spermatogenesis.

Keywords Infertility · *ARMC2* · Teratozoospermia · Intracytoplasmic sperm injection

Introduction

Male infertility is a worldwide problem which cannot be ignored. Among the complicated problems, asthenoteratozoospermia is a clinically common condition whose causes vary. The flagella ultrastructure defects are responsible for many asthenoteratozoospermia cases. Multiple morphological abnormalities of sperm flagella (MMAF) is a type of uncommon but severe asthenoteratozoospermia which is characterized by the multiple flagellar malformations: short, absent, coiled, angulation, irregular caliber [1].

MMAF is thought to be caused by genetic defects. So far, lots of genes were reported to be related with MMAF, such as *DNAH1* (MIM: 603332) [2], *CFAP43* (MIM: 617558), *CFAP44* (MIM: 617559) [3], *CFAP65* (MIM: 614270) [4], *CFAP251* (MIM: 618146) [5], *TTC21A* (MIM: 611430) [6], and *TTC29* (MIM: 618735) [7]. *ARMC2* (also termed *SPGF38*, MIM: 618424) is a gene located on chromosome 6 and encodes an 867-amino-acid testis specifically expressing protein. It was first reported related to MMAF in a cohort of 168 infertile men by Coutton C, et al., using whole-exome sequencing [8]. Benefited from the development and wide application of whole-exome sequencing (WES), novel mutations in *ARMC2* have been identified in a series of MMAF cases. However, only about 60% of patients suffering from MMAF are caused by previously identified mutations [9]; the reveal of new mutations is still necessary.

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Here, we used WES and identified a novel nonsense mutation (c.1264C > T) in a patient with MMAF. The patient harboring the *ARMC2* mutation presented a severe impair in sperm motility and flagella morphology. The results of co-immunoprecipitation (co-IP) showed the potential interaction between ARMC2 and various proteins. The ICSI outcome of patients with the *ARMC2* mutations was reported for the first time, and it presented satisfactory. Our finding provided new experiment fundamental and insight to the diagnosis and therapy of the MMAF.

Materials and methods

Study participants

The patient with primary male infertility was recruited from the First Affiliated Hospital of Xinjiang Medical University. The proband was a 28-year-old Han Chinese male who suffered from 5 years of infertility. It is worth noting that according to the proband dictation, his paternal and maternal grandmothers are sisters. The physical examinations and hormone examinations of the patient displayed normal results. No obvious abnormalities were detected in the bilateral spermatic veins upon palpation. This study was approved by the Ethics Committees of the Affiliated Suzhou Hospital of Nanjing Medical University and the First Affiliated Hospital of Xinjiang Medical University. Signed informed consent was provided by the patient and his family.

Semen parameter and sperm morphology analysis

Semen parameter and sperm morphology analysis were carried out according to the WHO laboratory manual for the examination and processing of human semen (5th edition). The Papanicolaou stained sperm slides were photographed by a Nikon Eclipse CI microscope (Nikon, Japan) for the sperm morphological images.

Electron microscopy evaluation

The seminal plasma was removed after centrifugation for 400 g × 15 min while the sperm cells were rinsed and fixed routinely. Samples for scanning electron microscopy (SEM) were sputter coated by an ionic sprayer meter (ACE200; Leica, Germany) and analyzed by SEM (Nova NanoSEM 450, FEI, USA) with an accelerating voltage of 5 kV. For transmission electron microscopy (TEM), the specimens were embedded in Epon 812 (SPI, USA); ultrathin sections were stained with uranyl acetate and lead citrate and observed and photographed by TEM (TECNAI-10, Philips, Netherlands) with an accelerating voltage of 80 kV.

Whole-exome sequencing, Sanger sequencing validation, and data processing

Genomic DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Germany). A minimum of 3 μg DNA of the patient was used to create the DNA libraries enriched by xGen Exome research panel v1.0 (Integrated DNA Technologies, Coralville, IA, USA). After bioinformatic analysis, we filtrated and analyzed the data. Taking into account the phenotypes and modes of inheritance, one homozygous mutation in *ARMC2* came into sight. Then, a direct Sanger sequencing was conducted to validate putative mutations. The sequence was amplified by polymerase chain reaction (PCR) with the primers of *ARMC2*. The sequences of the primers are listed in Table 1. PCR products were verified by agarose gel electrophoresis and subsequently sequenced by an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Expression plasmid construction

ARMC2 cDNA was amplified from the plasmid expressing *ARMC2* (Sino Biological, China, HG25854-UT) and inserted into pcDNA 3.1/V5-His plasmid (Thermo Fisher, K4800-01), which encodes a C-terminal V5 tag. PCR-based site-directed mutagenesis was used to make construct expressing *ARMC2* mutant R422X using ClonExpress One Step Cloning kit (Vazyme, China, C112). Both plasmids were verified by DNA sequencing.

Cell culture and transfection

HEK293 cells (ATCC, STR profiling) were culture in Dulbecco's modified Eagle's medium (HyClone, SH30243.01) with 10% fetal bovine serum (FBS) (Gibco, 10099–141) at 37 °C with 5% CO_2. Plasmids were transfected into the cells at ~80% confluence using ExFect Transfection Reagent (Vazyme,

Table 1 Sperm parameters of the proband with several semen analyses

Primer	Sequence		Tm
ARMC2-F	5′-CAGGATTTAGTCGTC CGTGTTG -3′		58 °C
ARMC2-R	5′-GGAAAGTTGGTTGTG TCTGCTG -3′		
Program	Cycles	Target (°C)	Hold (hh:mm:ss)
Initial-denature	1	95	3 mm
Denature	35	95	15 ss
Annealing		55	15 ss
Extension		72	30 ss
Final extension	1	72	5 mm

T101) based on the manufacturer's instructions. After 6 h of incubation, the cells were switched to fresh medium and incubated for 24 h before being used for Western blotting and proteomic analysis.

Western blotting

After 24 h of incubation, the cells were lysed in 25 mM Tris–HCl (PH 7.4), 150 mM NaCl, 1% NP-40, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF, NCM Biotech). Cell lysates were mixed with 5 × loading buffer, and analyzed by SDS-PAGE and Western blotting using a horseradish peroxidase (HRP)–conjugated anti-V5 antibody (1:5000, Invitrogen, R96125). After incubation with a solution with an enhanced chemiluminescent substrate (ECL) (Vazyme, E411-04), Western blots were exposed to a chemiluminescent imager (Tannon).

co-IP and mass spectrometry

The extracted proteins from WT and mutant *ARMC2* plasmids were incubated with 3 μg of target antibodies at 4 °C overnight, followed by adding 50 μL of protein A/G magnetic beads (LSKMAGAG10, Millipore) to each sample and incubating for 1 h at room temperature. The beads were washed with PBS for three times. Then, the mass spectrometry (MS) analyses were carried out. The peptides were resuspended in 0.1%formic acid and analyzed using a LTQ Orbitrap Velos mass spectrometer (Thermo Finnigan) as described before [10]. The minimum peptide length required was six. The mass tolerance for MS/MS fragment ions was set to 0.5 Da. Statistical significance was investigated by the unpaired two-tailed Student *t* test. Proteins with a *P* value less than 0.05 and a fold change greater than 1.5 were considered as differentially expressed between each group.

ICSI procedure

The patient's wife underwent a long protocol to induce ovulation. The total Gn dose was 3000 IU. A total of 0.2 mg of triptorelin acetate (Ferring Pharmaceuticals) was injected while the diameter of dominant follicle reached 18 mm, and 35 h later, the oocytes were retrieved. The obtained cumulus oocyte complex was washed and placed in a protein-containing fertilization fluid (Vitrolife, Sweden). After 5 h, the patient's sperms were injected into the prepared oocytes.

Results

The patient's sperm showed a typical MMAF phenotype

In the 4 times of routine clinical semen tests, the patient's sperms all showed low concentration with severe low motility, while the semen volumes were normal (Table 2). Under light microscopy, by contrast to the normal control, the sperms of the patient showed a typical MMAF phenotype. The sperm flagella demonstrated a variety of morphological abnormalities (short, coiled, bent, etc.), but it was worth noting that there were still few sperms with normal flagella in the man harboring *ARMC2* mutation. The results of SEM were consistent with the light microscopy. The sperm head, compared with flagella, showed much more normal. TEM was used to investigate the ultrastructure of sperm flagella and the central microtubule pairs were found absent in the sperm of the patient, and corresponding to the previous results, normal ultrastructure flagella also existed in the patient's sperms (Fig. 1).

Novel mutations in *ARMC2*

The mutation identified in the patient was a nonsense mutation c.1264C > T (p.R422X) located in exon 13. Meanwhile, we examined other MMAF-related genes (*DNAH1*, *CFAP43*, *CFAP44*, *CFAP69*, *FSIP2*, *TTC21A*, *TTC29*, and *WDR66*) and other loci in the *ARMC2* gene in the raw data, but there were no positive findings. Sanger sequencing revealed that the patient's parents both carried a heterozygous mutation in the same site (Fig. 2). The c.1264C > T mutation was classified as pathogenic according to the ACMG mutation classification guideline [11] with 1 very strong (PVS1) and 2 supporting (PM2 and PM3_Supporting) evidences. The c.1264C > T mutation was not recorded in the dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). The allele frequency of this mutation shown in Exome Aggregation Consortium (ExAC) (http://

Table 2 Sperm parameters of the proband with several semen analyses

	1	2	3	4
Sperm volume (mL)	3.5	4.0	4.0	4.8
Concentration (10^6/mL)	1.4	0.9	1.2	1.4
Total motility (%)	4.2	0	0*	0
Progressive motility (%)	3.2	0	0	0
Normal sperm morphology (%)	0	0	0	0

*Only one non-forward motile sperm was observed

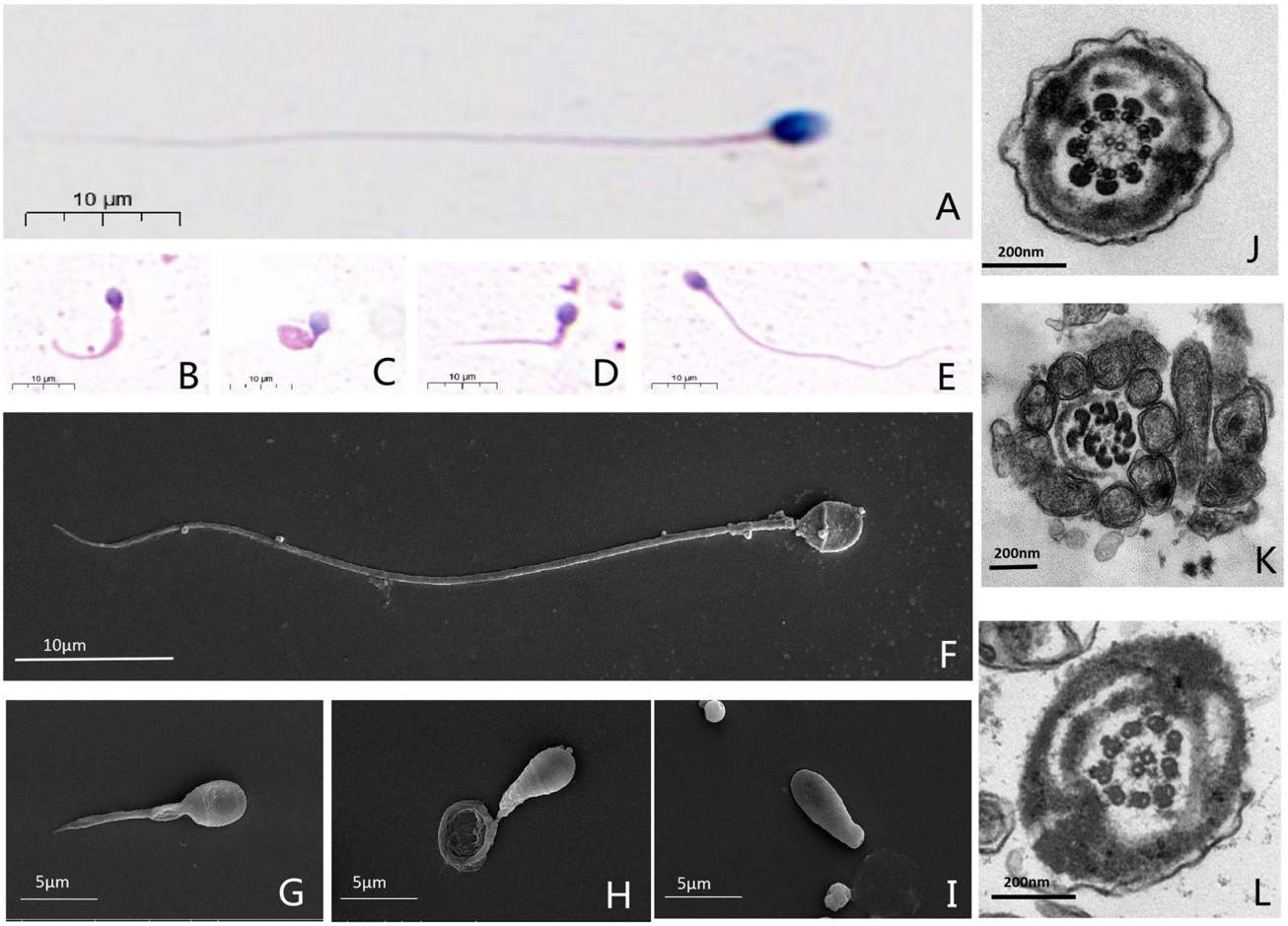

Fig. 1 Sperm morphology analyses for men with *ARMC2* mutation. Normal morphology spermatozoa from a healthy man were revealed by light microscopy (**A**) and SEM (**F**). While in sperm from the man harboring homozygous *ARMC2* mutation, multiple malformations can be observed, including short and irregular caliber (**B**, **G**), coiled (**C**, **H**), and absent (**I**) and bent (**D**) flagella. In addition, sperm with normal length can also be found in the *ARMC2*-deficient patient's sperm (**E**). Under TEM, the cross-sections of the sperm from normal control show a typical "9 + 2" structure of microtubules and the regularly arranged outer dense fibrous sheath (**J**). Most of the sperm from the man with *ARMC2* mutations show a severe disorder in the arrangement of the flagellar ultrastructure, especially the loss of the central pair of microtubules (**K**), and few are normal (**L**)

exac.broadinstitute.org/) was 0.00003229, and in Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/) was 0.0000279.

The *ARMC2* protein interacted with multiple proteins

The construction scheme and effect verification of the plasmid are shown in Fig. 3. After co-IP and mass spectrometry, 1912 differential proteins of WT and *ARMC2* mutant plasmid groups were detected in the precipitated products, and finally 69 of them were found to be different between the WT and *ARMC2* mutant groups. As shown in Fig. 4, the most downregulated protein in mutant groups was TEX10. Among the downregulated proteins, 5 proteins might be involved in the sperm function, namely CEP78, PGAM5, RHOA, FXR1, and SKIV2L2, which was indicating the ARMC2 might participate in multiple processes of spermatogenesis.

Successful ICSI outcome achieved

Eight oocytes were obtained, and 5 were used for ICSI. On day 1, three 2PN, one 1PN, and one 0PN embryos were observed. On day 3, three level 2 8-cell embryos were obtained and the other two also divided (Fig. 5). Two level 2 8-cell embryos were transplanted on day 3, and the remaining embryo was frozen. Ultrasound at 28 days showed clinical pregnancy. Finally, a successful birth was given to boy and girl twins in February 2021. The detailed data of ICSI treatment outcome is shown in Table 3.

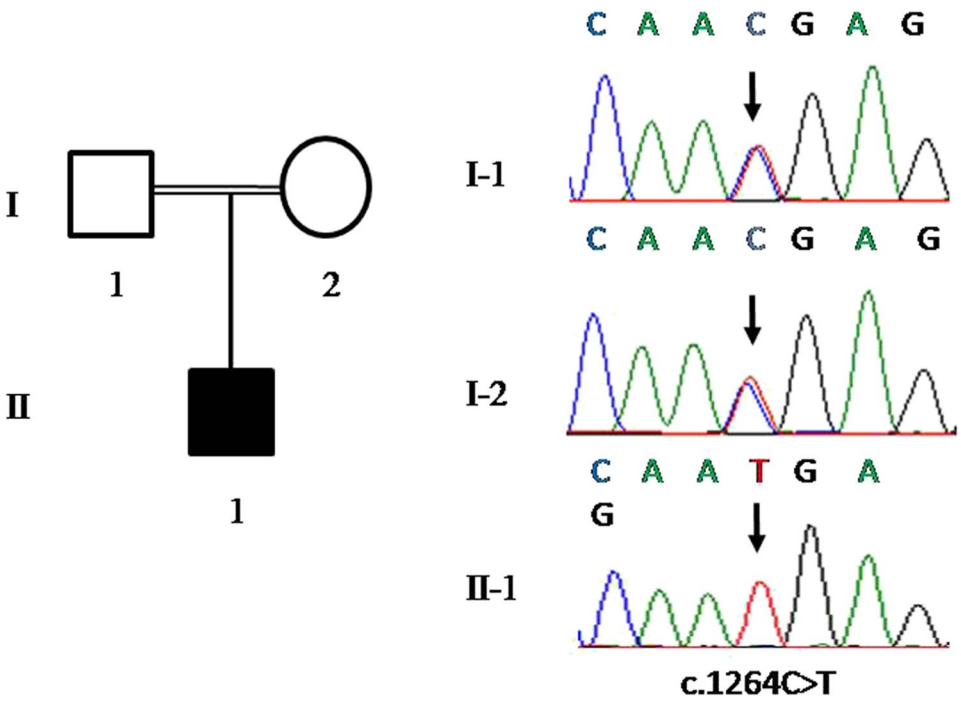

Fig. 2 Identification of novel mutations of *ARMC2* in a man with MMAF. The pedigree of the family showing the father (I-1) and mother (I-2) with their offspring numbered II-1; black-filled squares indicate the male individuals with MMAF. Sanger sequencing results are shown below and the mutation positions are indicated by black arrows

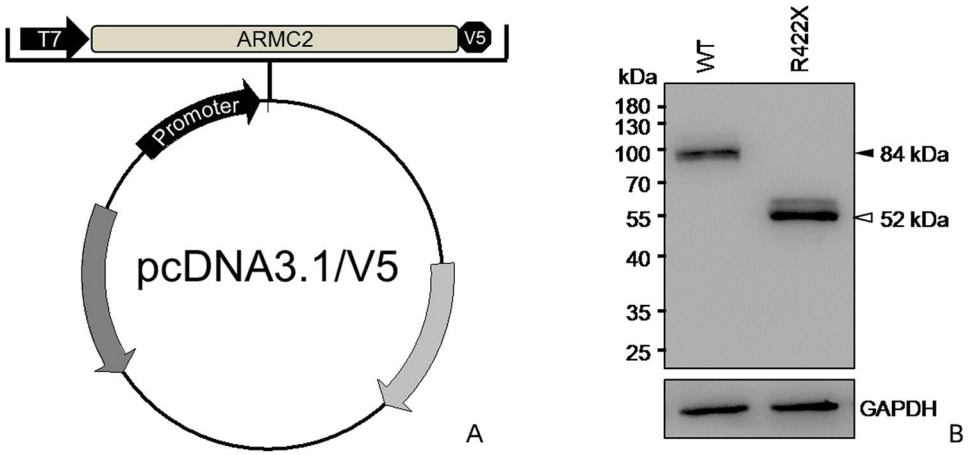

Fig. 3 Construction and expression detection of *ARMC2* mutant plasmid. **A** WT *ARMC2* cDNA and *ARMC2* mutant R422X were inserted into pcDNA 3.1/V5-His plasmid, which encodes a C-terminal V5 tag. **B** The result of WB shows the expression product of mutant plasmid was shorter than WT

Discussion

MMAF, termed as dysplasia of fibrous sheath (DFS) before [12], is a set of flagellogenesis dysfunction caused by several genes (e.g., *CFAP43*, *CFAP44*) [3]. Several *ARMC2* mutations (c.421 C > T, c.1023 + 1G > A, c.1284_1288delAA, c.2279 T > A, c.2353_2354delTT, c.182C > G) had been reported with MMAF in previous studies [8, 13], but no ICSI outcomes of these cases were revealed. Here, we identified a novel homozygous nonsense mutation in *ARMC2* from a Chinese patient with a typical MMAF phenotype. Notably, although the flagella of the patient harboring *ARMC2* mutation showed multiple abnormalities, there were still a few sperms with normal morphology, which could explain why motile sperm could still be found.

ARMC2, also termed as *SPGF38*, is a testis specifically expressing gene [14], which encodes a protein containing several armadillo (ARM) repeats [15]. ARM repeat was first characterized in the Drosophila segment polarity protein [16]. It was involved in lots of biological processes, including intracellular signaling, cytoskeletal regulation, and protein degradation or folding [17]. Among all the ARM repeat proteins, there were a subset especially associated with the flagella structure, such as SPGF6, which regulated the motility of flagella by stabilizing the central pair of microtubules in the classic "9 + 2" arrangement [18]. Mutations in these

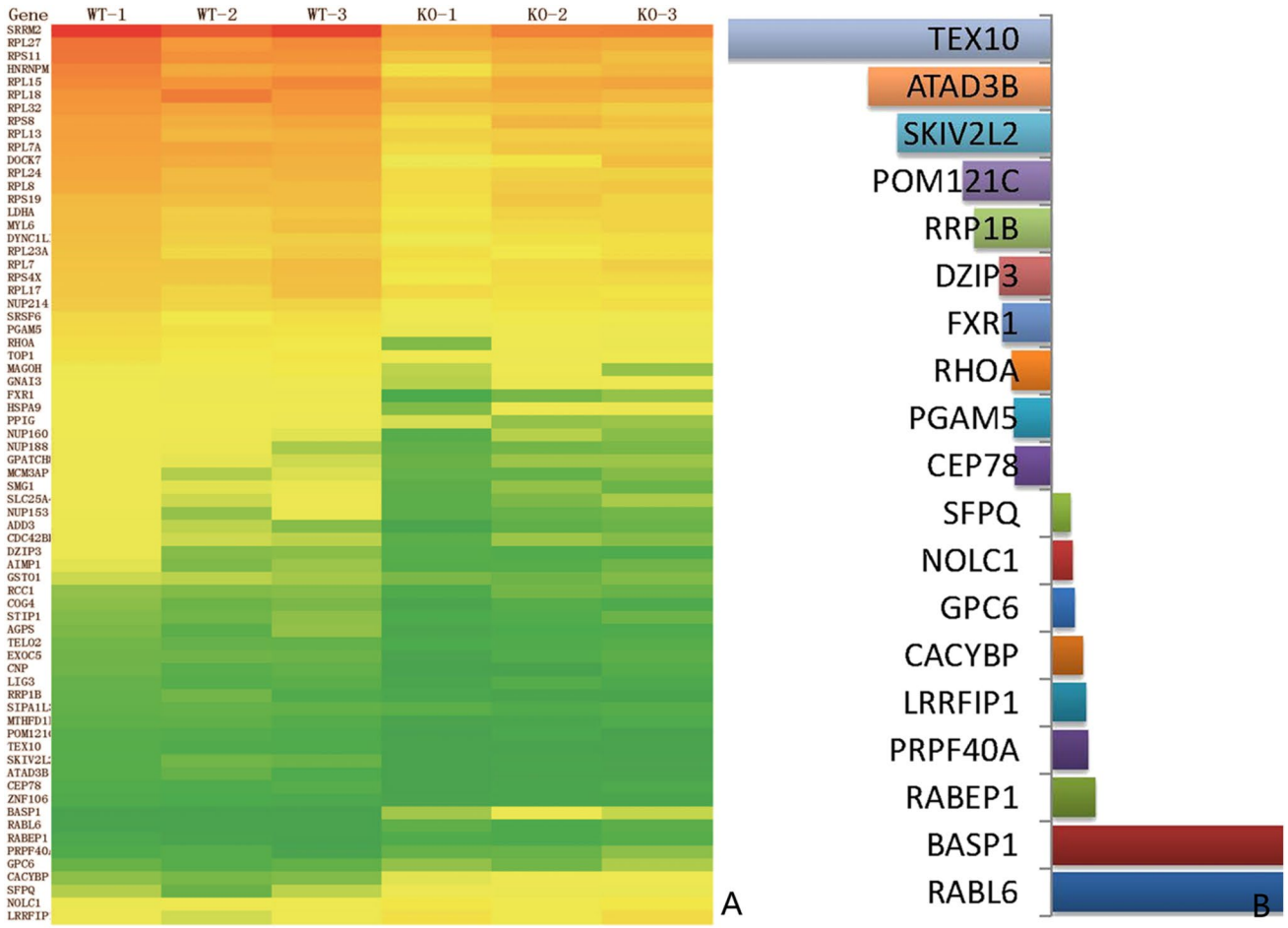

Fig. 4 ARMC2 interacts with multiple proteins. **A** Heat map from three independent proteomic analyses of proteins extracted from wild-type and *ARMC2*-mutant HEK293 cells. Red: proteins upregulated; green: proteins downregulated. **B** The top up (left) and down (right) regulated proteins

ARM repeat proteins, such as *SPAG6* and *ARMC3*, had been reported related to the male infertility caused by sperm flagella malformations [19, 20]. The localization of ARMC2 was presumed to be on the axonemal central pair complex (CPC), because in sperm from an individual harboring *ARMC2* mutations, staining of SPAG6, a CPC protein, was totally absent from the flagellum, while staining for AKAP4, DNALI1, DNAH5, RSPH1, and GAS8 showed no difference from normal control, which suggested other flagellar structures like fibrous sheath, dynein arms, or radial spokes were affected by mutations in *ARMC2* [8]. In our observation of the sperm in the patient harboring *ARMC2* mutation, the most common flagellar ultrastructure defect was the absence of central pair microtubules, which was consistent with previous conjectures on the location of ARMC2.

The co-IP and mass spectrum analysis revealed the interaction between ARMC2 and multiple proteins, such as CEP78, PGAM5, RHOA, FXR1, and SKIV2L2. CEP78 is a centrosomal protein implicated in ciliogenesis and ciliary length control, which is also important for sperm flagella. The mutations in the *CEP78* would result in retinal cone-rod dystrophy associated with hearing loss [21], and sometimes male infertility [22]. But in the study of Giulia Ascar et al. [22], no obvious ultrastructural abnormalities of cilia were observed in sperm of patients harboring *CEP78* mutations. PGAM5 is a mitochondrial protein which expresses in the testis and plays an important role in the function of mitochondria [23]. It was reported the cigarette smoking would significantly increase the DNA methylation level in more than one CpG in PGAM5, which could be potentially related to the impacts of smoking on the spermatogenesis [24]. RHOA was identified interacting with proteins involved in sperm capacitation and acrosome reaction [25]. FXR1 is widely distributed in human and mouse tissues and localized to mature spermatocytes. It was found to be associated with microtubules assembly [26], which might interact with ARMC2 in sperm flagella. SKIV2L2 had both the RNA-binding and ATPase activities and was mainly localized in

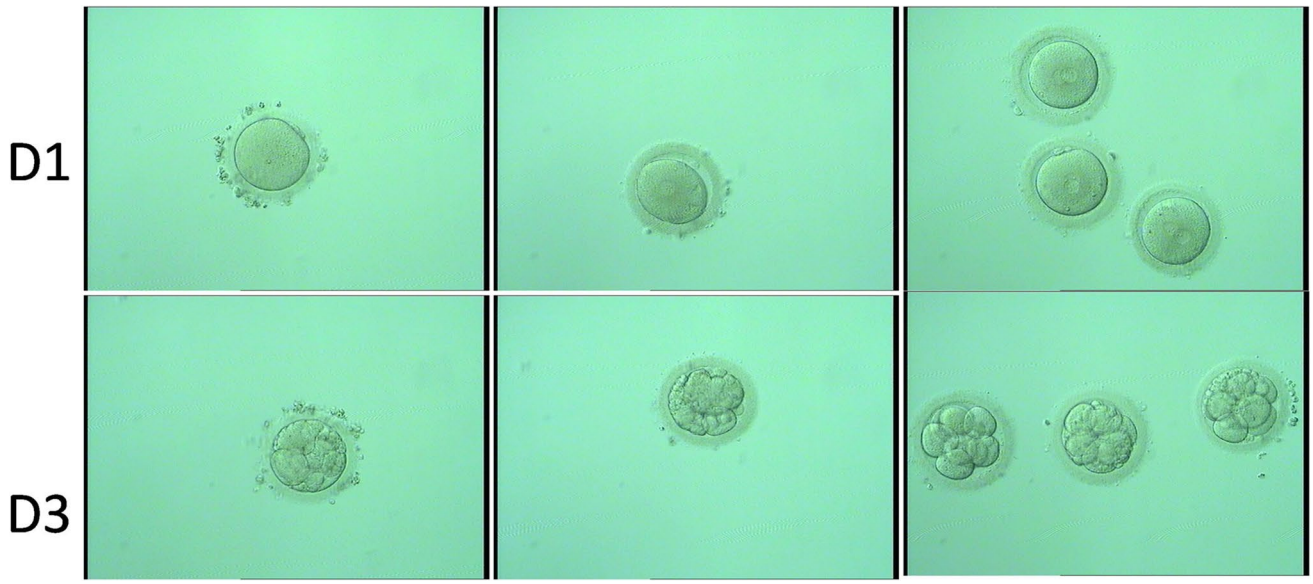

Fig. 5 Images of the embryos of the ICSI cycles. Line 1 shows that the 3 embryos (right) carried 2 PN; the other 2 were 0PN (left) and 1PN (middle). Line 2 shows the 3-day embryos; 3 embryos were qualified as good quality (right) and other 2 were also divided

Table 3 Clinical outcomes of ICSI cycles using spermatozoa from men with homozygous *ARMC2* mutation

Subject	1
Male age (year)	28
Female age (year)	28
Number of ICSI cycles	1
Number of oocytes obtained	8
Number of oocytes injected	5
Number (and rate) of fertilized oocytes	3 (60%)
Number (and rate) of cleavage embryos	3 (100%)
Number (and rate) of high-quality embryos	2 (66.67%)
Number of transfer cycles	1
Number of embryos transferred per cycle	2
Implantation rate	100%
Clinical pregnancy rate	100%
The live birth rate	100%
The gender and weight of the newborns (kg)	♂3.5/♀3.0

the nuclei of round spermatids. Its coding gene *SKIV2L2* was shown to be highly expressed in the spermatocytes at stages I to VI [27]. Nevertheless, we had not continued to explore the interaction mechanism of these proteins and their potential impact on spermatogenesis. Further experiments are still needed to observe the exact axonemal localization and explore the specific role of ARMC2 in spermatogenesis.

Nowadays, ICSI has become the most efficient, even the only approach to achieve a successful pregnancy for MMAF patients. The previous studies showed the MMAF patients harboring mutations in other genes, such as *DNAH1* and *CFAP251*, had a good prognosis after ICSI [28, 29]. However, there were still several failure ICSI cases that were reported, for example, of patients with *CEP135* [30] or *CFAP65* [31]. The impacts of the affected genes on ICSI outcome are supposed depended on the gene's function on fertilization and embryonic development. CEP135 is a centrosomal protein taking part in centriole biogenesis. The injection of sperms from patients harboring *CEP135* mutations might lead to irregular cleavage during embryo development or chromosomal aberrations, causing delays or stagnation of embryo development. Meanwhile, *CFAP65* was recently found related with the sperm head shaping and acrosome, which made its defect severely harmful to the fertility of the sperm [32]. The main function of ARMC2 was supposed to be one component of CPC, and the deficiency in ARMC2 seemed not harmful to the integrity of the sperm nuclear. So the ICSI outcomes of the patients harboring *ARMC2* mutations were predicted to be ideal, which was proved by our clinical observation. Here, the first successful ICSI outcome of a patient with the *ARMC2* mutation was reported, which indicated that ICSI was still the most effective approach to help MMAF patients, especially the patients harboring *ARMC2* mutations, to achieve successful fertilization.

In summary, we identified a novel bi-allelic mutation of *ARMC2* which resulted in male infertility via abnormal flagella formation. Our finding will provide a theoretical basis for genetic counseling and clinical treatment of MMAF caused by *ARMC2* mutations.

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Author contribution J.W. conceived and designed the experiments and wrote the manuscript. X.L. and J.Z. performed genetic analysis. C.Z. and W.W. performed bioinformatic analyses. Y.X., H.L., and S.Y. contributed to the discussion of the data. All authors have read and agreed to the published version of the manuscript.

Declarations

Ethics approval This study was approved by the Affiliated Suzhou Hospital of Nanjing Medical University and the First Affiliated Hospital of Xinjiang Medical University.

Consent to participate Written informed consent was obtained from all of the subjects and their family members participating in the study.

Conflict of interest The authors declare no competing interests.

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