GENETICS

Novel *DNAAF6* variants identified by whole-exome sequencing cause male infertility and primary ciliary dyskinesia

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Abstract

Purpose To identify the genetic cause of patients with primary ciliary dyskinesia (PCD) and male infertility from two unrelated Han Chinese families.

Methods We conducted whole-exome sequencing of three individuals with PCD and male infertility from two unrelated Chinese families, and performed a targeted look-up for *DNAAF6* variants in our previously reported cohort of 442 individuals (219 with isolated oligoasthenospermia and 223 fertile controls). Ultrastructural and immunostaining analyses of patients' spermatozoa were performed. The pathogenicity of the variants was validated using patient's spermatozoa and HEK293T cells. Intracytoplasmic sperm injection (ICSI) treatment was conducted in two patients.

Results We identified one novel hemizygous frameshift variant (NM_173494, c.319_329del: p.R107fs) of *DNAAF6* gene (previously named *PIH1D3*) in family 1 and one novel hemizygous missense variant (c.290G>T: p.G97V) in family 2. No hemizygous deleterious variants in *DNAAF6* were detected in the control cohort of 442 individuals. Ultrastructural and immunostaining analyses of patients' spermatozoa showed the absence of outer and inner dynein arms in sperm flagella. Both variants were proven to lead to DNAAF6 protein degradation in HEK293T cells. Both patients carrying *DNAAF6* variants underwent one ICSI cycle and delivered one healthy child each.

Conclusion We identified novel *DNAAF6* variants causing male infertility and PCD in Han Chinese patients. This finding extended the spectrum of variants in *DNAAF6* and revealed new light on the impact of *DNAAF6* variants in sperm flagella.

Keywords Primary ciliary dyskinesia · Asthenozoospermia · *DNAAF6* variant · Dynein arms · Sperm flagella

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Introduction

Malfunctioning cilia are associated with approximately one hundred human genetic disorders, with an accumulated incidence of 1:1000 [1]. As the hallmark disease of motile cilia, primary ciliary dyskinesia (PCD; MIM: 244400) [2, 3] is a genetically heterogeneous recessive disorder that affects an estimated 1:15,000 live births with typical symptoms characterized by neonatal respiratory distress, chronic sinusitis, bronchiectasis, and situs inversus [4, 5]. To date, approximately 43 disease-causing genes accounting for approximately 70% of PCD cases have been implicated in humans [6]. The variants in five axoneme-related genes (*DNAH5*, *DNAH11*, *DNAI1*, *CCDC39*, and *CCDC40*) predominate in PCD [7–11] and only three X-linked PCD genes have been reported, namely *OFD1*, *RPGR*, and *DNAAF6* [12–15]. Generally, males with PCD are infertile due to immotile spermatozoa [16]. However, since the male infertility is usually diagnosed after puberty,

sperm parameters are not systematically investigated in previously identified PCD cases, leaving not only the records of male infertility incomplete but also the role of PCD-related genes in flagellar function unclear [17, 18].

DNAAF6 (MIM:300933, previously named *PIH1D3*) is located on chromosome Xq22.3 and contains 8 exons, encoding a protein containing 214 amino acids and a PIH1 domain [15]. *DNAAF6* was identified as a dynein axonemal assembly factor (DNAAF) proposed to cooperate with heat shock protein (HSP) chaperones and other DNAAFs to promote subunit folding and cytoplasmic preassembly of dynein motors [19]. *Dnaaf6* defective mice only presented with male infertility and *Dnaaf6* defective spermatozoa were immotile and fragile while motile cilia remained unaffected [20]. Currently, three studies have reported that the loss-of-function variants in *DNAAF6* cause the classic PCD phenotypes with ciliary immotility in humans due to the lack of dynein arms (DAs) and the male patients present infertility. These studies only focus on the impact of *DNAAF6* variants in immotile cilia and respiratory tract phenotypes in patients [13, 15, 21]. However, no systematic studies yet exist on the ultrastructural defects of spermatozoa and asthenozoospermia phenotypes in human patients with *DNAAF6* variants. Moreover, with the development of assisted reproductive technology (ART), asthenozoospermia patients can achieve pregnancy through intracytoplasmic sperm injection (ICSI) [22]. The impact of *DNAAF6* variants in spermatozoa and the outcome of the ICSI treatment in *DNAAF6*-mutated male patients remain to be assessed.

In this study, we conducted a genetic analysis of three patients with PCD and male infertility from two unrelated Han Chinese families using whole-exome sequencing (WES), detecting two novel variants in *DNAAF6* in three patients. The spermatozoa from PCD patients with *DNAAF6* variants presented abnormal flagella with loss of DAs. We also validated the effects of these variants in patients' spermatozoa and HEK293T cells. Our results indicated that the *DNAAF6* variants might be responsible for PCD and male infertility.

Materials and methods

Patients and subjects

Three patients from two unrelated Han Chinese families were recruited to identify the causal genetic factor of infertility at the Reproductive and Genetic Hospital of CITIC-Xiangya (Changsha, Hunan, China). Family 1 included normal parents (first cousins married to each other, F1: I-1 and F1: I-2), their sons affected by PCD (F1: II-1, proband, and his brother F1: II-3), and their daughter devoid of known abnormalities (F1: II-2). Family 2 included normal parents (F2: I-1 and F2: I-2) and their son affected by PCD (F2: II-2).

A random cohort of 442 individuals (Han Chinese origin, described previously [23]), including 219 isolated oligoasthenospermia and 223 controls with normal fertility (had at least one offspring by natural fertilization), were used for variant screening. Abnormalities in somatic karyotypes and azoospermia factor (AZF) microdeletion on the Y chromosome were not detected. Other causes of infertility such as reproductive malformation, drug-use, and exposure to gonadotoxic factors were also excluded. Semen evaluations were performed according to World Health Organization (WHO, 2010) guidelines [24].

The present study was approved by the Institutional Ethics Committees of Central South University and the Reproductive Genetic Hospital of CITIC-Xiangya, China. All methods were performed in accordance with approved guidelines. Written informed consent was obtained from all individuals who participated in the study prior to commencement.

Whole-exome sequencing and bioinformatic analysis

Blood samples from participating individuals, including those of 3 individuals with PCD and male infertility were collected and genomic DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany). This genomic DNA was subsequently used for whole-exome sequencing (WES) analysis. Library construction, WES, and data analysis were carried out by the Beijing Genomics Institute (BGI, Shenzhen, China) as described previously [25]. Variant identification was performed using the Genome Analysis Toolkit (GATK). Sequencing variants including single-nucleotide variants (SNVs) and small insertions or deletions (INDELs) were annotated using ANNOVAR software [26].

The most promising candidate pathogenic variants in the two families were screened using the following criteria: (1) variants with a minor allele frequency below 5% as reported by the public databases (dbSNP, GO-ESP, 1000G, and ExAC); (2) variants predicted to be deleterious using multiple tools (PolyPhen-2 (genetics.bwh.harvard.edu/pph2), Mutation Taster (www.mutationtaster.org/), and SIFT (sift.jcvi.org/)); and (3) phenotype relevance using comprehensive expression data (high expression in ciliated tissues) and model organism data [27]. We performed a targeted look-up for *DNAAF6* variants in WES data from the 219 subjects with oligoasthenospermia and 223 subjects with normal fertility, described in our previous study [23].

Sanger sequencing

Specific PCR primers were designed to target the region of the variants in the candidate gene *DNAAF6* with sequences as follows: *PIH1D3*-F1: 5′-ACTGAGTGTGAAGAAGAACAGGAG-3′, *PIH1D3*-R1: 5′-CCTTCCGTGTGTCACTGTAGTTT-3′. Amplified PCR products were analyzed via 2.0% agarose gel electrophoresis to determine band size, followed by bi-directional

sequencing on an ABI 3730 automated sequencer (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA).

Transmission electron microscopy

Semen samples of normal fertile control and patients (F1: II-1 and F2: II-2) were treated as described previously [23]. Samples were fixed with glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) and osmium tetroxide, followed by OsO_4 and sucrose, then dehydrated using graded ethanol. Subsequently, the samples were embedded in Epon 812, dodecenylsuccinic anhydride, methyl-nadic anhydride, and dimethylaminomethyl phenol. Ultrathin (80 nm) sections were contrasted with uranyl acetate and lead citrate. An AHT7700 Hitachi electron microscope (Hitachi, Tokyo, Japan) and a MegaView Iii digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) were used to capture images.

Histological and immunofluorescence analysis

For hematoxylin and eosin (H&E) staining, sperm were dehydrated with graded ethanol, stained with H&E. For immunofluorescence analysis, the slides were incubated with primary antibodies (PIH1D3, DNAH5, DNALI1, AKAP4, TOMM20, RSPH1, SPAG6, and anti-acetylated tubulin monoclonal antibody, respectively) for 2.5 h at 37 °C. Detailed information on all antibodies is provided in Table S1. The slides were then incubated with secondary antibodies (Alexa Fluor® 488 anti-mouse IgG (A-21121) and Alexa Fluor® 555 anti-rabbit IgG (A31572)) for 1.5 h at 37 °C. Finally, all slides were stained using 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) for 5 min. An Olympus IX51 fluorescence microscope (Olympus, Tokyo, Japan) and VideoTesT-FISH 2.0 software (VideoTesT Ltd., St. Petersburg, Russia) were utilized to photograph fluorescence signals.

Expression vector construction

The full-length coding sequence of the human DNAAF6 (NM_173494) cDNA clone was purchased from GeneChem Co., Ltd. (Shanghai, China). Wild-type DNAAF6 was cloned to CMV-MCS-FLAG-SV40-Neomycin with FLAG-tag at the C-terminal end. Site-directed mutagenesis was performed to introduce both DNAAF6 variants (c.319_329del and c.290G>T) into the wild-type vector using a Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Wild-type and mutant clones were confirmed by Sanger sequencing.

Cell culture and transfection

Human embryonic kidney cells (HEK293T) obtained from China Center for Type Culture Collection (CCTCC) (Wuhan, China) were cultivated at 37 °C with 5% CO_2 in a 6-mL petri dish supplemented with 10% fetal bovine serum (Gibco™/Thermo Fisher Scientific, Gaithersburg, MD, USA). When cells had grown to ~ 80% confluence, DNAAF6 wild-type and mutant plasmids were transiently transfected using Lipofectamine® 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

Western blotting

Cells were collected after transfection for 48 h with DNAAF6 wild-type and mutant plasmids and homogenized with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific). Lysates were centrifuged at 13,000×g for 15 min at 4 °C and supernatants were collected and mixed with 5× sodium dodecyl sulfate (SDS) loading buffer and heated at 100 °C for 10 min. The proteins were separated via 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) then transferred onto a polyvinylidene difluoride membrane and incubated overnight at 4 °C with anti-FLAG antibody (AB0008, Abways, Shanghai, China) and anti-GAPDH antibody (ab8245, Abcam, Cambridge. UK). Then, the membranes were incubated with secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG, MultiSciences Biotech Co., Ltd., Hangzhou, China). Finally, the blots were revealed using the Pierce ECL Western Blotting Kit (Pierce Biotechnology, Rockford, IL, USA).

Intracytoplasmic sperm injection procedures

The wives of 2 individuals with DNAAF6 variants (F1: II-1 and F2: II-2) accepted intracytoplasmic sperm injection (ICSI) treatment. They underwent controlled ovarian hyperstimulation, oocyte retrieval, ICSI, and pregnancy confirmation as described previously [28, 29]. Viable spermatozoa for ICSI, selected from completely immotile spermatozoa, were stimulated using SperMagic® medium as our described previously [28, 29]. Pregnancy was confirmed via 2 hCG tests conducted 14 days following embryo transfer; clinical pregnancy was defined as, at minimum, a visible sac with a fetal heart beat detected via ultrasound screening after 28 days.

Results

Clinical findings

The first proband (F1: II-1) from a consanguineous family (Family 1) was 32 years old (Fig. 1a). Routine semen analysis of this subject revealed severe asthenoteratozoospermia with 100% immotile spermatozoa and comparatively low sperm count ($5.2–11.2 \times 10^6$/mL) (Table 1). The bilateral testicular

size, as measured by B ultrasonography, was normal. F1: II-1 exhibited classic PCD-like symptoms from early life based on his previous medical tests, including a positive history of chronic wet cough, nasal obstruction, pneumonia, and dextrocardia. However, F1: II-1 refused to a ciliary biopsy and nasal nitric oxide (nNO) test. The proband's brother (F1: II-3) was also affected by primary infertility and exhibited classic PCD-like symptoms similar to the proband. F2: II-2 from a non-consanguineous family (Family 2) was 44 years old (Fig. 1a). Routine semen analysis of the subject revealed severe asthenoteratozoospermia with 91.4% immotile spermatozoa and a comparatively low sperm count ($3.5–8.7 \times 10^6$/ mL) (Table 1). F2: II-2 also exhibited classic PCD-like symptoms, including a positive history of chronic wet cough, nasal obstruction, pneumonia, and bronchiectasia, but he refused to a ciliary biopsy and nasal nitric oxide (nNO) test. Routine physical examination of these patients yielded normal results, including height, weight, hair distribution, mental state, and external genital organs.

WES-based identification of DNAAF6 variants

WES was performed to identify the genetic source of PCD and male infertility in these patients. The results for both families were analyzed and filtered to exclude irrelevant variants according to the criteria described in Methods. Briefly, candidate variants were considered a priority when they met the following criteria: frequency below 5% in public databases, predicted to be deleterious, homozygous or compound heterozygous, and relevance to infertility or PCD phenotypes. After filtering, only two novel hemizygous variants (NM_173494, c.319_329del: p.R107fs for family 1 and c.290G>T: p.G97V for family 2) in DNAAF6, a known pathogenic gene for PCD [13, 15], fulfilled these criteria (Table 2). Neither hemizygous deleterious variants in DNAAF6 were assessed in our previously reported cohort of 442 individuals, including 219 with isolated oligoasthenospermia and 223 normal controls.

Sanger sequencing and pedigree analysis were performed for the patients to confirm the DNAAF6 variants. Patient F1: II-1 and his brother F1: II-3 had inherited the frameshift variant (c.319_329del: p.R107fs) from their mother with heterozygous condition; their father and sister with normal phenotype were wild type. Co-segregation analysis supported a X-linked recessive genetic model and genotype–phenotype correlation (Fig. 1b). The missense variant (c.290G>T: p.G97V) of which position is highly conserved in different species was identified in patient F2: II-2 (Fig. 1c), but pedigree validation was failed to be performed due to unavailability of the family members (Fig. 1d). These results indicated that the two variants in DNAAF6 were associated with PCD and male infertility.

Patients with DNAAF6 variants lack dynein arms

We performed H&E staining to observe the morphological features of the mutated spermatozoa from F1: II-1 and F2: II-2. Most spermatozoa exhibited abnormal tail morphologies, including short, coiled, and curled tails (Fig. 2a). Transmission electron microscopy (TEM) analysis showed lack of inner dynein arms (IDAs) and outer dynein arms (ODAs) in the mutated spermatozoa from F1: II-1 and F2: II-2, while exhibiting normal "9 + 2" microtubule pairs (Fig. 2b). Immunostaining analyses of spermatozoa (F1: II-1) using the ODA marker DNAH5 and the IDA marker DNALI1 further confirmed the absence of ODA and IDA (Fig. 2c, d). We then assessed flagellum structure by immunofluorescence staining with AKAP4, TOMM20, RSPH1, and SPAG6—markers of the fibrous sheath, mitochondrial sheath, radial spokes, and central pair complex, respectively. The immunostaining of mutant spermatozoa (F1: II-1) with AKAP4, TOMM20, RSPH1, and SPAG6 yielded results similar to the normal controls (Fig. 3a–d). Our results showed that DNAAF6 variants only caused the absence of dynein arms (DAs) in flagella; other flagellum components including fibrous sheath, mitochondrial sheath, and radial spokes were not directly affected by variants in DNAAF6.

Significantly decreased DNAAF6 protein in patient spermatozoa and in HEK293T cells

To assess the functional effects of the identified DNAAF6 variants in spermatozoa, we analyzed DNAAF6 expression by immunofluorescence analysis on spermatozoa from controls and patient F1: II-1. DNAAF6 was found to be localized on the midpiece of flagella in normal spermatozoa, but absent in DNAAF6-mutated spermatozoa (Fig. 4a). To further validate the effects caused by DNAAF6 variants in vitro, we constructed two mutant expression plasmids and transfected wild-type and mutant vectors into HEK293T cells. Western blot showed both c.290G>T (p.G97V) and c.319_329del (p.R107fs) variants caused DNAAF6 protein degradation, compared with that with wild-type proteins (Fig. 4b). These results suggested the missense variant (c.290G>T: p.G97V) and the frameshift variant (c.319_329del: p.R107fs) both

Fig. 1 Two DNAAF6 variants were identified in three patients with PCD and male infertility phenotypes. **a** Pedigree analysis of the three patients from two Han Chinese families. Black squares indicate male patients; black arrow, proband (P); circles with black spot, female heterozygous carriers; parallel lines, infertility. **b** Sanger sequencing results of the two variants from the two families. Red arrow indicates missense variant site; black line, frameshift variant site. NC, normal control. **c** Conservative analysis of the missense variant (c.290G>T: p.G97V). Red arrow, mutant amino acid site. **d** Positions of all reported variants in the DNAAF6 protein structure model. Green box, PIH domain; black, variants identified from other studies; red, variants identified in this study
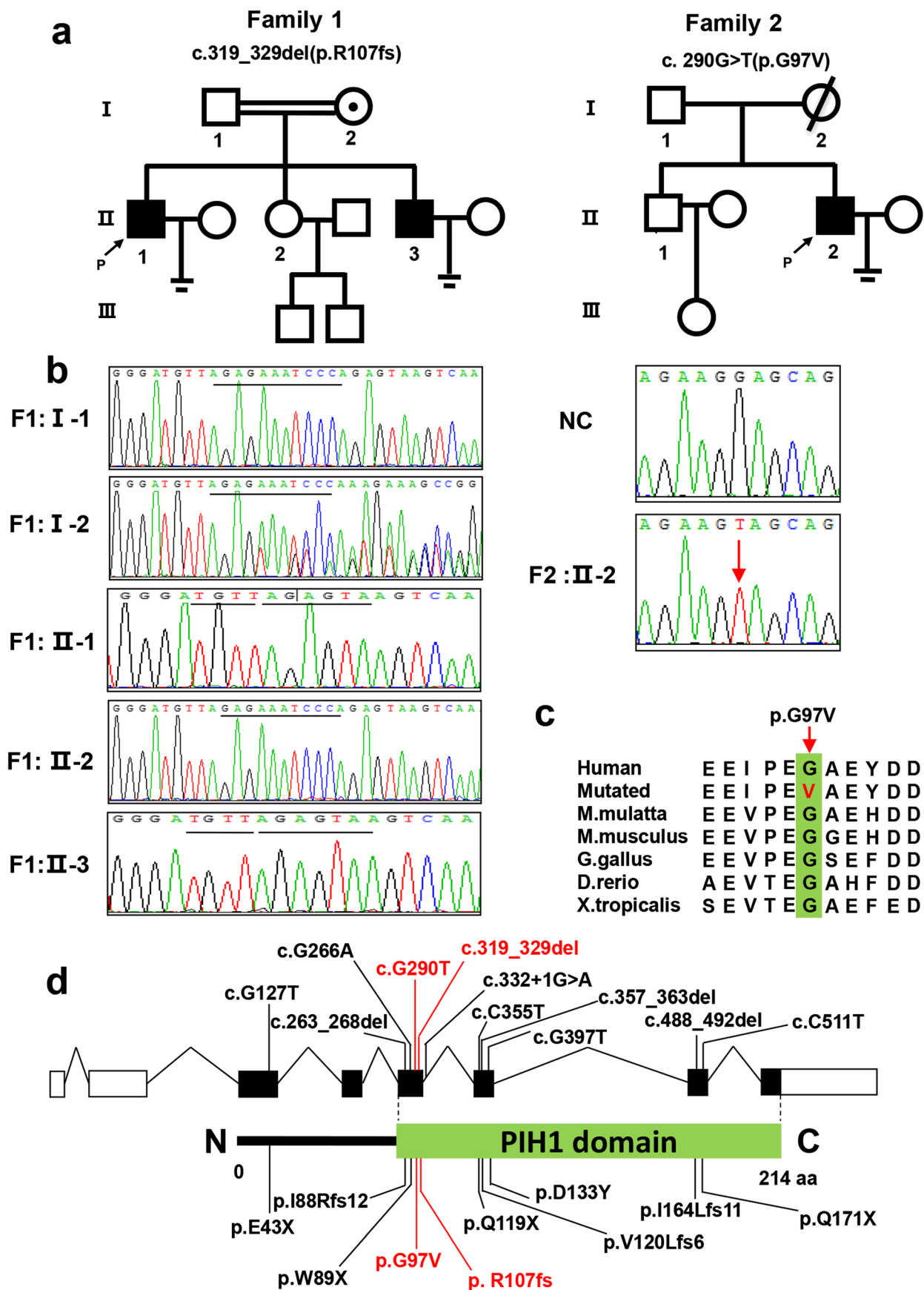
Table 1 Semen routine examination and intracytoplasmic sperm injection outcomes of the PCD couples

Family	*Age	†Semen volume (mL)	†Sperm count (×10^6/mL)	†Progressive motility (%)	Number of MII oocytes	Number of fertilized oocytes	Sperm retrieval technique	Number of transferred embryos	Characteristics of transferred embryos	Outcome of pregnancy
F1: II-1	M 32 / F 28	2.5–3.8	5.2–11.2	0	13	9	Ejaculation	2	8-cell grade	Single birth
F2: II-2	M 44 / F 35	1.5–2.8	3.5–8.7	8.6	15	13	Ejaculation	2	8-cell grade	Single birth

*Age of the couple. *M*, male; *F*, female

†Semen parameters were evaluated according to the World Health Organization (WHO, 2010) guidelines

Table 2 Variants identified by whole-exome sequencing in combination with cilia-related gene-filtering for the patients

Patient	Gene	Position	RefSeq ID	AA alteration	Function	MinDepth	dbSNP ID	†1000 G	†GO-ESP	†ExAC	*Mutation Taster	*SIFT	*PolyPhen-2
F1: II-1 / F1: II-3	*DNAAF6*	ChrX: 106462185	NM_173494	c.319_329del:p.R107fs	frameshift	69	-	-	-	-	D	-	-
F2: II-2	*DNAAF6*	ChrX: 106462157	NM_173494	c.G290T:p.G97V	missense	49	-	-	-	0.00001201	D	D	D

*Mutation assessment by Mutation Taster, SIFT, and PolyPhen-2. *D*, disease causing; -, not available

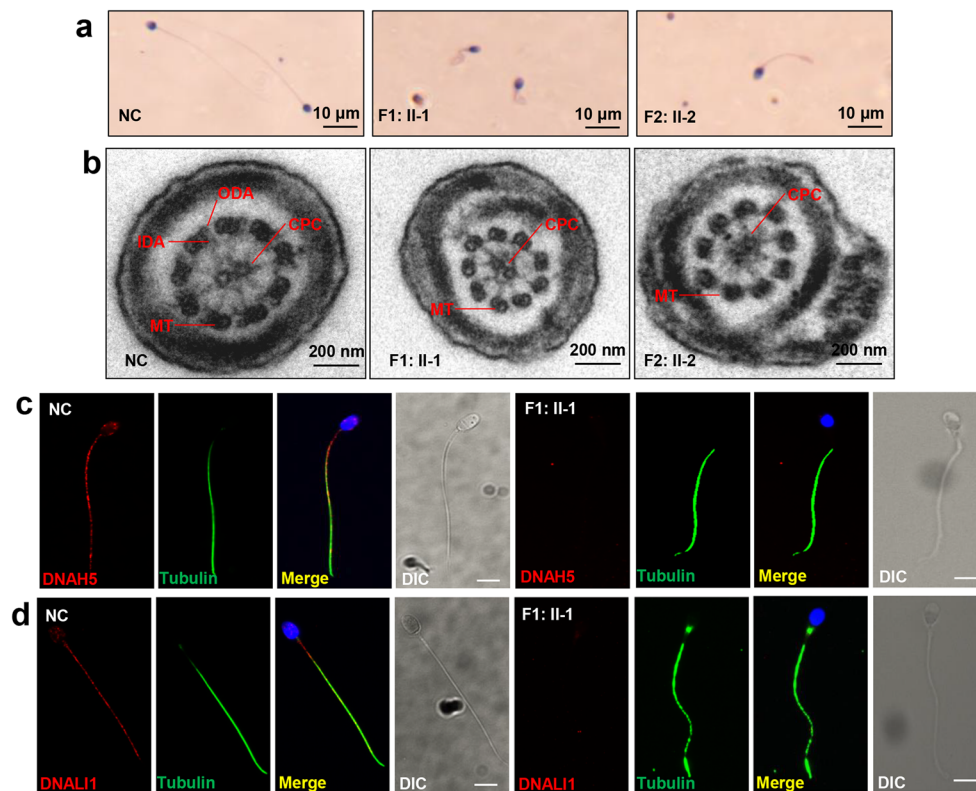†Frequency of corresponding mutations in 1000 Genomes, GO-ESP, and total ExAC Browser

Fig. 2 Abnormal morphology and ultrastructure of mutant spermatozoa. **a** H&E staining of spermatozoa from both patients (F1: II-1 and F2: II-2) and normal control (NC) indicated coiling tails and curly tails in *DNAAF6* mutant spermatozoa. Scale bars, 10 μm. **b** TEM analysis indicated loss of IDAs and ODAs and normal microtubule pairs (9 + 2) in the spermatozoa from both patients. Scale bars, 200 nm; CPC, central pair complex; MT, peripheral microtubule doublet; ODA, outer dynein arms; IDA, inner dynein arms. **c, d** Immunofluorescence analysis showed significant decline with anti-DNAH5 and anti-DNALI1 antibodies (red) in the mutant spermatozoa from F1: II-1 compared with the normal control. The spermatozoa were stained with anti-α-tubulin antibody (green) as loading controls and 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI, blue) as a nuclear marker. Scale bars, 5 μm; DIC, differential interference contrast

caused extremely unstable proteins in spermatozoa and HEK293T cells.

ICSI outcome of both patients

Furthermore, ICSI was conducted for two couples using ejaculated spermatozoa with comparatively normal morphology. Both couples underwent one ICSI treatment cycle each; thirteen metaphase II oocytes were injected and nine oocytes were fertilized (2PN/injected oocytes = 69%) in family 1 (F1: II-1) and fifteen metaphase II oocytes were injected and thirteen oocytes were fertilized (2PN/injected oocytes = 87%) in family 2 (F2: II-2) (Table 1). Two embryos were subsequently transferred into the partner's uterus and one embryo resulted in clinical pregnancy in both families. Both families ultimately delivered one healthy child each.

Discussion

In this study, two novel hemizygote variants (c.290G>T: p.G97V and c.319_329del: p.R107fs) in *DNAAF6* were identified in three patients with PCD and male infertility from two unrelated Han Chinese families by WES and Sanger sequencing. TEM and immunostaining analyses of patients' spermatozoa showed the absence of ODA and IDA in sperm flagella. Both variants impaired the stability of the DNAAF6 protein in HEK293T cells as well as in patient spermatozoa. These results revealed that both variants in *DNAAF6* were associated with the PCD phenotypes and asthenozoospermia. Our study is also the first to report Chinese PCD patients with *DNAAF6* variants.

DNAAF6 is a dynein axonemal assembly factor with only one PIH domain that participates in a co-chaperone complex with KTU/DNAAF2 and DYX1C1/DNAAF4 to regulate the dynein arm preassembly in cytoplasm by direct interaction with HSP90 and DNAI2 [13, 19]. To date, three groups have reported that variants in *DNAAF6* caused PCD with outer and inner dynein arm defects. They identified a total of twelve variants in ten patients, including four nonsense variants, three frameshift variants, one splicing variant, one missense variant, and three gross deletions [13, 15, 21]. In this study, the frameshift variant (c.319_329del: p.R107fs) was predicted to produce truncated protein. The missense variant (c.290G>T:
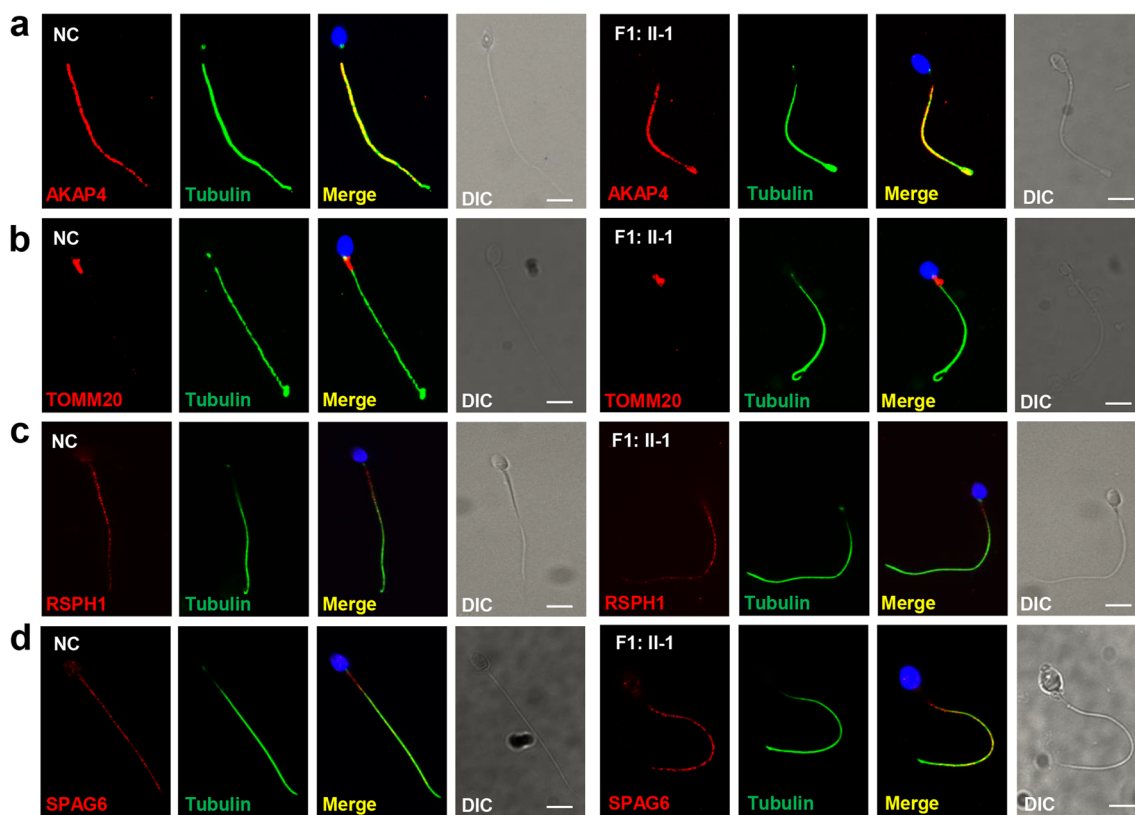
Fig. 3 No abnormalities were found in other flagellum components. Immunostaining analyses with **a** anti-AKAP4, **b** anti-TOMM20, **c** anti-RSPH1, and **d** anti-SPAG6 antibodies (red) showed no significant abnormalities in fibrous sheath, mitochondrial sheath, radial spokes, and central pair complex of spermatozoa from F1: II-1 compared with the normal control (NC). Anti-α-tubulin antibodies (green) were used as loading controls and DAPI (blue) as a nuclear marker. Scale bars, 5 μm; DIC, differential interference contrast

p.G97V) from F2: II-2 was located in a highly conserved region in the PIH domain [15]. This domain is a key non-catalytic subunit of the R2TP-HSP90 chaperoning complex [19]. Both variants were validated to impact the function of DNAAF6 protein in cells. These results suggested that the missense variant was also a main pathogenic variant type and that the region near the PIH domain may be the pathogenic hot spot combining all known variants.

We observed *DNAAF6* staining only in the midpiece flagellum of human sperm, which is similar to the location of CCDC103, another cytoplasmatic dynein assembly factor [30]. In mice, *Dnaaf6* was detected only in the cytoplasm of late pachytene spermatocytes to round spermatids [20]. We speculated that DNAAF6 also plays a role during flagellar development and was retained to the midpiece of flagella in chromatin condensation and the elimination of excess cytoplasm, while also participating in dynein arm preassembly in the cytoplasm of round sperm cells. The molecular pathways of *DNAAF6* and other DNAAFs in sperm cells require further investigation.

For PCD patients, basal brushing biopsies were performed as a classical measurement to PCD diagnosis in previous reports [31–33]. However, it is known to cause trauma to patients. Our results showed that variants in *DNAAF6* affected both IDAs and ODAs in the sperm flagella rather than only ODAs as previously described, the ultrastructural defects in *DNAAF6*-mutant spermatozoa showed no difference with those in *DNAAF6*-mutant cilia from other reports [13]. This result suggested that male PCD patients could be diagnosed by sperm flagella ultrastructural examination combined with known PCD pathogenic genes related to defects of both cilia and sperm flagella. It is essential to identify the effects of PCD pathogenic genes on spermatozoa, as such an assessment offers not only a potential non-invasive diagnostic method to PCD patients but also the possibility of treating male infertility.

From a clinical point of view, ICSI is currently the only way for couples to conceive when the male partner has been diagnosed with PCD and male infertility, due to the lack of empirical medicinal therapy to improve semen parameters. Specifically, the estimated fertilization and pregnancy rates among PCD patients were 55–65% and 35–45% when relying on ejaculated or testicular sources, respectively [34], while the overall live birth rate was only 39%. In this study, patients carrying *DNAAF6* variants experience successful ICSI outcomes, indicating that ICSI may be a good choice for patients with *DNAAF6* variants who want to have children. However, ICSI cycles are administered prior to genetic analysis on both patients; going forward, protocols should include variant screening of *DNAAF6* in female partners and their offspring as well.
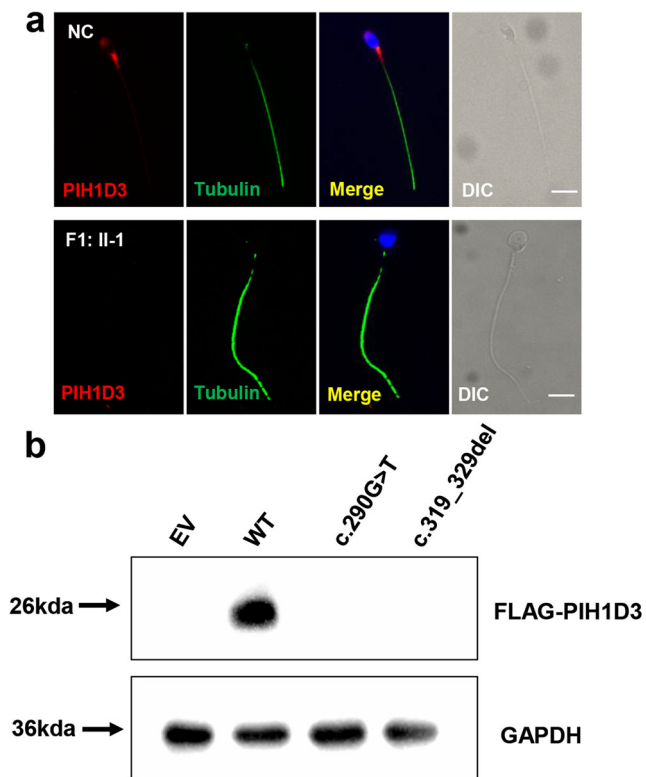
Fig. 4 Expression levels of mutant DNAAF6 proteins in vitro and in spermatozoa. **a** DNAAF6 immunostaining in human spermatozoa from normal controls (NC) and F1: II-1 with anti-DNAAF6 (red) and anti-α-tubulin (green) antibodies. DNAAF6 protein located in midpiece of normal sperm flagella, but absent in mutant spermatozoa. Scale bars, 5 μm; DIC, differential interference contrast. **b** The effects of the variants on DNAAF6 protein levels by western blot in HEK293T cells transfected with empty vector (EV), wild-type (WT), and mutant *DNAAF6* vectors. *GAPDH* was used as the loading control

Overall, we validated two novel pathogenic variants in *DNAAF6* from two unrelated Han Chinese families with PCD and male infertility. Patients who carried *DNAAF6* variants have good ICSI outcomes. Our findings extend the mutational spectrums of *DNAAF6* and have important implications for genetic counseling of patients with PCD and male infertility.

Compliance with ethical standards

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