Asian Journal of Andrology (2023) 25, 512–519
www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Male Infertility

Novel compound heterozygous mutations in *DNAH1* cause primary infertility in Han Chinese males with multiple morphological abnormalities of the sperm flagella

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This study aimed to identify genetic causes responsible for multiple morphological abnormalities of the sperm flagella (MMAF) in the Han Chinese population. Three primary infertile males with completely immobile sperm and MMAF were enrolled. Whole-exome sequencing and Sanger sequencing were performed to identify disease-causing genes. Subsequently, morphological and ultrastructural analyses of sperm flagella were investigated. The probable impact of genetic variants on protein function was analyzed by online bioinformatic tools and immunofluorescence assay. Three patients with dynein axonemal heavy chain 1 (*DNAH1*) gene compound heterozygous variations were identified. *DNAH1* c.7435C>T, p.R2479X and c.10757T>C, p.F3586S were identified in the patient from Family 1, c.11726_11727delCT, p.P3909fs and c.12154delC, p.L4052fs were found in the patient from Family 2, and c.10627-3C>G and c.11726_11727delCT, p.P3909fs existed in the patient from Family 3. Four of these variations have not been reported, and all the mutations showed pathogenicity by functional effect predictions. The absence of the center pair and disorganization of the fibrous sheath were present in sperm flagella at the ultrastructural level. Moreover, the expression of DNAH1 was absent in spermatozoa from the participants, validating the pathogenicity of the variants. All three couples have undergone intracytoplasmic sperm injection (ICSI), and two couples of them became pregnant after the treatment. In conclusion, the newly identified *DNAH1* mutations can expand the mutational and phenotypic spectrum of MMAF genes and provide a theoretical basis for genetic diagnosis in MMAF patients. It is recommended to conduct genetic screening in male infertility patients with MMAF and provide rational genetic counseling, and ICSI might be an optimal strategy to help with fertilization and conception for patients with *DNAH1* mutations.

Asian Journal of Andrology (2023) **25**, 512–519; doi: 10.4103/aja202292; published online: 09 December 2022

Keywords: *DNAH1*; intracytoplasmic sperm injection; male infertility; MMAF; mutation

INTRODUCTION

Infertility is a global health concern that affects 8%–12% of couples of reproductive age,[1] and male-related factors contribute to approximately 50% of infertility cases.[2] Spermatozoa contain highly specialized structures required for fertilization, such as the sperm head and flagellum. Among them, the sperm flagellum is an evolutionarily conserved organelle that maintains sperm motility and is indispensable for fertilization.[3] The axoneme, the main structure of the sperm flagellum, is composed of nine microtubule doublets and a central pair of microtubules in a "9+2" pattern. Hence, morphological or functional defects of the sperm flagellum are a crucial cause of male fertility. Multiple morphological abnormalities of the sperm flagella (MMAF), a phenotypic variant of primary ciliary dyskinesia (PCD),[4] are characterized by a mosaic of spermatozoa with short, absent, bent, coiled, and/or irregular flagella.[5] MMAF patients often exhibit extreme asthenozoospermia with immobile sperm and can be easily diagnosed by semen morphology analysis.[6] Severe disorganization of the axonemal structure at the ultrastructure level can also be observed by transmission electron microscopy.[5]

Genetic abnormalities could be responsible for some male infertility cases, and up to 50% of MMAF cases are likely to be due to genetic causes.[7] PCD, also called immotile-cilia syndrome, is a genetically heterogeneous disorder, as is MMAF.[8] To date, mutations in 18 genes, such as dynein axonemal heavy chain 1 (*DNAH1*), cilia- and flagella-associated protein 43 (*CFAP43*), and sperm flagellar 2 (*SPEF2*), have been found to be closely related to the MMAF phenotype and male infertility.[9] Moreover, a large number of identified genes were linked to human male fertility phenotypes.[10] Remarkably, mutations in the identified MMAF genes account for only 30%–60% in different cohorts,[9] and in most cases, the exact functions of these MMAF-related proteins remain unclear. In addition, the genetic origin of MMAF has still not been identified in the Han Chinese population. Expanding the mutational and phenotypic spectrum of MMAF genes and exploring the molecular mechanism related to flagellum assembly and function

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Received: 08 August 2022; Accepted: 21 October 2022

are beneficial to genetic counseling and treatment in male infertility patients with MMAF.

DNAH1 was the first identified gene responsible for MMAF and was found in 2014.[6] In our study, three primary infertile males with completely immobile sperm caused by MMAF were enrolled. We performed whole-exome sequencing (WES), a useful tool for disease-causing variant discovery,[11] combined with Sanger sequencing to identify new *DNAH1* mutations or novel genetic causes of MMAF. This study expands the mutant library of the *DNAH1* gene and lays a foundation for diagnostic screening kits for MMAF.

PARTICIPANTS AND METHODS

Participants

Three Han Chinese males diagnosed with MMAF were enrolled in this study between January 2018 and December 2021 from the Reproductive Medicine Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Details of their history of infertility and family were obtained. Peripheral blood samples from patients and their families were collected for genetic analyses. None of the patients were from consanguineous families or suffering from PCD-associated symptoms, such as bronchitis and pneumonia. This study was approved by the Ethical Committee of Tongji Hospital (Approval No. TJ-IRB20200722), and informed written consent was provided by the patients and their family members.

WES and mutation analysis

The details of the genetic analysis procedure have been well described previously.[12,13] Genomic DNA was extracted from peripheral blood samples for WES to identify potential disease-causing mutations according to the manufacturer's instructions (69506, Qiagen, Hilden, Germany). The exomes were captured and enriched using an Agilent SureSelect Human All Exon Kit (Agilent, Beijing, China), and next-generation sequencing (NGS) was performed on the Illumina HiSeq X-TEN platform (Illumina, San Diego, CA, USA). After quality control, the obtained raw FASTQ files were aligned to the human genome reference sequence (hg19/GRCh37) using Burrows–Wheeler Aligner (BWA) software.[14] DNA sequence variants were detected using Genome Analysis Toolkit (GATK) software and then annotated with ANNOVAR software. Candidate variants identified in the participants were validated by Sanger sequencing analyses conducted on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The conservation of the amino acids in the mutational sites was analyzed among multiple species using MEGA software (https://www.megasoftware.net/). The allele frequency of the mutations in the general population was assessed using the 1000 Genomes (http://www.1000genomes.org/data) and gnomAD databases (http://gnomad-sg.org/), and analyses of the probable impact of single-nucleotide variants (SNVs) on protein function were performed using Sorting Intolerant From Tolerant (SIFT; http://sift.bii.astar.edu.sg/), Polymorphism Phenotyping (Polyphen-2; http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutaiontaster.org/), and MutationAssessor (http://mutationassessor.org/). The three-dimensional structure of the wild-type and missense mutation DNAH1 protein was predicted using SWISS-MODEL (https://swissmodel.expasy.org/) based on homology modeling, and PYMOL software (https://pymol.org/2/) was utilized to analyze the effect of the mutation.

Sperm analysis and morphological assessment

Sperm analysis was performed using a combination of manual Papanicolaou sperm staining and a computer-assisted sperm analysis (CASA) system (BEION S3-3, version 4.20, BEION, Shanghai, China).[15] Freshly ejaculated semen was collected in sterile containers by masturbation after sexual abstinence for 2–7 days. Semen samples were assessed under light microscopes (Olympus, Tokyo, Japan) after liquefaction for half an hour at 37°C. Semen volume, semen concentration, progressive motility, and morphology were evaluated based on the standard of the WHO guidelines (5th edition) under light microscopes.[16] The low reference limits were 1.5 ml for semen volume, 15×10^6 ml^{-1} for sperm concentration, 32% for progressive motility, and 4% for normal forms. For each participant, at least 200 spermatozoa were examined for semen characteristics analysis and morphological assessment. Each patient underwent semen analyses at least three times. Pentoxifylline (Vitrolife, Gothenburg, Sweden), which can induce sperm motility by inhibiting phosphodiesterase activity and thus increasing intracellular cyclic adenosine monophosphate (cAMP) levels, was used to select immotile but viable spermatozoa.[17]

Scanning and transmission electron microscope analysis

The prepared spermatozoa samples were immobilized with 2.5% phosphate-buffered glutaraldehyde at 4°C for 2 h and washed with 0.1 mol l^{-1} phosphate buffer (PB; pH 7.4) three times. The samples were then postfixed with 1% osmium tetroxide in 0.1 mol l^{-1} PB for 1–2 h at room temperature in the dark, followed by dehydration using graded ethanol and isoamyl acetate. For scanning electron microscopy (SEM), the specimens were attached to metallic stubs and coated with gold particles after drying and then observed and photographed by SEM (SU8100, HITACHI, Tokyo, Japan) at 10 kV. For transmission electron microscopy (TEM), after infiltration with 1:1 acetone and SPI-CHEM EMBed 812 resin (90529-77-4, SPI Supplies, West Chester, Pennsylvania, USA) for 4 h at 37°C, the specimens were embedded in pure EMBed 812 overnight at 37°C and then polymerized for 48 h at 60°C. Ultrathin sections with a thickness of 70 nm were cut and collected onto 150 mesh cuprum grids followed by counterstaining with 2% uranium acetate and 2.6% lead citrate. The specimens were observed and photographed by TEM (HT7800, HITACHI) at 80 kV.

Immunofluorescence assay

The spermatozoa were fixed in 4% paraformaldehyde and blocked in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 (GC204003, ServiceBio, Wuhan, China) for 1 h at room temperature, followed by incubation with 1:100 primary rabbit polyclonal anti-DNAH1 antibody (1:100 dilution; DF13457, Affinity Biosciences, Changzhou, China) overnight at 4°C. After washing, the samples were incubated with anti-rabbit Cy3-conjugated secondary antibodies (1:100 dilution; GB21303, ServiceBio) for 1 h at room temperature, followed by incubation with 100 μg ml^{-1} isothiocyanate-labeled peanut agglutinin (FITC-PNA; L7381, Sigma, Shanghai, China) for 1 h. The nuclei were stained with Hoechst 33258 (G1011, ServiceBio) for 10 min. Images were captured with a confocal microscope (FV1000, Olympus) as previously described.[18]

RESULTS

The basic characteristics and semen parameters of the involved patients

The three infertile males included in this study were of Chinese Han nationality in Hubei Province (**Table 1**). The patient from Family 1 (II-1, 33 years old) was diagnosed with primary infertility for 3 years. The patient from Family 2 (II-1, 31 years old) was diagnosed with primary infertility for 2 years. The patient from Family 3 (II-1, 34 years old) was diagnosed with primary infertility for 6 years and had an elder brother who delivered two children naturally. The ovarian reserve of females in

the three couples was within the normal range, and none of the couples had any history of drinking, smoking, or any PCD-associated symptoms (**Table 1**). In addition, all couples had normal chromosomal karyotypes (46,XX and 46,XY). The semen parameters of the three MMAF patients are summarized in **Table 1**. All three MMAF patients exhibited decreased sperm concentrations, a reduced percentage of sperm with normal morphology, and completely immobile sperm, despite semen volumes within the normal ranges. Hence, the three MMAF males were diagnosed with severe oligo-astheno-teratozoospermia.

Morphological and ultrastructural analyses of sperm flagella from MMAF patients

The morphology of sperm from MMAF patients was assessed with Papanicolaou staining (**Figure 1a**). It was shown that the sperm flagella displayed multiple morphological abnormalities, such as absent, short, bent, or coiled tails, compared to morphologically normal sperm under a light microscope. Subsequently, the abnormal morphology of spermatozoa was confirmed using SEM (**Figure 1b**). The ultrastructural morphology of sperm flagella from an MMAF patient (Family 2 II-1) was further investigated using TEM (**Figure 1c**–**1e**). A normal spermatozoa flagellum exhibited microtubule doublets with clear radial spokes and a center pair, which was surrounded by a certain number of dense fibers in cross-section. It was obvious that the "9+2" structure of the microtubules in sperm flagella from MMAF patients was disordered, especially the malformation or absence of the center pair (**Figure 1c** and **1d**). Moreover, the fibrous sheath surrounding microtubules was severely disorganized with morphologically abnormal mitochondria (**Figure 1e**).

Identification of DNAH1 variations

To identify the genetic causes responsible for MMAF, WES of DNA samples of the patients was performed (**Figure 2a**). Five *DNAH1* heterozygous variations were identified in the 3 cases, and no other variations were found in other candidate genes related to male infertility. All genes were mapped to GRCh37/hg19. Specifically, 3p21.1-3-52413978C>T and 3p21.1-3-52428611T>C were identified in II-1 from Family 1, 3p21.1-3-52430998CCT>C and 3p21.1-3-52432930CT>T were found in II-1 from Family 2, and 3p21.1-3-52428478C>G and 3p21.1-3-52430998CCT>C were identified in II-1 from Family 3 (**Table 2**). Except for 3p21.1-52430998CCT>C (c.11726_11727delCT, p.P3909fs), the other four mutations of 3p21.1-52413978C>T (c.7435C>T, p.R2479X), 3p21.1-52428611T>C (c.10757T>C, p.F3586S), 3p21.1-52432930CT>T (c.12154delC, p.L4052fs), and 3p21.1-52428478C>G (c.10627-3C>G, not available [NA]) had not been reported before. The abovementioned mutations were further confirmed by Sanger sequencing (**Figure 2b**). In Family 1, the nonsense mutation 3p21.1-52413978C>T was from the father, and the missense mutation 3p21.1-52428611T>C came from the mother. In Family 2, the frameshift mutation 3p21.1-52430998CCT>C was from the mother, and the other frameshift mutation 3p21.1-52432930CT>T was from the father. In Family 3, the frameshift mutation 3p21.1-52430998CCT>C was inherited from the father with a splice site mutation 3p21.1-52428478C>G from the mother. The elder brother (Family 3 II-2) had no mutations and delivered two children spontaneously. The locations of these variations in the *DNAH1* gene and DNAH1 protein are presented in **Figure 2c**. Sequence alignment revealed that affected amino acids at the mutational sites were highly evolutionarily conserved across different organisms (**Figure 2d**). These variations showed pathogenicity by the functional effect predictions of online bioinformatic analyses (**Table 2**). Nonsense and frameshift mutations mean that the mRNA is likely to be degraded, and the effect of missense mutations on protein function is unclear. The missense mutation 3p21.1-52428611T>C occurred in exon 67 of *DNAH1*, subsequently resulting in the replacement of phenylalanine (Phe, F) by serine (Ser, S) at position 3586. F3596 in the wild type can form hydrogen bonds with S3584 and F3590, while the *DNAH1*-F3586 mutation did not affect the formation of hydrogen bonds (**Figure 2e**).

Table 1: Baseline characteristics and semen parameters of patients

Characteristic	*Reference values*	*Family 1 II-1*	*Family 2 II-1*	*Family 3 II-1*
Male age (year)	NA	33	31	34
Male basal FSH (mIU ml^{-1})	1.4–18.1	1.39	4.90	NA
Male basal LH (mIU ml^{-1})	1.5–9.3	4.54	0.90	NA
Female age (year)	NA	31	31	32
Female BMI (kg m^{-2})	NA	28.6	24.5	25.0
Female AMH (ng ml^{-1})	0.32–8.64	5.36	1.50	2.54
Female basal FSH (mIU ml^{-1})	2.5–10.2	7.15	5.18	8.85
Female basal LH (mIU ml^{-1})	1.9–12.5	4.31	4.02	0.90
Female AFC	NA	10+7	3+2	1+4
Infertility type	NA	Primary	Primary	Primary
Infertility duration (year)	NA	3	2	6
Sperm volume (ml)	>1.5	2.1	4.6	3.3
Sperm concentration (×10^6 ml^{-1})	>15	6.8	4.8	8.2
Progressive motile sperm (%)	>32	0	0	0
Motile sperm (%)	>40	0	0	0
Morphologically normal sperm (%)	>4	0.5	0.7	0.5
Abnormal flagella (%)				
Short	NA	49.5	21.1	32.9
Coiled	NA	12.5	20.4	36.2
Bent	NA	0	0	4.8
Multiple	NA	4.5	0	0.5

FSH: follicle-stimulating hormone; LH: luteinizing hormone; BMI: body mass index; AMH: anti-Müllerian hormone; AFC: antral follicle count; NA: not available

Table 2: Analysis of dynein axonemal heavy chain 1 variations identified by whole-exome sequencing

Patient	*Mutation*	*Position*	*Location*	*Amino acid change*	*Zygosity*	*Allele frequency*	*Functional effect predictions*			
							SIFT	*Polyphen-2*	*MutationTaster*	*MutationAssessor*
Family 1	c.7435C>T	3p21.1-52413978	Exon 48	p.R2479X	Heterozygous	0.00001292	NA	NA	Disease causing	NA
II-1	c.10757T>C	3p21.1-52428611	Exon 67	p.F3586S	Heterozygous	0.00002032	Deleterious	Probably damaging	Disease causing	High
Family 2	c.11726_11727delCT	3p21.1-52430999_52431000	Exon 73	p.P3909fs	Heterozygous	0.0001007	NA	NA	Disease causing	NA
II-1	c.12154delC	3p21.1-52432930	Exon 75	p.L4052fs	Heterozygous	NA	NA	NA	Disease causing	NA
Family 3	c.10627-3C>G	3p21.1-52428478	Intron 66	NA	Heterozygous	$4.037e^{-6}$	NA	NA	NA	NA
II-1	c.11726_11727delCT	3p21.1-52430999_52431000	Exon 73	p.P3909fs	Heterozygous	0.0001007	NA	NA	Disease causing	NA

DNAH1: dynein axonemal heavy chain 1.; NA: not available; SIFT: Sorting Intolerant From Tolerant

Location of DNAH1 in spermatozoa from MMAF patients

The expression and localization of DNAH1 were visualized in an MMAF patient (Family 2 II-1) and a healthy control using an immunofluorescence assay on semen smears (**Figure 3**). Immunostaining of DNAH1 was observed throughout the flagella of sperm from healthy controls, while the expression of DNAH1 was absent in spermatozoa from the MMAF patient with two frameshift mutations bearing *DNAH1* variations, validating the pathogenicity of the variants.

In vitro fertilization (IVF) treatment and clinical outcome

The clinical outcomes of the three couples involved in this study are presented in **Table 3**. The couple from Family 1 underwent one intracytoplasmic sperm injection (ICSI) cycle and had two embryos transferred on Day 3, and they became pregnant. The couple from Family 2 underwent three ICSI cycles. They obtained 19 oocytes and 7 available embryos. They had three embryos transferred on Day 3 in total, and fortunately, they conceived in the third cycle. The couple from Family 3 had undergone three ICSI cycles with a total of 22 oocytes retrieved. They had five embryos transferred on Day 3 in three frozen-thawed transfer cycles, but there was, regretfully, no pregnancy. They are now still endeavoring in another ICSI attempt.

DISCUSSION

In this study, we reported three Han Chinese males with primary infertility who were diagnosed with MMAF from three independent families and identified five *DNAH1* heterozygous variations by WES and Sanger sequencing, including a nonsense mutation (c.7435C>T, p.R2479X), a missense mutation (c.10757T>C, p.F3586S), two frameshift mutations (c.11726_11727delCT, p.P3909fs; c.12154delC, p.L4052fs), and a splice region mutation (c.10627-3C>G, NA). Bioinformatic analysis indicated that the mutations were probably pathogenic, and some of them can result in the absent expression of DNAH1 protein in spermatozoa flagella.

DNAH1, encoding an inner dynein arm (IDA) heavy chain, was the first identified genetic cause of MMAF and is predominantly expressed in the human testis.[6] DNAH1 is an axonemal component and is required for the formation of IDAs, which are multiprotein adenosine triphosphatase (ATPase) complexes that are indispensable for flagellar beating.[19] In *Chlamydomonas*, the loss of IDAs has changed the amplitude rather than the frequency of cilia beating,[20] whereas in mammals, the function of IDAs is mostly associated with the ciliary motility of respiratory cells.[21] The absence or dysfunction of DNAH1, caused by pathogenic mutations in *DNAH1*, is thought to weaken the link between the outer doublet and the radial spokes in the axoneme of sperm, resulting in severe instability of the central singlet microtubules and the subsequent defects of the central pair, which present as a disorganized "9+0" structure in the axoneme of sperm flagella.[22] In our study, the results of TEM analyses demonstrated abnormalities in the sperm flagella ultrastructure in MMAF patients, particularly the absence of a central pair and a disordered outer fibrous sheath, which supports the important function of DNAH1 protein in sperm flagella. In addition, DNAH1 is expressed in both sperm flagella and cilia, while patients carrying *DNAH1* mutations are more likely to exhibit MMAF-related infertility without other PCD-related symptoms,[4] indicating that DNAH1 function is more essential in sperm flagella than in cilia.[6] Several studies have reported that *DNAH1* mutations are responsible for male infertility associated with the MMAF phenotype both in humans[6,22–25] and mice.[26] In this study, we identified five novel compound heterozygous mutations in *DNAH1* in three patients with

Table 3: *In vitro* fertilization outcomes of patients

Patient	*Cycles (n)*	*Oocyte retrieved (n)*	*MII oocytes (n)*	*2PN (n)*	*Available embryos on Day 3 (n)*	*Transferred embryos (n)*	*Clinical pregnancy*
Family 1 II-1	1	16	14	11	11	1	Yes
Family 2 II-1	1	9	8	2	2	2	No
	2[a]	4	4	1	1	1	No
	3	6	5	5	4	2	Yes
Family 3 II-1	1[a]	10	7	4	3	2	No
	2[a]	8	7	1	1	1+1[b]	No
	3	3	2	1	1	1	No

[a]ICSI cycles in other IVF centers. [b]One frozen-thaw embryo from the previous cycle. MII: metaphase II; PN: pronuclei; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection

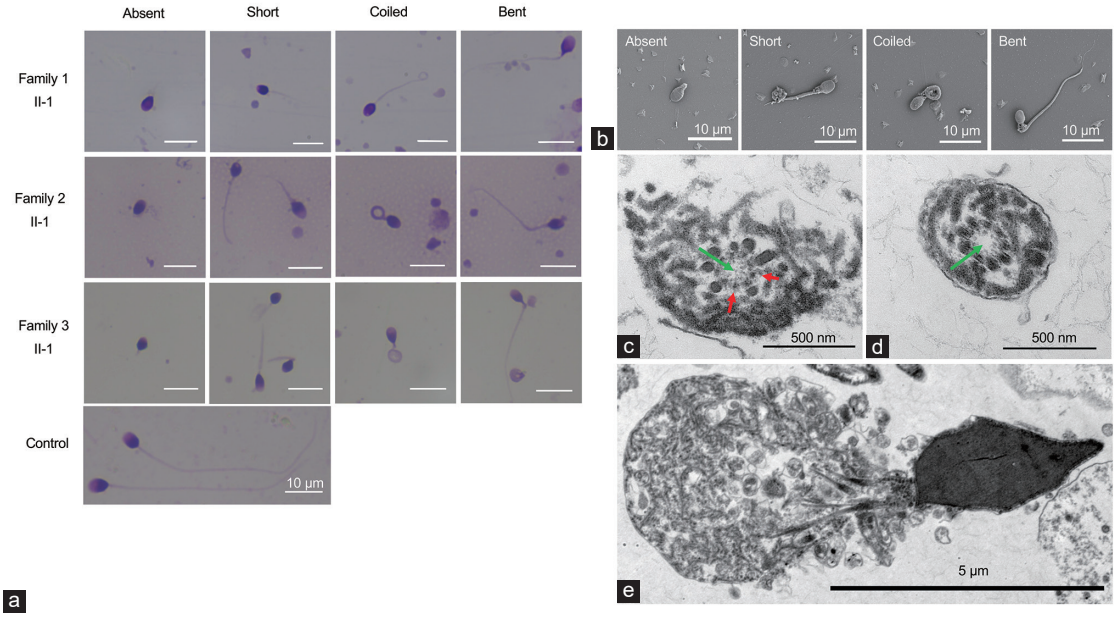

Figure 1: Morphological assessment of sperm from MMAF patients bearing *DNAH1* mutations. (**a**) Light microscopy of sperm using the Papanicolaou staining. The sperm flagella of MMAF patients exhibited multiple morphological abnormalities, including absent, short, coiled, and bent tails, compared to those of healthy donors. Scale bars = 10 μm. (**b**) Scanning electron microscopy of sperm. Scale bars = 10 μm. (**c**–**e**) Transmission electron microscopy of sperm. (**c**) The number of dense fibers is inadequate (red arrow), and the central pair was missing (green arrow) with a disorganized mitochondrial sheath. Scale bar = 500 nm. (**d**) The "9+2" structure of the microtubules is disordered with a complete absence of the central pair (green arrow). Scale bar = 500 nm. (**e**) In the longitudinal section, the arrangement of the fibrous sheath is disordered with abnormal morphology of the mitochondria. Scale bar = 5 μm. MMAF: multiple morphological abnormalities of the sperm flagella; *DNAH1*: dynein axonemal heavy chain 1.

MMAF, which reinforced the association between *DNAH1* mutations and male infertility. Thus, pathogenic variations in *DNAH1* are a critical genetic cause of MMAF-related primary male infertility.

In the present study, five *DNAH1* mutations were identified. The nonsense mutation 3p21.1-52413978C>T at exon 47 found in Family 1 may introduce the stop codon c.7435C>T, p.R2479X in the *DNAH1* coding sequence and result in the premature termination and deletion of the functional domain, including AAA4, AAA5, AAA6, and coiled-coil domains in the stalk-like structure. In East Asian populations, the missense mutation 3p21.1-52428611T>C at exon 67 (c.10757T>C, p.F3586S) does not occur based on the gnomAD database. The replacement of phenylalanine by serine at position 3586 did not affect the formation of hydrogen bonds, indicating that this missense mutation may not greatly affect folding and stability. Thus, it was speculated that the alteration of DNAH1 expression in Family 1 II-1 might be attributed to the combined effect of 3p21.1-52413978C>T at exon 47. Of note, in two patients (Family 2 II-1 and Family 3 II-1), the *DNAH1* 3p21.1-52430998CCT>C deletion at exon 73 was identified. This mutation (c.11726_11727delCT, p.P3909fs) is a common loss-of-function mutation in East Asian populations with an allele frequency of 0.001389 according to the gnomAD database.[27–30] A recent literature review analyzed reported cohorts of Chinese men with MMAF, and it was found that this mutation showed a high prevalence ranging from 7% to 75% in Chinese patients.[25] This mutation is pathogenic and might also have been influenced by a founder effect in East Asian populations. The frameshift mutation 3p21.1-3-52432930CT>T deletion at exon 75 resulted in c.12154delC, p. L4052fs and it has not been reported before in the gnomAD database. Although the results of the immunofluorescence assay showed that the expression of DNAH1 was absent in spermatozoa from Family 2 II-1, whether it was affected by the mutation of the 3p21.1-3-52432930CT>T deletion remains unclear, considering that this mutation coexisted with another frameshift mutation (3p21.1-3-52430998CCT>C deletion) in Family 2. More genetic screening in MMAF patients is needed to expand the *DNAH1* mutant library.

Clinically, except for a mosaic of flagellar abnormalities, the three patients with *DNAH1* mutations enrolled in this study exhibited abnormal semen parameters, especially completely immobile sperm. A recent study showed that the prevalence of oligo- and

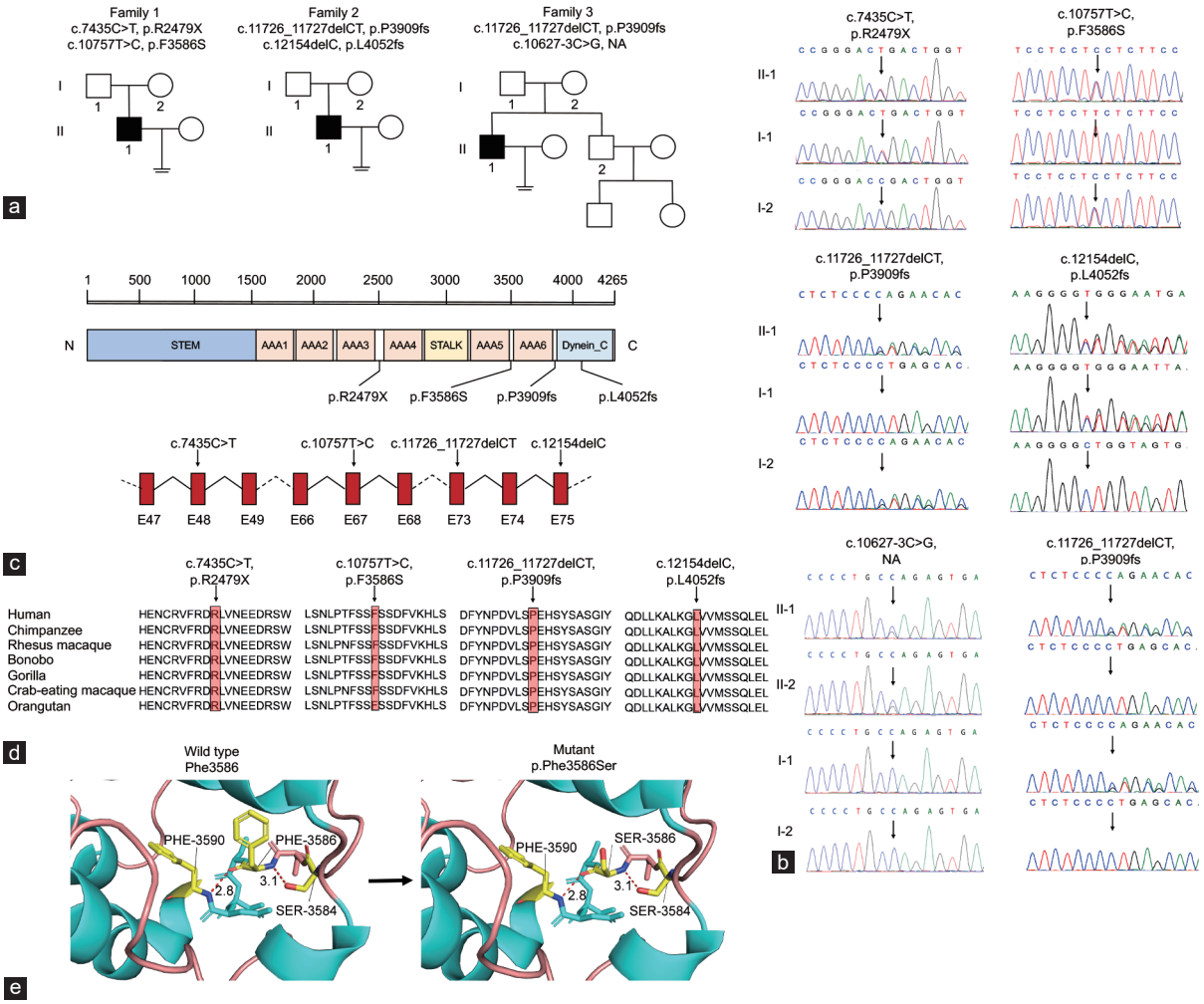

Figure 2: Identification of mutations in *DNAH1*. (**a**) Pedigrees of three MMAF patients bearing *DNAH1* variants. The black squares represent the affected male individuals. The patient in Family 1 carried 1 nonsense mutation (c.7435C>T, p.R2479X) and 1 missense mutation (c.10757T>C, p.F3586S). The patient in Family 2 carried 2 different frameshift mutations: c.11726_11727delCT, p.P3909fs and c.12154delC, p.L4052fs. The patient in Family 3 carried 1 frameshift mutation (c.11726_11727delCT, p.P3909fs) and 1 splice region mutation (c.10627-3C>G, NA). (**b**) Sanger sequencing results of the mutations. (**c**) Locations of the mutations in the *DNAH1* gene and protein. (**d**) Interspecies alignment. Amino acids in the mutational sites are highly conserved during evolution among multiple species. (**e**) The modeling structural prediction of the wild type and a missense mutation (p.F3586S) of the DNAH1 protein. MMAF: multiple morphological abnormalities of the sperm flagella; *DNAH1*: dynein axonemal heavy chain 1; NA: not available.

necrozoospermia was 52.31% and 68.97% in patients with *DNAH1* mutations, respectively, which was higher than that in healthy controls (<15%), and 24.1% of them were without viable sperm.[24] Similarly, the knockout of the mouse dynein heavy chain 7 (*MDHC7*) gene, the mouse ortholog gene to human *DNAH1*, in mice resulted in 62% of completely immobile sperm.[31] Therefore, it was reasonable to conjecture that *DNAH1* mutations might result in immobile sperm. Moreover, the sperm concentration of the MMAF individuals in our study was lower than the low limit of the reference value. In a previous study, a persistent reduction in sperm concentration was observed in such patients during a long period over five years.[24] More evidence and longer follow-up are needed to reinforce this hypothesis.

Currently, for MMAF patients with identified pathogenic mutations, it was reported that ICSI was the only practicable method.[32] Several studies have indicated that the outcomes of ICSI in patients with genetic mutations are favorable.[29,33,34] In the current study, two patients achieved clinical pregnancy successfully by ICSI, and the other patient is still attempting ICSI treatment. Our results supported that ICSI was an optimal management strategy with a good prognosis for *DNAH1* mutation patients. Similar results were presented in another study that involved 12 participants with *DNAH1* mutations. Four of them obtained embryos in ICSI cycles, and one of the two couples with embryo transfer delivered successfully.[25] Another study reported a comparable fertilization rate in *DNAH1* mutation patients following ICSI.[35] Based on the above successful cases, couples suffering from *DNAH1* mutation are encouraged to undergo ICSI treatment. Moreover, during genetic counseling, they should be informed that their newborns will inherit at least one of these heterozygous variants from the fathers. However, some studies have suggested elevated levels of chromosomal abnormalities and DNA fragmentation in spermatozoa from males with teratozoospermia.[36,37] In an *in vivo* experiment, it was found that *MDHC7*-deficient mice could fertilize, but the number of embryos at the 8-cell stage decreased.[31] Furthermore, centriole function appears unaffected in men with *DNAH1* variants since many successful deliveries have been reported in patients with *DNAH1* mutants. Therefore, closer long-term follow-up is required

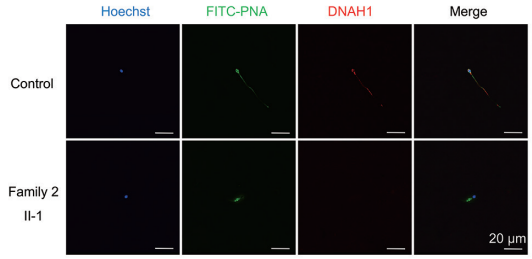

Figure 3: Immunofluorescence assay of human spermatozoa from healthy controls and patients bearing *DNAH1* mutations. DNAH1 staining (red) can be observed throughout the sperm flagella from healthy controls, while the expression of DNAH1 protein was completely absent in an MMAF patient (Family 2 II-1). The acrosome of sperm was stained by FITC-PNA (green) and the nuclei were marked by Hoechst (blue). Representative images of the immunofluorescence assay are shown (scale bars = 20 μm). MMAF: multiple morphological abnormalities of the sperm flagella; *DNAH1*: dynein axonemal heavy chain 1; FITC-PNA: isothiocyanate-labeled peanut agglutinin.

to evaluate the safety of ICSI in patients with MMAF, and whether *DNAH1* mutations have an impact on the success rate of ICSI remains to be further confirmed.

The current study has several clinical implications for patients with the MMAF phenotype caused by genetic mutations. Therapeutic methods to improve semen quality might be useless and not recommended for such patients. Although ICSI treatment might be optimal for patients with *DNAH1* mutations, it is still unclear whether it can impair the success of ICSI. When conducting genetic counseling, genetically diagnosed patients should be aware of the risk that their male offspring might also inherit the same pathogenic variants. Thus, genetic screening is recommended for their female partner. In addition, for MMAF patients, the prognosis of ICSI treatment may differ based on the results of genetic diagnosis. Thus, systematic genetic screening can help physicians not only definitely diagnose MMAF but also provide precise instructions and treatments based on the types of genetic mutations. Furthermore, expanding the mutational and phenotypic spectrum of MMAF genes can lay a foundation for diagnostic screening kits for MMAF.

In conclusion, we identified five *DNAH1* pathogenic mutations in three Han Chinese males with MMAF and primary infertility, which expanded the mutant library in China and provided a theoretical basis for genetic diagnosis in MMAF patients. *DNAH1* mutations might lead to MMAF and completely immobile sperm. It is recommended to conduct genetic screening in male infertility patients with MMAF and provide rational genetic counseling, and ICSI might be an optimal strategy to help with fertilization and conception for MMAF patients.

AUTHOR CONTRIBUTIONS

MW drafted the manuscript and performed most of the experiments. QYY and JPZ help with data analyses and carried out some of the experiments. HPT and JH participated in data curation. LJ and LXZ designed the study, participated in the whole process of this study, and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

This study was supported by the Health Commission of Hubei Province Scientific Research Project (WJ2021M110) and the National Key Research and Development Project (2018YFC1002103).

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