Original Article

Novel bi-allelic mutations in *DNAH1* cause multiple morphological abnormalities of the sperm flagella resulting in male infertility

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Background: Male infertility is a major health concern and approximately 10–15% of cases are caused by genetic abnormalities. Defects in the sperm flagella are closely related to male infertility, since flagellar beating allows sperm to swim. The sperm of males afflicted with multiple morphological abnormalities of the flagella (MMAF) possess severe defects of the sperm flagella, may impair sperm motility and lead to male infertility. Currently, known genetic defects only account for MMAF in about 60% of patients and need more intensive efforts to explore the relationship between genes and MMAF.

Methods: The whole-exome sequencing (WES) was performed to analyze the genetic cause of the MMAF patient. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to observe the morphology of sperm cells and to identify the ultrastructural characteristics of the flagella in the patient. The expression of DNAH1 was analyzed by sperm immunofluorescence staining.

Results: We identified the negative effects produced by the *DNAH1* mutations c. 8170.C>T (p. R2724*) and c. 4670C>T (p. T1557M) on *DNAH1* expression and the development of sperm flagella.

Conclusions: Our findings suggest that *DNAH1* is associated with the formation of sperm flagella and homozygous loss-of-function mutations in *DNAH1* can impair sperm motility and cause male infertility.

Keywords: Multiple morphological abnormalities of the flagella (MMAF); whole-exome sequencing (WES); male infertility

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Introduction

Male infertility is a major health concern and approximately 10–15% of cases are caused by genetic abnormalities (1). Defects in the sperm flagella are closely related to male infertility, since flagellar beating allows sperm to swim. The sperm of males afflicted with multiple morphological abnormalities of the flagella (MMAF) presents severe defects, including bent, short, coiled, absent, and/or irregular flagella that may fully or partially impair sperm motility. The cytoskeleton of motile flagella possesses a (9+2) axonemal configuration, consisting of nine outer microtubule doublets (ODs) and two central microtubules (CPs) surrounded by fibrous sheath (FS) and outer dense fiber (ODF) (2). The typical features of MMAF include an abnormal ultrastructure of axons and absence of ODF and the FS in the flagella, as well as stump or short tails and dysplasia of the fibrous sheath (DFS), causing partial or complete asthenozoospermia.

Developmental disturbances may be responsible for abnormalities in mitochondrial sheath, failure of the flagella to properly elongate, and the characteristic absence of CPs in MMAF with patients. The development of MMAF has been linked to many genes, including *CFAP65*, *CFAP44*, *CFAP43*, *CFAP69*, *DNAH1*, *CFAP251*, *AK7*, *CEP135*, *QRICH2*, *FSIP2*, *TTC21A*, *SPEF2*, *ARMC2*, *WDR66*, *AKAP4*, and *CCDC39* (3-19). However, known genetic defects account for only 60% of patients with MMAF. The causes of the remaining MMAF cases remain unknown, which could indicate uncharacterized genetic defects. Therefore, the relationship between genetic abnormalities and MMAF should be further scrutinized.

DNAH1 (MIM 603332), which encodes an inner dynein arm heavy chain, is the first axonemal gene responsible for infertility phenotype without any of the other symptoms usually observed in primary ciliary dyskinesia (PCD) and is also highly associated with immotile sperm. The DNAH1 protein connects the outer doublet and radial spokes of sperm, while the central doublets are localized and stabilized by the radial spokes (20). Pathogenic mutations or the absence of *DNAH1* can result in the absence or dysfunction of the central doublets in the axoneme. Loss-of-function mutations in *DNAH1* are known to lead to MMAF in humans and mice, seeing that they are harmful to the organization and biogenesis of the sperm axoneme (6).

There is limited information on the pathogenic variants of *DNAH1* in the Chinese population, and understanding this association may lead to a greater understanding of MMAF. We discovered a hereditary homozygous *DNAH1* mutation in a patient with MMAF and successfully identified a novel homozygous nonsense mutation in *DNAH1* by whole-exome sequencing (WES). We identified the negative effects produced by the *DNAH1* mutations c. 8170.C>T (p. R2724*) and c. 4670C>T (p. T1557M) on *DNAH1* expression and the development of sperm flagella. Our findings suggest that *DNAH1* is related to the formation of sperm flagella, and that homozygous mutations in *DNAH1* can damage sperm motility and lead to male infertility. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tau-20-1434).

Methods

Study participants

A 28-year-old Han Chinese man was diagnosed with infertility 2 years before our study, who was from a consanguineous family. The patient's wife did not have any fertility-related disorders, and his parents were also evaluated. The control sample included 200 unrelated Han Chinese male volunteers, who had naturally conceived at least one child and had normal sperm quality according to the World Health Organization index (WHO-5) (sperm concentration ≥15 million/mL; progressively motile sperm ≥32%; normal sperm morphology ≥4%). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of West China Second University Hospital (No. 2019019) and informed consent was taken from the patient and all members of his family.

WES and Sanger sequencing

The DNA of the patient was analyzed using WES. Briefly, genomic DNA was separated from peripheral blood samples using the FitAmp Plasma/Serum DNA Isolation Kit (EpiGentek), exon capture was performed using the the Agilent SureSelect Human All Exon V6 Kit, and an Illumina HiSeq X system was used for sequencing. ANNOVAR was used for functional annotation and further filtering was performed using the 1000 Genomes Project, Human Gene Mutation Database (HGMD), database of single nucleotide polymorphisms (dbSNP), and Exome Aggregation Consortium (ExAC). After filtering, functional predictions for the retained nonsynonymous single nucleotide variants

(SNVs) were performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), SIFT (https://sift.bii.a-star.edu.sg), MutationTaster, and CADD. Using Sanger sequencing, the patient's parents, as well as the control subjects, were tested for the candidate pathogenic gene variants identified in the patient. The ProFlex PCR System (Thermo Fisher Scientific) was used for polymerase chain reaction (PCR) amplification and the DNA sequencing of PCR products was conducted on an ABI377A DNA sequencer (Applied Biosystems). The PCR primers were: DNAH1-1: 5'-GGC CAACCTCATGGCTGCTTACACAG-3' and 5'-CCAAG GGCCTGAGCTTCAGCAAGAC-3', DNAH1-2: 5'-CC GTGAGGAGGCCTCAGAGGAAACTC-3' and 5'-CTG TCCTTGAGGTTCTGGGACAAGC-3'.

Immunofluorescence staining

The sperm samples were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and sealed with 5% bovine serum albumin (BSA); they were then incubated with primary antibodies (1:50, DNAH1, sc-102481, Santa Cruz; 1:50, CL48866031, α-tubulin, Proteintech) overnight at 4 ℃. On the next day, samples were washed with 1× PBS three times, incubated with DyLight 488-labeled secondary antibodies (1:800, 1927937, Thermo Fisher Scientific) for 1 h at 25 ℃, and then the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were obtained using laser scanning confocal microscopy (Olympus).

Electron microscopy

The sperm samples were prepared using Sperm Washing Medium (SpermRinse, Vitrolife), mounted onto slides, and evaluated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Results

MMAF phenotype identified in an infertile patient

The proband, a 28-year-old man from a consanguineous family, had been diagnosed with infertility 2 years before our study. His semen samples were submitted to examination three times. As shown in *Table 1*, the sperm count was a very low, and most cells were immobile because of the completely defective flagella. These abnormalities were confirmed by Papanicolaou staining. Specifically, spermatozoa from the patient displayed typical MMAF phenotypes, including short, absent, coiled flagella with an irregular caliber, rather than the long, thin tails of normal sperm (*Figure 1A*). A more vivid MMAF phenotype was revealed via SEM (*Figure 1B*), and the patient's spermatozoa were further analyzed using TEM. Remarkably, the normal sperm flagella consists of 9+2 axons, including nine ODs and two CPs, ODF and FS; this patient's microtubules showed a significant disorder in the axons and other surrounding components. The abnormalities included missing CPs, and disordered OD arrangements in the midpiece, principal, and end pieces (*Figure 1C*). Therefore, we speculate that the observed MMAF phenotype is the cause of infertility in this patient.

Bi-allelic loss-of-function mutations of DNAH1 in an infertile male with MMAF

To elucidate the genetic cause of MMAF, our study performed WES analysis on this affected male subject. Based on the autosomal-recessive inheritance of MMAF, variants were excluded if the following conditions were met: (I) in the ExAC Browser, gnomAD or the 1000 Genome Project, minor allele frequency greater than or equal to 1%, because the pathogenic variants that cause MMAF are rare in humans; (II) no harmful variant detected using SIFT, PolyPhen-2, or Mutation Taster tools; (III) except for the canonical splice sites, all variants located in noncoding exons, 30 or 50 untranslated regions, or intronic sequences; (IV) the variant is heterozygous. As a result, we identified two *DNAH1* bi-allelic pathogenic variants in the MMAF-affected man: [c. 8170.C>T (p. R2724*) and c. 4670C>T (p. T1557M)] (*Figure 2A*), and these mutations were further verified through Sanger sequencing of this patient and his parents (*Figure 2B*). Notably, these bi-allelic pathogenic variants in *DNAH1* were not detected in the 200 normal controls, and the two mutation sites are 100% conserved among many species (*Figure 2C*). Therefore, we conclude that these bi-allelic variants in DNAH1 were the cause of MMAF in this patient.

Effects of the bi-allelic variants on the DNAH1 protein

To determine the negative effects of the two variants on *DNAH1* expression, we performed immunofluorescence analysis on the sperm samples of patients and normal controls using an anti-DNAH1 antibody. As expected, full-length *DNAH1* was detected in the sperm flagella of the

Table 1 Semen parameters of patient and normal control

Semen parameters	Patient	Normal control	Normospermic parameters
Sperm volume (mL)	4.8	3.5	≥1.5
Sperm concentration (million/mL)	1.8	61	≥15
Motility sperm (%)	1	52	≥40
Vitality (%)	1	71	≥58
Normal spermatozoa (%)	0.5	75	≥4
Absent flagella (%)	31	2.3	–
Short flagella (%)	25	9	–
Coiled flagella (%)	13.5	8.5	–
Bent flagella (%)	3	3.7	–
Flagella of irregular caliber (%)	27	1.5	–

control group (*Figure 3A*). However, DNAH1 staining was hardly detected in the spermatozoa of the patient, indicating that the absence of *DNAH1* expression was probably caused by the bi-allelic variants. To uncover the mechanism that led to a lack of *DNAH1* expression, we analyzed the characteristics of the bi-allelic variants. The c. 8170. C>T (p. R2724*) mutation, which results in a truncated protein owing to a premature termination codon, can be clearly linked to protein degradation. On the other hand, c. 4670C>T (p. T1557M) is a missense mutation that, may lead to DNAH1 protein degradation by mechanisms other than affecting the transcription or translation processes. Considering that *DNAH1* is a vital structural protein, we hypothesized that this mutation may change protein conformation. Therefore, we predicted the conformational changes in the DNAH1 protein induced by this mutation using SWISSMODEL4.0 (https://swissmodel.expasy.org/) and PyMoL software 4.1 (1.3r1, DeLano Scientific LLC). Strikingly, the molecular modeling showed that the spatial structure of *DNAH1* was completely changed by this mutation, including random coils, α-helices and β-sheets (*Figure 3B*). Altogether, our results showed that the deficiency in *DNAH1* expression was induced by the bi-allelic loss of function mutations that led to an MMAF phenotype in this infertile man.

Discussion

In 2001, asthenozoospermia owing to *DNAH1* mutations were identified (4). MMAF became a recognized disorder in 2014, and several pathogenic variants of *DNAH1* associated with sperm flagella development were recognized as potential contributors to MMAF (5,7,21-24). We identified bi-allelic loss-of-function mutations in *DNAH1* from an infertile male with MMAF by WES. This mutation led to the absence of DNAH1 protein in sperm flagella resulting in abnormal flagellar development and irregular sperm ultrastructure.

The *DNAH1* gene is composed of 79 exons, located on chromosome 3p21.1, with a size of 13,126 bp. In spermatozoa, *DNAH1* is involved in the formation of the internal dynamic armis, which consists of seven molecular complexes arranged in a 3-2-2 fashion into three different types of internal dynamic arms (IDA1 to IDA3). DNAH1 is an integral part of IDA3, which provides an anchoring site for radial spoke 3 (RS3). Missing outer dynein arms suggest that DNAH1 may localize between RS3 and outer dynein arms on microtubules (25). The absence of DNAH1 results in the lack of an RS3 anchoring site, which weakens the attachment of the CPs. The radial spokes are considered to be related to the location/stability of the central bimodal. When DNAH1 is absent or dysfunctional, the axon is severely disorganized, usually as a (9+0) structure (6). CP microtubule abnormalities are the most frequently observed ultrastructural abnormalities in genetically uncharacterized MMAF patients (26). Additionally, Abnormal sperm head morphology was present in 21.8% of MMAF patients with *DNAH1* mutations (21). In *DNAH1* knockout mice, TEM revealed a missing globule of the IDA3 head, resulting in a 3-2-1 arrangement (6). DNAHc1 (the ortholog of DNAH1) knockout mice showed decreased sperm motility and infertility (27). When most of sperm are alive but

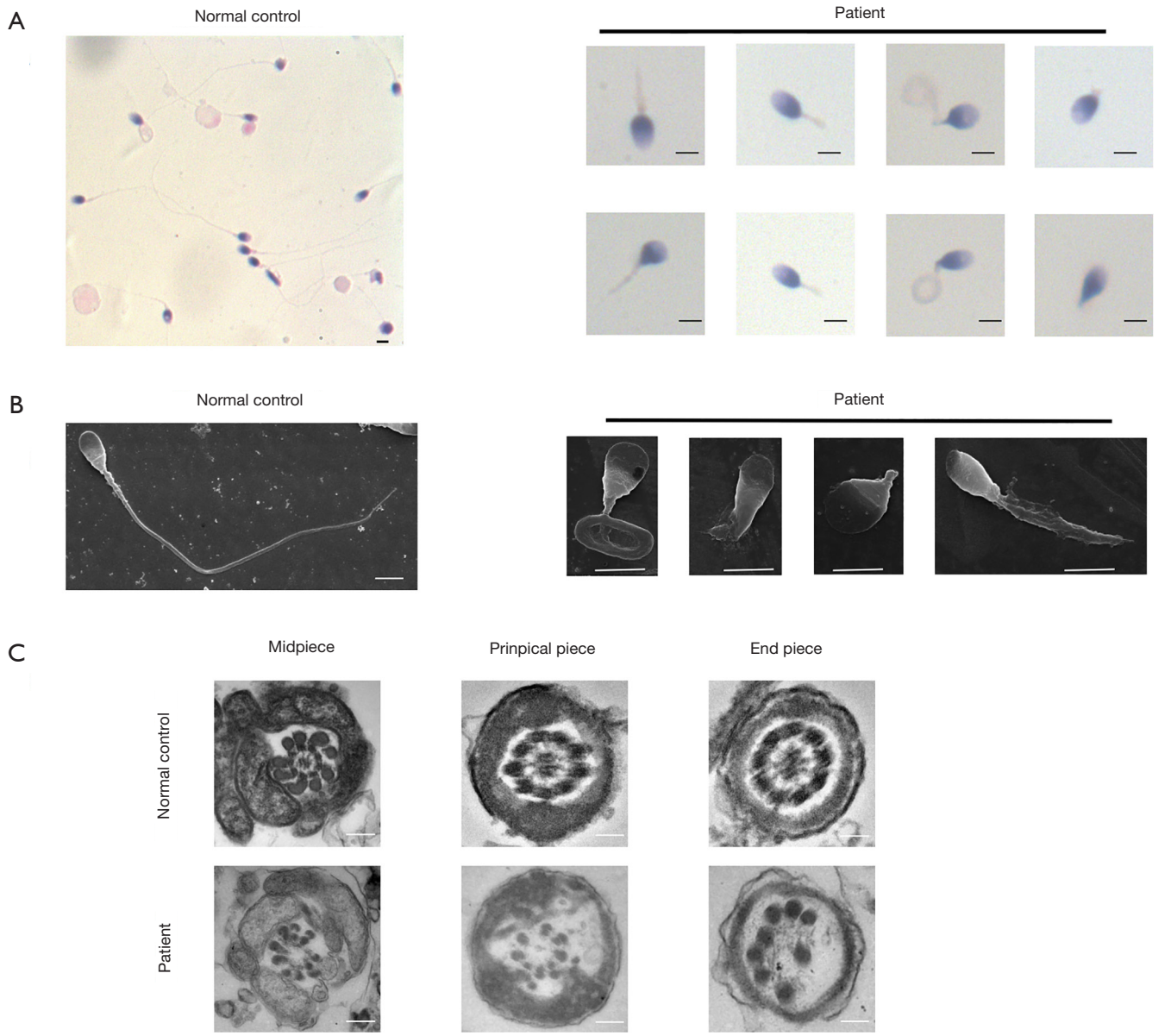

Figure 1 The MMAF phenotype was identified in the patient. (A) Papanicolaou staining of patient sperm cells showed short, absent, coiled flagella and irregular caliber (scale bars, 5 μm). (B) The patient sperm flagella showed typical MMAF phenotype by SEM (scale bars, 5 μm). (C) TEM results of the patient showed the abnormal ultrastructure of sperm flagellum: the central microtubule was missing, and the outer microtubule doublet was disordered arrangement in midpiece, principal piece, and end piece (scale bars, 100 nm). MMAF, multiple morphological abnormalities of the flagella; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

immotile, this is usually due to structural defects in the sperm flagellum (26). Variants introns 31 and 73, and in exons 23 and 78 of the *DNAH1* gene resulted in severe asthenospermia in MMAF without any PCD symptoms (6).

Mutated *DNAH1* gene leads to various morphological abnormalities of sperm flagellum, causing male infertility (6,20,28,29). Mutant *DNAH1* variants are the first gene abnormalities to be universally acknowledged as causing MMAF and are estimated to account for up to a third of MMAF cases (6). The combination of *DNAH1* gene mutations is supported by pedigree analysis: 52430998CCT>C in exon 73, 52409336C>T in exon

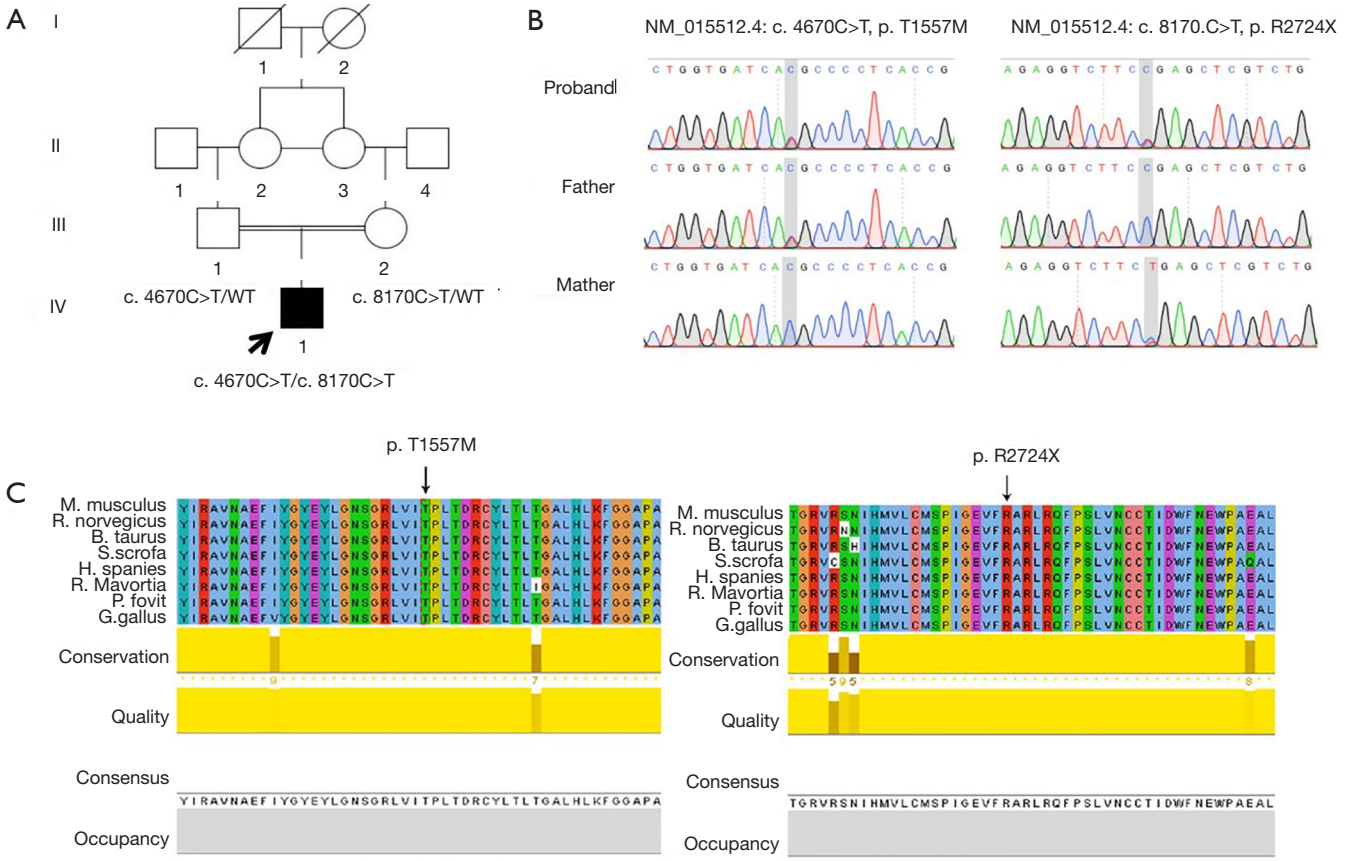

Figure 2 The bi-allelic pathogenic mutations of *DNAH1* in a consanguineous family. (A) Family pedigree. Squares represent male pedigree members, circles represent female pedigree members, and the black square represents the proband. Open symbols represent unaffected members. (B) Sanger sequencing of (c. 4607C>T) and (c. 8170C>T) were confirmed by PCR-sequencing in this family. (C) Multiple sequence alignment of the DNAH1 protein for different species. The black arrow denotes the position of the variants. PCR, polymerase chain reaction.

45, and 52428484G>T in exon 67 alone were related with MMAF and infertility in the ethnic Han Chinese population (22). A study reported that the homozygous frame-shift deletion DNAH1: exon73:c.11726_11727delCT caused MMAF, impairing sperm motility (23). This frame-shift mutation resulted in the disorder of downstream amino acid sequence of 3909 aa and truncation of the DNAH1 protein. This severely damaged the dynein heavy chain domain at the c-terminal of the protein and the tertiary structure of DNAH1, resulting in its dysfunction (23). Three asthenoteratozoospermia patients, 2.09% of the patients tested using bioinformatics analyses, carried four pathogenic *DNAH1* variants (5). The other two mutations were missense mutations, which were c. 6446T>G (g. 52404762T>G) and c. 7205C>A (g. 52412624C>A), may adversely affect protein function (5). Mouse with dynein heavy chain 7 (MDHC7) defects revealed that disruption of the *MDHC7* gene caused asthenozoospermia and reduced the frequency of cilia beats, but was not associated with any serious defects in axonemal structure. The data we present here in accordance with the previous studies of human HDHC7 (6,28).

The progressive motility of sperm with *DNAH1* mutations is higher than with other genetic causes for MMAF, ranging from 0% to 13% (25). Three independent semen tests performed within 6 months did not find any progressively motile sperm in our patient's samples; however, he had normal sperm survival rates. *DNAH1* is highly expressed in testis and is necessary for the formation of the inner dynein arms (6). The absence of *DNAH1* is very harmful to the organization and biogenesis of the axoneme. This result was expected given the important

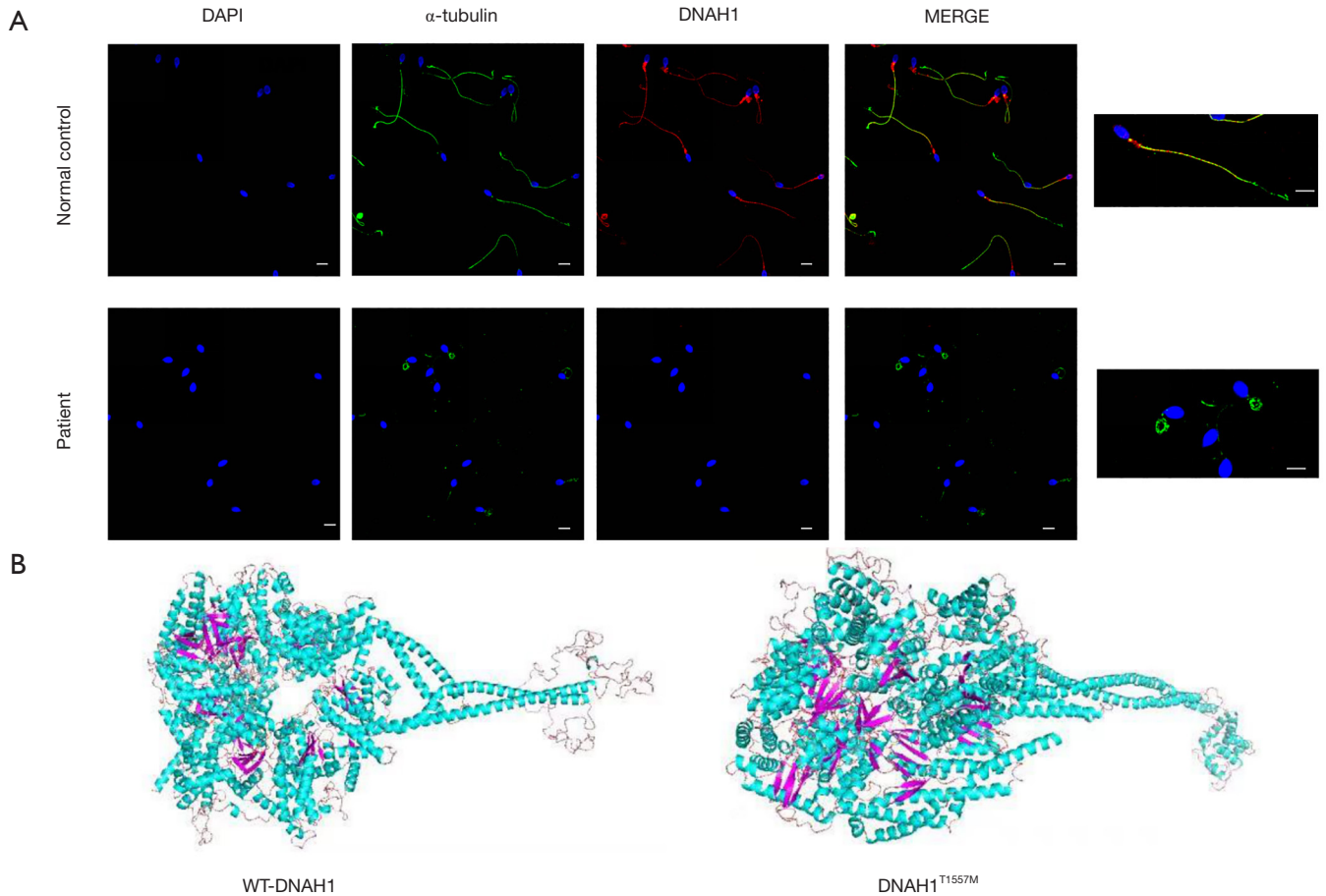

Figure 3 The influence of the bi-allelic mutations on DNAH1 protein. (A) Immunofluorescence staining in the sperm cells reveals an absence of DNAH1 protein in the patient compared to the normal control. (green, α-tubulin; red, DNAH1; blue, DAPI; scale bars, 5 μm). (B) The spatial structure of DNAH1 was totally changed by the c. 4670C>T (p. T1557M) mutation, including random coils, α-helix and β-sheet.

role of inner dynein arms in sperm motility. Although other dynein heavy chains may compensate for the absence of functional cilia owing to *DNAH1* mutations in other structures, *DNAH1* is critical for the proper functioning sperm flagella (6). Sperm flagellates and cilia, although similar in structure, differ significantly in axonal tissue and biogenesis (6). The role of DNAH1 in cilia may not be as crucial as it is in sperm flagella, and this may be evidenced by its lower levels of normal expression in other structures, including the trachea, compared to the testis. This allows the differentiation between *DNAH1* mutant MMAF patients and PCD patients because PCD typically has other severe symptoms in addition to infertility. Collectively, this supports our findings that *DNAH1* is essential for flagellar development in sperm.

In conclusion, in this study, we identified two novel bi-allelic pathogenic mutations of *DNAH1* gene in an infertile male, thus expanding the known spectrum of *DNAH1* gene mutations, further helping identify the pathogenesis and causative variation affecting MMAF-induced male infertility, finally providing beneficial diagnosis and prognosis for male infertility.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of West China Second University Hospital (No. 2019019) and informed consent was taken from the patient and all members of his family.

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