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GENETICS

Bi-allelic variants in *DNAH10* cause asthenoteratozoospermia and male infertility

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Abstract

Purpose Multiple morphological abnormalities in the sperm flagella (MMAF) comprise a severe phenotype of asthenoteratozoospermia with reduced or absent spermatozoa motility. Whereas dozens of candidate pathogenic genes for MMAF have been identified, the genetic cause in a large proportion of patients is unknown. We attempted to identify novel genetic explanations for MMAF.

Methods We performed whole-exome sequencing of patients with MMAF to identify pathogenic variants. The phenotypes of spermatozoa in patients carrying *DNAH10* variants were investigated using haematoxylin and eosin staining, scanning electron microscopy, and transmission electron microscopy. The expression and location of DNAH10 and other spermatozoa structure–related proteins were analyzed using immunofluorescence assays.

Results We found one homozygous frameshift *DNAH10* variant (NM_207437: c.2514delG:p.L839*) and one compound heterozygous *DNAH10* variant (NM_207437: c.10820 T > C:p.M3607T; c.12692C > T:p.T4231I) in two patients with MMAF. These variants were absent or rare in the general population. Haematoxylin and eosin staining and scanning electron microscopy revealed the significant disruption of sperm flagella in the patients. In addition, ultrastructural analysis by transmission electron microscopy showed significant inner dynein arm (IDA) deficiency in sperm flagella. Using immunofluorescence assays, we found a significant reduction in IDA-related proteins including DNAH10 and DNAH1.

Conclusions We identified putative novel pathogenic variants in *DNAH10* for MMAF, which might advance the genetic diagnosis and clinical genetic counselling for male infertility.

Keywords Male infertility · Flagellum · Asthenoteratozoospermia · *DNAH10*

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Introduction

Infertility is defined as the failure to achieve pregnancy after 12 months of normal intercourse without the use of contraception[1]. Infertility severely affects human reproductive health worldwide and male infertility accounts for approximately half of these abnormal phenotypes[2]. Asthenoteratozoospermia is an important type of male infertility characterized by the absence or loss of the motor ability of sperm, which consequently have reduced or no ability to interact with mature oocytes and complete the process of fertilization. Multiple morphological abnormalities of the flagella (MMAF) comprise one subtype of asthenoteratozoospermia, which is characterized by abnormal flagella including absent, short, coiled, bent, and irregular calibre flagella[3].

Typically, mature sperms consist of a head, neck, and tail flagella. The axoneme, which is made up of one central pair of microtubules surrounded by nine microtubules doublets (the so-called (9 + 2 structure), forms the flagellar core structure of flagella [4, 5]. Together with other structures, including dynein arms, radial spokes, and the nexin dynein regulatory complex, the 9 + 2 structure forms an axoneme[6]. In the flagella, the outer dynein arm (ODA) and inner dynein arm (IDA) are two types of axonemal dynein arms that hydrolyze ATP converting chemical energy into mechanical energy and regulate flagellar movement[6]. The dynein arm consists of dynein axonemal heavy chains (DNAH), dynein axonemal intermediate chains (DNAI), dynein axonemal light intermediate chains (DNALI), and dynein axonemal light chains (DNAL). Mutations in the *DNAH* and *DNAI* gene families have been associated with human disorders, including airway symptoms, left–right laterality disturbances, obstructive lung disease, and male infertility[6, 7]. The *DNAH* gene family comprises 13 members, including *DNAH1-3*, *DNAH5-12*, *DNAH14*, and *DNAH17*. Nine of these, *DNAH1*[3], *DNAH2*[8], *DNAH5*[9], *DNAH6*[10], *DNAH7*[11], *DNAH8*[12], *DNAH9*[13], *DNAH11*[14], and *DNAH17*[15, 16], are associated with ciliary disorders. The *DNAH10* encodes an IDA component of the flagella and is highly expressed in the human testis, which suggests its indispensable role in maintaining the normal function of sperm[17].

Recently, Tu et al. identified six variants of *DNAH10* in five patients with primary male infertility due to MMAF[18]. In this study, we found additional three *DNAH10* variants in two patients with typical MMAF phenotypes. We validated the pathogenicity of the bi-allelic variants using haematoxylin and eosin (H&E) staining, immunofluorescence staining, scanning electron microscopy (SEM), and transmission electron microscopy (TEM), which revealed abnormal spermatozoa morphology and ultrastructure.

Materials and methods

Sample collection

Patients with MMAF and infertility were recruited from the First Affiliated Hospital of Anhui Medical University. The spouses of the patients had a normal reproductive system but did not achieve a clinical pregnancy during more than 12 months of unprotected intercourse. We did not find primary ciliary dyskinesia associated abnormal symptoms, including airway symptoms, left–right laterality disturbances, obstructive lung disease, sinusitis, and otitis media. All patients showed normal somatic karyotypes (46, XY) and no pathogenic Y chromosome microdeletions (Table S1). We collected whole peripheral blood samples of the patients and performed WES to detect potential pathogenic variants, as described in our previous study[19]. This research was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University, and patients agreed to participate in the study and signed the informed consent form.

Semen and spermatozoa analysis

We collected and examined the semen and spermatozoa according to the World Health Organization (WHO) guidelines (5th Edition)[20]. Semen of patients was collected after more than 3 days of abstinence and was evaluated after liquefaction at 37 °C for 30 min. The standard parameters, including semen volume, sperm concentration, sperm motility, sperm progressive motility, and sperm morphology, were tested during routine clinical diagnosis. More than 200 spermatozoa from each individual were used to evaluate the percentages of sperm with morphological abnormalities.

Table 1 *DNAH10* bi-allelic pathogenic variants in male patients

Patient	AY0229	AY0150	AY0150
cDNA mutation	c.2514delG	c.10820 T > C	c.12692C > T
Protein alteration	p.L839*	p.M3607T	p.T4231I
Variant type	Homozygous	Heterozygous	Heterozygous
1000G_eas	0	0	0
gnomAD_exome_eas	0	0	0
gnomAD_gnome_eas	0	0	0
SIFT	-	-	-
PolyPhen-2	-	P	P
MutationTaster	-	D	D
CADD	-	25.7	25.1

NCBI accession number of DNAH10 is NM_207437. Abbreviations: *1000G_eas*, East Asian population of 1000 Genomes Project; *gnomAD*, the Genome Aggregation Database; *P*, possibly damaging; *D*, damaging; -, not available

Genetic risk factor identification by using WES and Sanger sequencing

We extracted DNA from whole peripheral blood of the patients. Exome targets were captured using Roche KAPA HyperExome Probes which covered the RefSeq, CCDS, Ensemble, GENCODE, and ClinVar databases, and sequenced with the MGISEQ-2000 platform. The original FASTQ data were mapped to the human genome (hg19) using BWA software, and SAMtools and GATK were used to call genetic variants. We annotated variants using allele frequency databases (1000G, gnomAD_exome, gnomAD_genome), deleterious prediction tools (SIFT, PolyPhen-2, Mutation Taster and CADD), and the GTEx database based

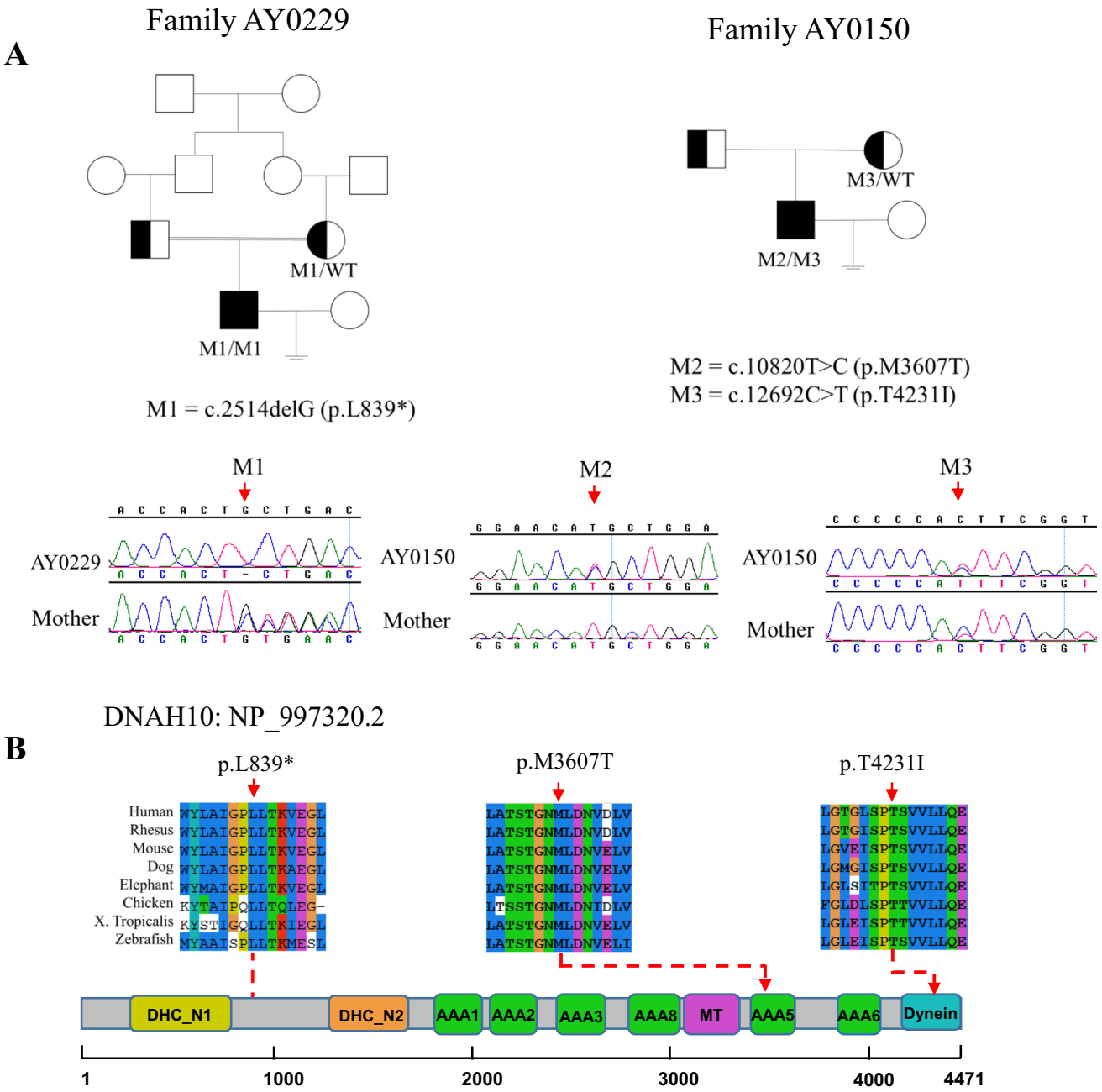

Fig. 1 Bi-allelic variants of DNAH10 in male patients. **A** Pedigrees of the two families carrying *DNAH10* variants (M1–M3). Male patients with multiple morphological abnormalities in the sperm flagella (MMAF) are indicated by a black square. The variants are indicated by a red arrow. WT, wild type.. **B** Upper panel: Conservation of mutated residues across eight species. Lower panel: Schematic representation of DNAH10 protein domains. The positions of the related amino acids encoded by *DNAH10* variants are indicated by red dotted lines

on ANNOVAR[21], VarCards[22], and dbNSFP[23]. Common variants with allele frequencies > 0.01 were excluded. We focused on loss-of-function variants including splicing (≤ 2 bp), stop-gain, stop-loss, and frameshift indel variants, as well as deleterious missense variants. Variants that were predicted to be deleterious by more than three of four tools including SIFT, PolyPhen-2, Mutation Taster, and CADD (score > 20) were defined as deleterious variants. The pathogenic variants of testis-specific expressed genes were used for further analysis. We defined testis-specific expression as an average expression value of ≥ 5 reads per kilobase per million map reads in the human testis and 2-folds higher than the average expression value in other tissues based on the GTEx. We screened putative bi-allelic variants or X-linked variants of the patients according to the recessive[7] or X-linked[24] model of MMAF. Sanger sequencing was used to validate the inheritance patterns (Table S2).

Immunofluorescence analysis

To observe the expression and location of DNAH10 and related proteins, we used immunofluorescence staining, as described in our previous studies[19, 24, 25]. First, spermatozoa were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% triton X-100 for 10 min, and blocked with serum for 1 h. Second, they were incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies at 37 °C for 1 h. The nuclei of spermatozoa were counter-stained with DAPI at 37 °C for 5 min. In between the two steps, the cells were washed with phosphate-buffered saline (PBS). Finally, the cells were washed with ddH_2O to remove the salts in PBS and to prevent crystal formation. The following primary antibodies were used in this study: anti-DNAH10 (bs-11022R, Bioss), anti-DNAH1 (Ab-122367, Abcam), anti-DNAI1 (BS90420, Bioworld), anti-AKAP4 (HPA020046, Sigma-Aldrich), anti-SPAG6 (HPA038440, Sigma), and anti-acetylated alpha-tubulin (T6793, Sigma). The following secondary antibodies were used in this study: anti-rabbit-Alexa Fluor-594 for anti-DNAH10, anti-DNAH1, anti-AKAP4, anti-SPAG6, and anti-mouse-Alexa Fluor-488 for anti-acetylated alpha-tubulin. Spermatozoa nuclei were stained with Hoechst. Images were detected with an LSM 800 confocal microscope (Carl Zeiss AG).

Electron microscopic analysis of spermatozoa

To analyze morphological and ultrastructural abnormalities of spermatozoa in detail, SEM and TEM were used as previously described[19]. Samples for SEM were fixed with 2.5% glutaraldehyde at 4 °C for 2 h. The fixator was removed and the cells were rinsed, dehydrated, and dried with PBS, an ethanol gradient, and hexamethyldisilazane. We observed the cells under a Nova Nano 450 scanning electron microscope (Thermo Fisher).

For TEM analysis, we fixed spermatozoa with 1% osmium tetroxide and dehydrated them with graded ethanol. The spermatozoa were infiltrated with acetone and SPI-Chem resin and embedded with Epon 812. We cut the samples using an ultramicrotome and stained the sections using uranyl acetate and lead citrate. We observed the stained sections under a TecnaiG2 Spirit 120 kV transmission electron microscope.

Results

Bi-allelic pathogenic variants of DNAH10 in men with MMAF

We performed WES of specimens from male patients with MMAF and found bi-allelic pathogenic variants of *DNAH10* in two unrelated patients (Table 1, Fig. 1). Patient AY0229 was from a consanguineous family and carried a homozygous frameshift variant (NM_207437: c.2514delG:p.L839*) which was absent in the general population (1000G, gnomAD_exome_eas, gnomad_genome_eas). Patient AY0150 harboured two missense variants (NM_207437:c.10820 T > C: p.M3607T; NM_207437:c.12692C > T:p.T4231I), which were determined to be rare or absent in the general population. These two missense variants were also predicted to be deleterious by the PolyPhen-2, MutationTaster, and CADD tools. Sanger sequencing was performed to validate the inheritance patterns of these variants. AY0229 and his biological mother were homozygous and heterozygous carriers of c.2514delG:p.L839*, respectively. Moreover, AY0150 was a heterozygous carrier of both variants (NM_207437: c.10820 T > C:p.M3607T; NM_207437:c.12692C > T:p.T4231I) and his mother was a heterozygous carrier of

Table 2 Semen and spermatozoa parameters of male patients

Patient	AY0229	AY0150	Reference
Volume (ml)	2	2	≥1.5
Concentration (10^6/ml)	145	43.5	≥15
Motility (%)	6.3	13.3	≥40
Progressive motility (%)	3.8	2.4	≥32
Flagella analysis			
Normal (%)	35.3 (71)	23.0 (46)	> 23
Short (%)	27.4 (55)	34.5 (69)	< 1
Absent (%)	3.5 (7)	4.5 (9)	< 5
Coiled (%)	19.9 (40)	21 (42)	< 17
Angulation (%)	8.0 (16)	12 (24)	< 13
Irregular caliber (%)	6.0 (12)	5 (10)	< 23

The reference ranges of morphologically abnormal spermatozoa observed in fertile individuals [33]. The corresponding sperm number was in bracket

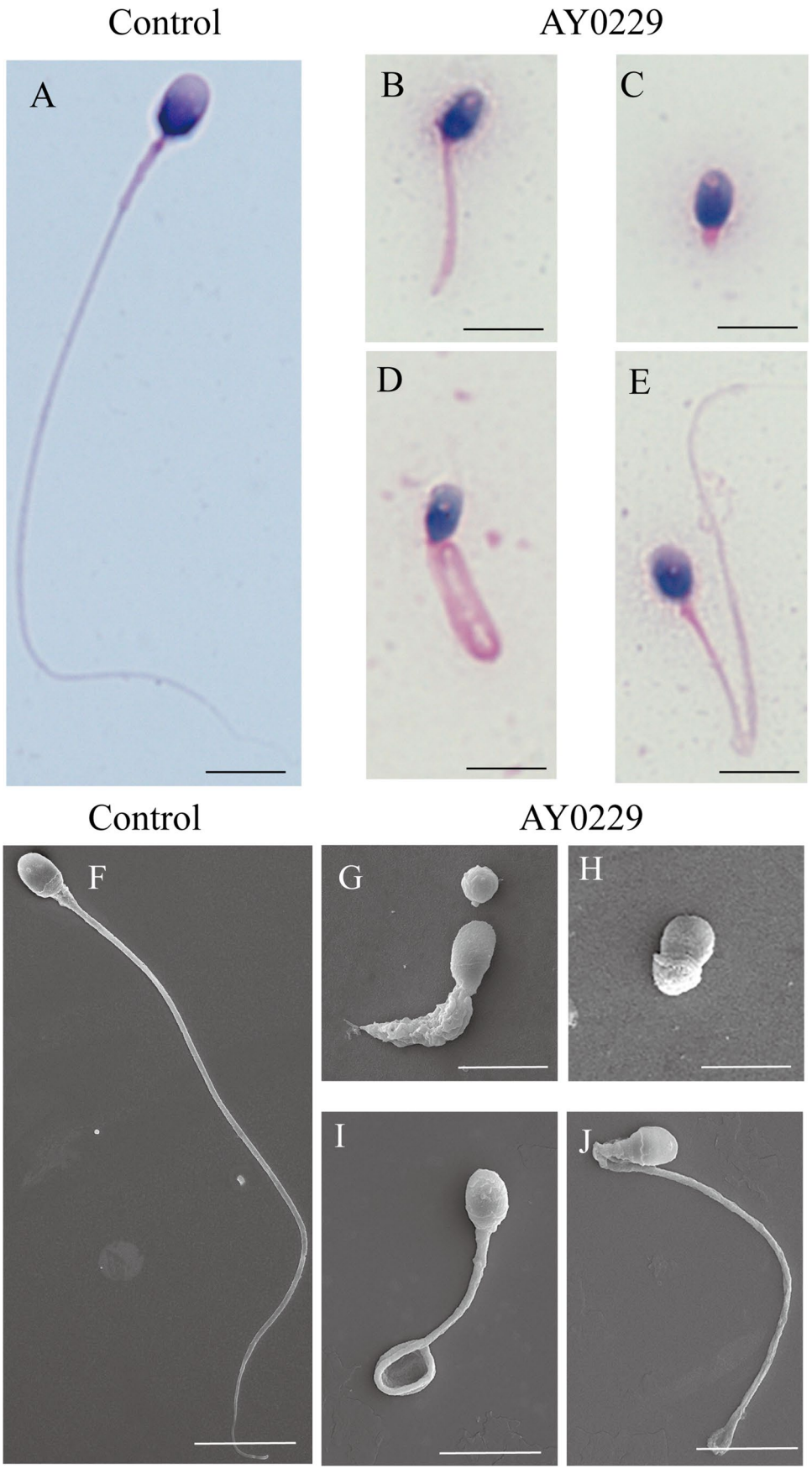

Fig. 2 Morphology of spermatozoa in a patient carrying a *DNAH10* variant. **A**–**E** and **F**–**J** show haematoxylin and eosin staining and scanning electron microscopy images of the spermatozoa, respectively. **A** and **F** Typical spermatozoa from unaffected control. **B** and **G** Short flagella. **C** and **H** Absent flagella. **D** and **I** Coiled flagella. **E** and **J** Angulated flagella. Representative images of patient AY0229 are shown. Scale bars = 5 μm

NM_207437:c.12692C > T:p.T4231I. Owing to the lack of paternal DNA samples from two families, we could not validate the inheritance mode. We presumed that the two fathers were heterozygous carriers of NM_207437: c.2514delG:p.L839* and NM_207437:c.10820 T > C:p.M3607T, respectively, conforming with the recessive inheritance mode.

DNAH10 (dynein, axonemal, heavy chain 10; OMIM: 605,884) is located on human chromosome 12q24.31 and contains the 77 exons encoding IDA heavy chain protein[17]. Based on GTEx and Human Protein Atlas data, DNAH10 is highly expressed in the testis. Previously, it was found that the knockdown of *DNAH10* results in motility defects in *Trypanosoma brucei*[26].

Asthenoteratozoospermia phenotypes in men carrying bi-allelic DNAH10 variants

We further explored the semen parameters of patients carrying bi-allelic *DNAH10* variants (Table 2). Compared to that of the reference standard, the sperm concentration of the patients was normal. However, we found a significant reduction in sperm motility (AY0229:6.3%; AY0150:13.3%) and progressive motility (AY0229:3.8%; AY0150:2.4%). We performed morphological analysis based on H&E staining. Compared to unaffected controls, the spermatozoa of patients carrying *DNAH10* variants exhibited disrupted sperm flagella, including absent, coiled, short, angulated, and irregular calibre flagella (Table 1, Fig. 2A–E, Figure S1). Abnormal sperm flagella represented 65% (130/201) and 77% (154/200) of total flagella in AY0229 and AY0150, respectively. Coiled flagella and short flagella accounted for 73% (95/130) and 72% (111/154) of these abnormalities, respectively. We validated the abnormal flagella of spermatozoa in AY0029 using SEM and found consistent phenotypes (Fig. 2F–J).

To validate the disruptive function of DNAH10 variants in sperm, we explored the location and expression of DNAH10 protein in patients and unaffected individuals using immunofluorescence. We found that DNAH10 was specifically expressed in the sperm flagella of unaffected individuals but was significantly reduced in the patients carrying bi-allelic *DNAH10* variants (Fig. 3). These results indicated that MMAF might be due to a defect in DNAH10.

Dysfunctional IDAs and related protein DNAH1 in spermatozoa of patients

We further investigated the sperm flagellar ultrastructure in patients carrying *DNAH10* variants and unaffected controls by using TEM. Compared with the typical "9 + 2 structure" as observed in the controls, the axonemal ultrastructure from the patient carrying *DNAH10* variant exhibited significant IDA deficiency (Fig. 4). We further investigated whether defects in DNAH10 affect other sperm flagella

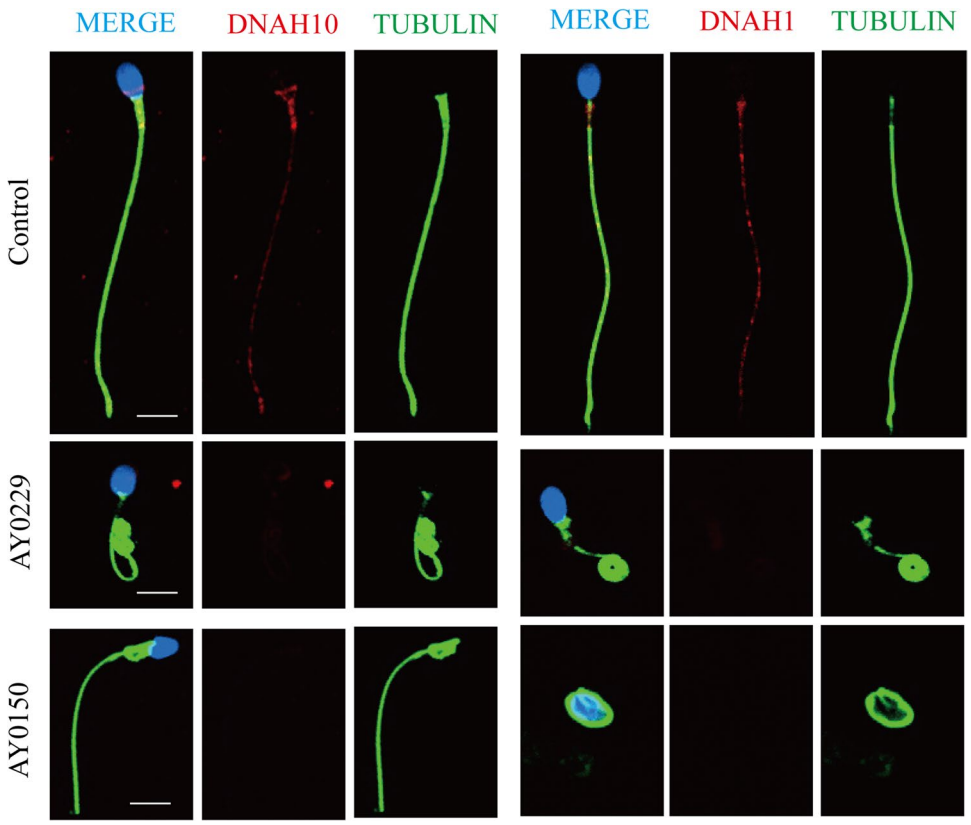

Fig. 3 Expression and location of DNAH10 and DNAH1 in spermatozoa from patients and unaffected control. **A** Spermatozoa were stained with anti-DNAH10 (red) and anti-α-tubulin (green). **B** Spermatozoa were stained with anti-DNAH1 (red) and anti-α-tubulin (green). Cell nuclei were stained with DAPI. Scale bars = 5 μm

ultrastructure–related proteins, including DNAH1 (IDA), DNAI1 (ODA), SPAG6 (central pair of microtubules, CP), and AKAP4 (fibrous sheath). The expression of DNAH1 was significantly reduced, which was consistent with the defective IDA phenotype (Fig. 3). The expression of AKAP4, DNAI1, and SPAG6 in the spermatozoa of patients was similar to that in the unaffected control (Figures S2-S4).

Discussion

The high contribution of genetic factors to male infertility provides an opportunity to decode related genetic aetiologies[27]. Candidate genes with recessive inheritance patterns have been widely accepted to contribute to the aetiology of male infertility[7]. In this study, we attempted to identify pathogenic variants involved in male infertility using high-through sequencing and found three pathogenic variants of *DNAH10* in two unrelated patients. The *DNAH10* variants were extremely rare or absent in the 1000G and gnomAD databases. The DNAH10 was specifically highly expressed in the testis at both the RNA and protein level. Functional studies revealed that the variants in *DNAH10* result in MMAF phenotypes.

IDA plays an indispensable role in spermatozoa motility. There are six IDA-related proteins including DNAH1-3, DHAN6, DNAH7, and DNAH12 in the dynein of the axoneme[28]. DNAH1, DNAH2, and DNAH6 are highly expressed in the testis and lungs and are involved in male infertility or primary ciliary dyskinesia[3, 8, 10]. Bi-allelic variants in *DNAH1*[3] and *DNAH2*[8] lead to male infertility

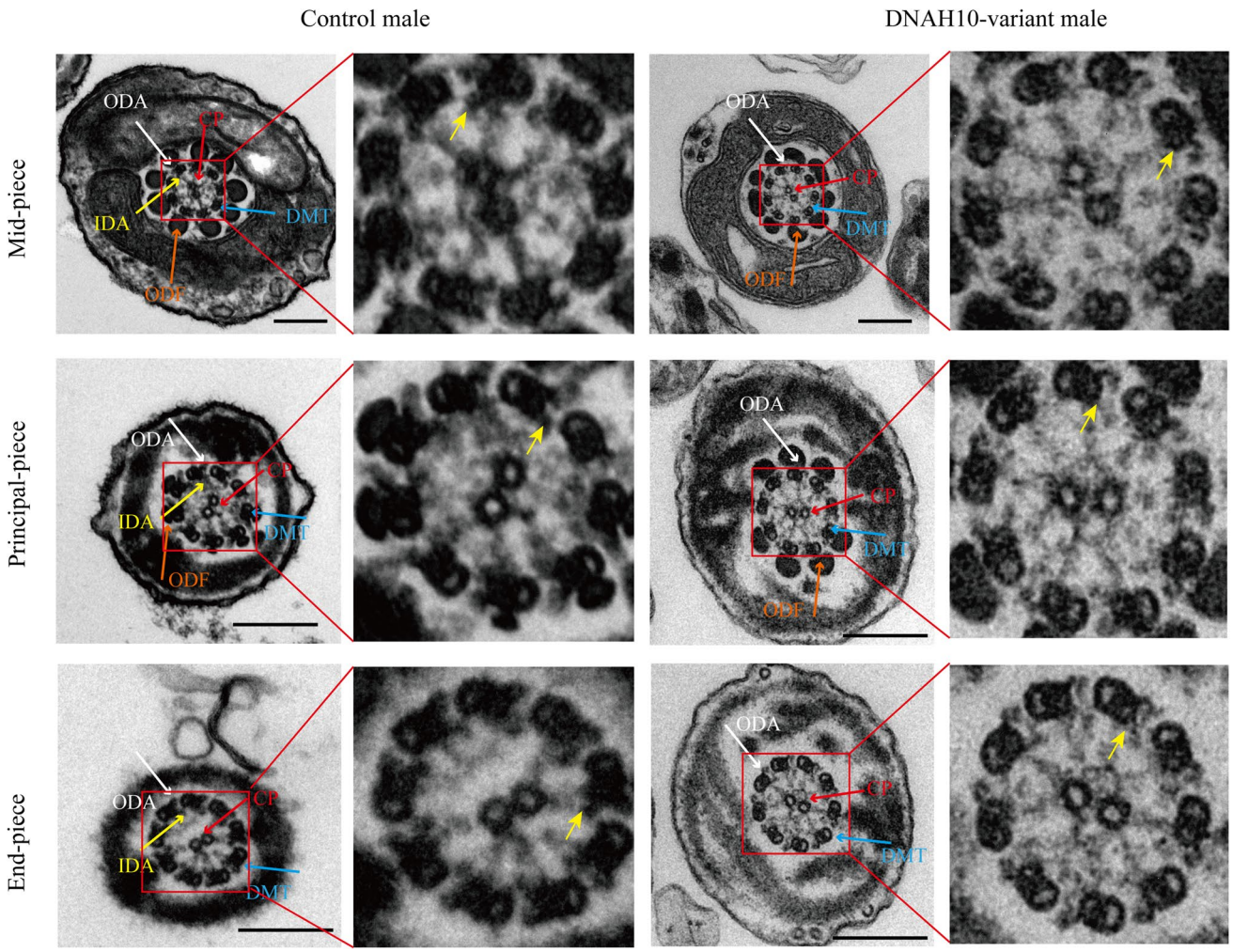

Fig. 4 DNAH10 deficiency associated with sperm inner dynein arms (IDA) defects. Transmission electron microscopy analysis of spermatozoa obtained from patient AY0229 and an unaffected control. Abbreviations: DMT, peripheral doublet microtubules (indicated by blue arrows); CP, central pair of microtubules (indicated by red arrows); ODA, outer dynein arms (indicated by white arrows); IDA, inner dynein arms (indicated by yellow arrows); ODF, outer dense fibres (indicated by orange arrows). Most of the sperm IDA of the patient was defective (yellow arrows in enlarged figures). Scale bars = 200 nm

without other primary ciliary dyskinesia-related symptoms, whereas variants in *DHAN6* are found in patients with male infertility[29, 30] and other primary ciliary dyskinesias (PCD), such as heterotaxy[10]. Tha association between DNAH10 and cilia has been reported in *Trypanosoma brucei*, in which motility defects occurred due to the knockdown of *DNAH10*[26]. Recently, Tu et al. identified six variants of *DNAH10* in five patients with primary male infertility due to MMAF[18]. All the patients carrying *DNAH10* variants did not exhibit the occurrence of other PCD symptoms. These results might indicate the different functions of IDA proteins or related transcripts due to the different locations of variants[18, 31, 32].

Variants in both *DNAH1* and *DNAH2* result in abnormal morphology in more than 90% of spermatozoa in patients, and the ultrastructure of spermatozoa exhibits IDA loss, mitochondrial sheath defects, and microtubule doublet disruption[3, 8]. In this study, we found that more than 60% of the sperm presented with abnormal flagella in two unrelated patients carrying bi-allelic *DNAH10* variants. However, ultrastructural analysis revealed the IDA deficiencies in these two patients carrying *DNAH10* variants, which differs from the obvious mitochondrial sheath defects and microtubule doublet disruptions in patients carrying *DNAH1* and *DNAH2* variants. Currently, Tu et al. found IDA deficiencies and the disorganization of the axonemal structure including a missing CP and microtubule doublets in patients carrying bi-allelic *DNAH10* missense variants, which was slightly different compared to that in the patients in this study. However, two patients carrying bi-allelic *DNAH10* frameshift variants did not exhibit significant loss of CP and microtubule doublets which indicated that these disruptions might not be the major results of *DNAH10* variants[18]. In addition, immunofluorescence analysis of spermatozoa of patients carrying *DNAH2* and *DNAH10* variants only revealed significant deficiencies in IDA-related proteins DNAH10, DNAH2, DNAH1, and DNALI1. These findings again indicated that the abnormal structure of doublet microtubules, mitochondrial sheath defects, and CP are not directly affected by IDA-related proteins.

In conclusion, we identified bi-allelic variants of *DNAH10* in two male patients with MMAF. We observed reduced motility and the abnormal morphology of spermatozoa in these two patients. Ultrastructural and immunofluorescence analyses revealed deficiencies in IDA and the related proteins DNAH10 and DNAH1. Taken together, our results link *DNAH10* gene variants with MMAF and expand the genetic spectrum of male infertility.

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Author contribution XH, YC designed and supervised the study. KL, GW, WJ, YG, FT, CX, WY, HY, SZ, HG, QT, QS, DT, ZZ participated in literature search and clinical data collection. KL, GW, ML, RH performed genetic analysis, functional assay, and figure and table preparation. KL and GW wrote the manuscript with input from all authors. BS, HW, YX, PZ, FT, ZW, XH, YC participated in the review of the manuscript and offered valuable advices. All authors read and approved the final manuscript.

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Availability of data and material The data of this study will be shared upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

Web resources dbNSFP: http://database.liulab.science/dbNSFP

GTEx: https://gtexportal.org/
Human Protein Atlas: https://www.proteinatlas.org/
1000 Genomes Project: http://www.internationalgenome.org
gnomAD: https://gnomad.broadinstitute.org
CADD: https://cadd.gs.washington.edu/snv
Mutationtester: http://www.mutationtaster.org/
PolyPhen: http://genetics.bwh.harvard.edu/pph2/
SIFT: https://sift.bii.a-star.edu.sg

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