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Identification of novel biallelic *LRRC6* variants in male Chinese patients with primary ciliary dyskinesia and infertility

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Abstract

Purpose The aim of this study is to identify the genetic cause of primary ciliary dyskinesia (PCD) and male infertility in two unrelated Han Chinese families.

Methods We performed whole-exome sequencing in two unrelated male Han Chinese patients suffering from infertility and PCD to identify the pathogenic variants. Ultrastructural and immunostaining analyses of patient's spermatozoa were performed to characterize the effect of the variants. The pathogenicity of the variants was validated using patient's spermatozoa by western blotting and immunostaining analysis. Intracytoplasmic sperm injection (ICSI) was conducted in the affected families.

Results Three variants in leucine-rich repeat containing 6 (*LRRC6*) [patient 1(compound heterozygote): NM_012472: c.538C > T, (p.R180*) and c.64dupT, (p.S22Ffs*19); patient 2 (homozygote): c.863C > A, (p.P288H)] were identified in two unrelated patients with PCD and male infertility. These variants were predicated deleterious and were absent or rare in human population genome data. *LRRC6*-mutant spermatozoa showed a highly aberrant morphology and ultrastructure with lacked inner and outer dynein arms. The LRRC6 protein was present along the normal sperm flagella, and was significantly decreased in the mutated spermatozoa. Interestingly, both patients were able to conceive through ICSI and birthed a healthy baby.

Conclusion Our results extend the *LRRC6* variant spectrum and provide reproductive guidance to families suffering from PCD-linked infertility caused by *LRRC6* variants.

Keywords Primary ciliary dyskinesia · Male infertility · *LRRC6* variant · Sperm flagella

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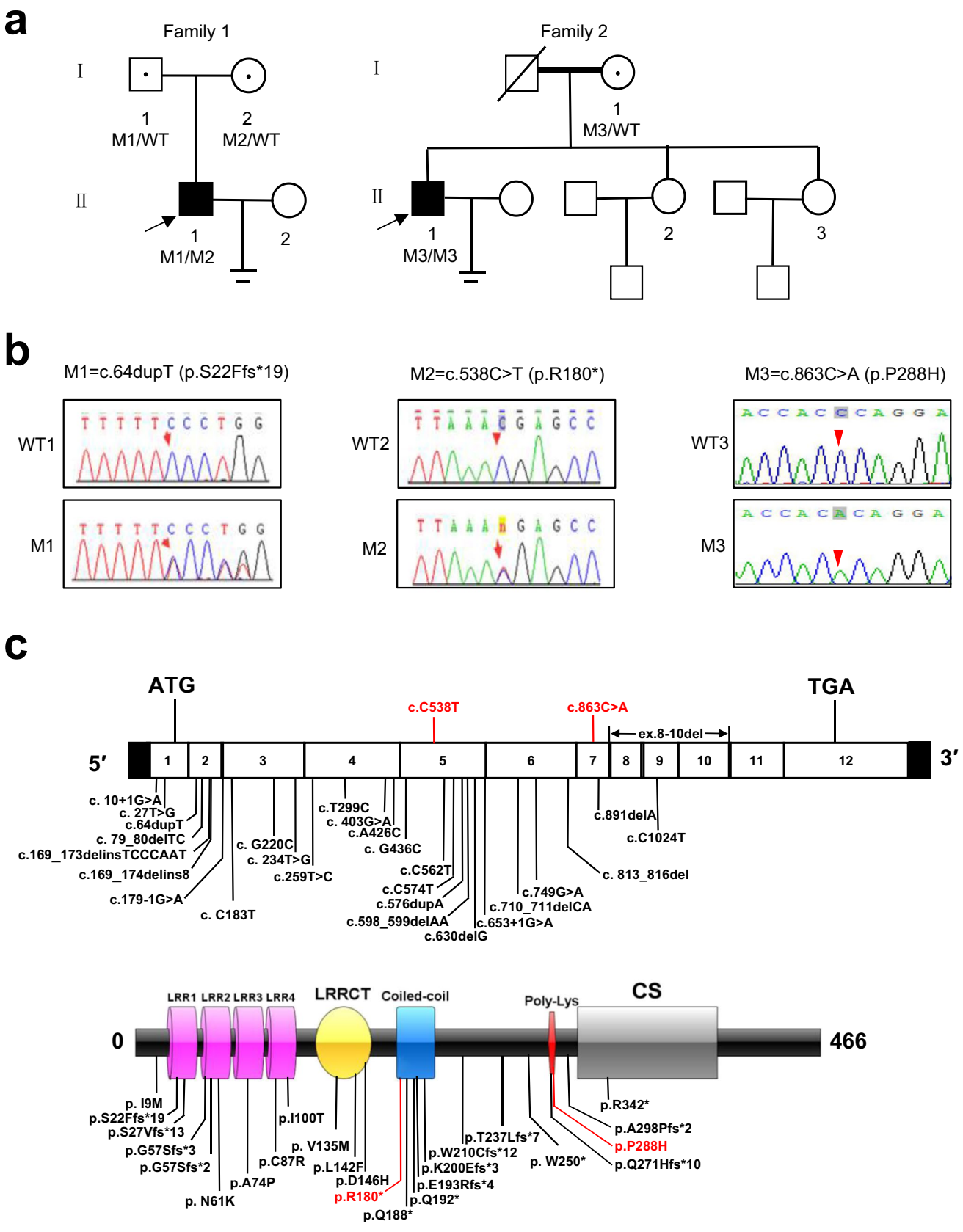
Introduction

Male infertility is a major medical and public health concern affecting more than 5% of couples worldwide. Asthenozoospermia is a common cause of male infertility, characterized by sperm with reduced motility and progressive motility < 32% [1]. As the sperm flagella and motile cilia share a similar axonemal structure, asthenozoospermia due to malformations of the axonemal structure is often associated with primary ciliary dyskinesia (PCD, MIM: 244,400) [2, 3]. PCD affects an estimated 1:15,000 live births and is characterized by abnormal ciliary and flagellar movement, leading to several multisystem disorders [4, 5], such as neonatal respiratory distress, chronic sinusitis, bronchiectasis, hydrocephalus, situs inversus (also known as Kartagener syndrome), and male infertility [6, 7]. To date, at least 40 pathogenic genes are associated with PCD and explain pathogenesis in more than 70% of cases; however, only half of the variants are known to cause male infertility [2,


a
Family 1
I
1
M1/WT
2
M2/WT
II
1
M1/M2
2
Family 2
I
1
M3/WT
II
1
M3/M3
2
3
b
M1=c.64dupT (p.S22Ffs*19)
M2=c.538C>T (p.R180*)
M3=c.863C>A (p.P288H)
WT1
M1
WT2
M2
WT3
M3
c
ATG
TGA
c.C538T
c.863C>A
ex.8-10del
5′
3′
1
2
3
4
5
6
7
8
9
10
11
12
c. 10+1G>A
c. 27T>G
c.64dupT
c. 79_80delTC
c.169_173delinsTCCCAAT
c.169_174delins8
c.179-1G>A
c. C183T
c. G220C
c. 234T>G
c.259T>C
c.T299C
c. 403G>A
c.A426C
c. G436C
c.C562T
c.C574T
c.576dupA
c.598_599delAA
c.630delG
c.653+1G>A
c.710_711delCA
c.749G>A
c. 813_816del
c.891delA
c.C1024T
LRR1 LRR2 LRR3 LRR4
LRRCT
Coiled-coil
Poly-Lys
CS
0
466
p. I9M
p.S22Ffs*19
p.S27Vfs*13
p.G57Sfs*3
p.G57Sfs*2
p. N61K
p.A74P
p.C87R
p.I100T
p. V135M
p.L142F
p.D146H
p.R180*
p.Q188*
p.Q192*
p.E193Rfs*4
p.K200Efs*3
p.W210Cfs*12
p.T237Lfs*7
p. W250*
p.R342*
p.A298Pfs*2
p.P288H
p.Q271Hfs*10

◂**Fig. 1** Biallelic variants in *LRRC6* were identified in male primary ciliary dyskinesia patients suffering from infertility. **a** Pedigrees of two families affected by *LRRC6* variants. Squares represent male family members; circles represent female family members; and black arrows represent probands, and equal signs indicate infertility. The symbol with a dot in the center represents carrier status. **b** Chromatograms produced by Sanger sequencing of patients' variants in *LRRC6*. The red arrows indicate the variant sites. **c** The positions of *LRRC6* variants identified in patients. LRRC6 domains and motifs are indicated in the colored squares. Red indicates the two novel variants identified in this study, and black indicates reported variants

8]. Since male infertility occurs after puberty, sperm parameters are not systematically investigated in male PCD cases, leaving not only the records of male infertility incomplete but also the role of these genes in flagellar function unclear [9, 10].

LRRC6 encodes a leucine-rich repeat-containing protein and was firstly identified as a dynein axonemal assembly factor (*DNAAF*) causing lack of inner dynein arms and outer dynein arms in axonemes of cilia and flagella in PCD patients in 2012 [11]. The *Lrrc6*-mutant mouse model validated this finding subsequently [12]. In human, approximately 27 pathogenic *LRRC6* variants have been reported and all affected adult male PCD patients had asthenozoospermia and were infertile [13, 14]. However, the majority of the reports focused on respiratory symptoms and ciliary phenotype, overlooking male infertility [11, 15–26].

Here, we identified two novel variants in *LRRC6* in two unrelated Han Chinese PCD patients with male infertility by whole-exome sequencing (WES) and Sanger sequencing. In vitro analyses were performed to identify the pathogenicity of the *LRRC6* variants. Additionally, this study represents the successful pregnancy resulting from intracytoplasmic sperm injection (ICSI) due to male infertility caused by *LRRC6* variants.

Materials and methods

Patients

Two infertile men (patient 1 and patient 2) from two unrelated Han Chinese families visited the Reproductive & Genetic Hospital of CITIC-Xiangya for fertility treatment. Patient 1 (family 1, II-1) and his phenotypically normal partner were unable to conceive after more than two years without contraception. Patient 2 (family 2, II-1) is from a consanguineous family and is affected by infertility for 7 years. Both patients exhibited classic PCD-like respiratory symptoms from early life including chronic cough, recurrent nasal obstruction, and pneumonia. Neither karyotype abnormalities nor Y-microdeletions in the azoospermia factor regions were detected. Malformation in the patients' reproductive tract, drugs, and exposure to gonadotoxins were excluded as causes of the patients' infertility. Peripheral blood samples from the family members and spermatozoa from the patients were collected for genetic testing. Each individual involved gave informed consent, and this study was approved by the ethics committees of the Reproductive and Genetic Hospital of CITIC-Xiangya.

Hematoxylin and eosin staining and immunofluorescence analysis

Semen samples from the patients and normal control were evaluated using hematoxylin and eosin (H&E) staining and immunofluorescence analysis as previously described. Briefly, semen smears were dehydrated in graded ethanol, visualized by H&E staining, and then dehydrated again with graded ethanol followed by dimethylbenzene treatment for 5 min twice. For immunofluorescence analysis, the slides were incubated with primary antibodies (LRRC6, DNAI1, DNALI1, SPAG6, RSPH1, TOMM20, AKAP4, and monoclonal anti-acetylated tubulin) 2.5 h at 37 °C, and then incubated with secondary antibodies [Alexa Fluor® 488 anti-mouse IgG (A-21121, 1:400) and Alexa Fluor® 555 anti-rabbit IgG (A31572, 1:400)] for 1.5 h at 37 °C. Detailed information on all antibodies is provided in Supplementary table S1. Slides were stained with 2-(4-aminophenyl)-1H-indole-6-carboxamidine (DAPI) for 5 min. Immunofluorescence analyses were performed by Olympus IX51 fluorescence microscope (Olympus, Tokyo, Japan) and VideoTesT-FISH 2.0 software (VideoTesT Ltd., St. Petersburg, Russia).

Transmission electron microscopy

The semen samples were treated as previously described [27]. Briefly, samples were fixed in 0.25% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) followed by secondary fixation in 1% osmium tetroxide for 1 h. Then, the sperm samples were subjected to postfixation with OsO4 and sucrose and embedded in Epon 812, dodecenylsuccinic anhydride, methyl nadic anhydride, and (dimethylaminomethyl)phenol. The contrast of 70–90-nm-thick sections of the embedded samples was enhanced with uranyl acetate and lead citrate. Images were captured by Hitachi HT7700 electron microscope (Hitachi, Tokyo, Japan) and MegaView III digital camera (Munster, Germany) as previously described [28].

Whole-exome sequencing and bioinformatic analysis

Genomic DNA was extracted from the peripheral blood samples of the patients' family by QIAamp DNA Blood

Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. WES of the patients were performed by the Beijing Genome Institute (Shenzhen, Guangdong, China) using the HiSeq2000 sequencing platform (Illumina, San Diego, California, USA) as previously described [29]. WES data were analyzed using the Genome Analysis Toolkit (GATK Broad Institute/Massachusetts Institute of Technology, Cambridge, MA, USA). Briefly, raw reads were aligned to the National Center for Biotechnology Information (NBCI) GRCh37 reference genome using the Burrows-Wheeler Aligner after removal of adaptors [30]. Next, PCR duplicates were removed followed by sorting using Picard (http://broadinstitute.github.io/picard/). Variant identification was performed using GATK's VariantEval tool following the recommended best-practices, including variant calling with HaplotypeCaller and variant quality score recalibration annotation using the variant identification tool ANNOVAR [31].

Inclusion criteria for candidate genes were:(1) a variant frequency below 5% in three public databases [1000 Genomes Project, NHLBI GO Exome Sequencing Project, and Exome Aggregation Consortium (ExAC)]; (2) variant was homozygote or compound heterozygote and predicted to be deleterious by MutationTaster, SIFT, and PolyPhen-2; and (3) the function of candidate gene was associated with cilia and flagella.

Sanger sequencing

The candidate variants of *LRRC6* were validated by Sanger sequencing using appropriate primers listed in Supplemental Table 2. PCR amplification was performed by Ex Taq DNA Polymerase (Bio-Rad, Hercules, CA, USA) and then bidirectional sequencing was performed using the Applied Biosystems 3730 automated sequencer (Forster City, California, USA).

Western blotting

Proteins were extracted from the spermatozoa samples using RIPA lysis buffer (Beyotime Biotechnology) supplemented with Protease Inhibitor Cocktail (Thermo Fisher Scientific, USA). Proteins were identified and isolated using 12% sodium dodecyl sulfate (SDS) and a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) system, blotted to polyvinylidene difluoride membranes, and incubated overnight at 4 °C with anti-LRRC6 antibody at a dilution of 1:250 (HPA028058; Sigma-Aldrich). The membranes were then incubated with goat anti-mouse IgG (GAM007-100; MultiSciences), or goat anti-rabbit IgG (GAR007-100; MultiSciences) secondary antibodies diluted to 1:5000. The blots were visualized using an ECL Western blotting kit (Pierce Biotechnology, Rockford, IL, USA).

Ovarian stimulation and intracytoplasmic sperm injection procedure

For partners of the two patients, a recombinant follicle-stimulating hormone was administrated to stimulate follicular development. Oocytes were retrieved 36 h after ovulation was induced using human chorionic gonadotropin (hCG). Semen samples from these two patients were obtained simultaneously through masturbation and the hypo-osmotic swelling test was used to select viable spermatozoa for intracytoplasmic sperm injection (ICSI). Single viable sperm was injected into metaphase II oocyte and the fertilized oocytes were individually cultured. Two embryos in the blastocyst stage were transferred transcervically 5–6 days after oocyte retrieval.

Results

Clinical characterization of patient

The patient 1 (Fig. 1a) at age of 31 years old presented with completely immotile spermatozoa and a comparatively low sperm count (5.6–21.6 × 10^6/mL) (Table 1). He also exhibited classic PCD-like respiratory symptoms from early life including chronic cough, recurrent nasal obstruction, and pneumonia. His wife and parents did not have PCD-related symptoms (Fig. 1a). HRCT scans of the patient revealed diffuse bronchiectasis and no indications of situs inversus (Fig. S1). Nasal nitric oxide concentration of the patient was

Table1 Semen routine examination and intracytoplasmic sperm injection outcomes of the PCD couples

Patient	Age* (year)	Semen volume[a] (ml)	Sperm count[a] (*10^6)	Progressive motility(%)[a]	Number of MII oocytes	Number of fertilized oocytes	Sperm retrieval technique	Number of transferred embryos	Outcome of pregnancy
Patient 1	M:31/F:31	2.5–4.6	5.6–21.6	0	13	7	Ejaculation	2	Single birth
Patient 2	M:30/F:30	1.2–1.7	12.92–47.72	0	15	13	Ejaculation	2	Single birth

* Age of the couple. M: Male, F: Female

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

Table2 Detailed description of the bi-allelic mutations in *LRRC6* identified in two infertile men

Patient	Gene	Position	RefSeq ID	AA Alteration	Mutation type	Min Depth	dbSNP ID	Status	1000 Ga	GO-ESP[a]	ExAC[a]	Mutation Taster[*]	SIFT[*]	Poly-Phen2[*]	Co-segregated
Patient1	*LRRC6*	Chr8: 133,645,101	NM_012472	c.C538T; p.R180X	nonsense	136	rs979934112	Het	NA	NA	NA	D	NA	NA	Yes
	LRRC6	Chr8: 133,673,819	NM_012472	c.64dupT; p.S22Ffs*19	Frameshift	92	NA	Het	NA	NA	0.000008256	D	NA	NA	Yes
Patient2	*LRRC6*	Chr8: 133,634,908	NM_012472	c.863C > A; p.P288H	Missense	61	rs76147813	Hom	0.00399361	NA	0.0021	D	D	D	Yes

[*]Mutation assessment by Mutation taster, SIFT and PolyPhen-2. D, disease causing; *Hom* homozygous, *Het* heterozygous, *NA* not available

[a]Frequency of corresponding mutations in 1000 Genomes, GO-ESP and total ExAC Browser

26 parts per billion (ppb). This is lower than the PCD-specific nasal nitric oxide cut-off value of 287 ppb [32]. These test results suggested that the patient suffers from PCD with male infertility. The patient 2 (Fig. 1a) was 31 years old and from a consanguineous family. Routine semen analysis of this patient revealed completely immotile spermatozoa with normal sperm counts. He also suffered from PCD symptoms, such as chronic nasitis, recurrent respiratory infections from childhood, and bronchiectasis. However, the PCD-related medical records were lost.

Identification of LRRC6 variants

WES was performed to identify the pathogenic genes of both patients. Single-nucleotide polymorphisms (SNPs) and indels were filtered according to the criteria and procedures described in "Methods" section. Three candidate variants in *LRRC6* (c.538C > T: p.R180* and c.64dupT: p.S22Ffs*19 in patient 1; c.863C > A:p.P288H in patient 2) were remained after filtering (Fig. 1b and c). Notably, two (c.538C > T: p.R180* and c.863C > A: p.P288H) of the three variants were the first time identified. All variants were predicted to be deleterious and completely absent or existed at very low allele frequencies in public databases (Table 2). Subsequent Sanger sequencing and pedigree analysis confirmed these variants and the compound heterozygote variants in *LRRC6* was inherited from their unaffected parents (pedigree analysis was failed due to unavailable DNA samples from the family members of patient 2), consistent with an autosomal recessive mode of inheritance (Fig. 1b). Therefore, we speculated that these novel variants of *LRRC6* were the causes of PCD with male infertility.

Significantly decreased LRRC6 protein in LRRC6 mutant spermatozoa

We performed Western blotting analysis to compare LRRC6 expression in the spermatozoa between the patient 1 (due to not enough sperm protein of patient 2) and normal control, and the results revealed a significant degradation of *LRRC6*-mutant protein in patient's spermatozoa (Fig. 2a). Immunostaining of *LRRC6* revealed a punctate pattern on normal sperm flagella but absent or significantly decreased in *LRRC6*-mutant spermatozoa (Fig. 2b). These results suggested that the variants in *LRRC6* were deleterious.

Abnormal morphology and ultrastructure of mutated spermatozoa

We investigated morphological and ultrastructural defects in patients' spermatozoa. Under light microscopy, compared

with those of normal control, most mutant spermatozoa exhibited multiple tail abnormalities, including short, curled, coiled, or looped tails (Fig. 3a). When observed by transmission electron microscopy (TEM), the spermatozoa from patient 1 exhibited a lack of inner and outer dynein arms but with a normal microtubule arrangement (Fig. 3b). Immunostaining of the outer dynein arm marker DNAI1 and the inner dynein arm marker DNALI1 confirmed that both patients' spermatozoa lacked outer and inner dynein arms (Fig. 3c and d). Immunostaining of the other flagellar structures, including radial spoke (RSPH1), mitochondrial sheath (TOMM20), central microtubules (SPAG6), and fibrous sheath (AKAP4) revealed no abnormalities in both patients (Fig. 4a–d). Taken together, these data suggested these *LRRC6* variants affected the dynein arm assembly of sperm flagella [11, 16, 33].

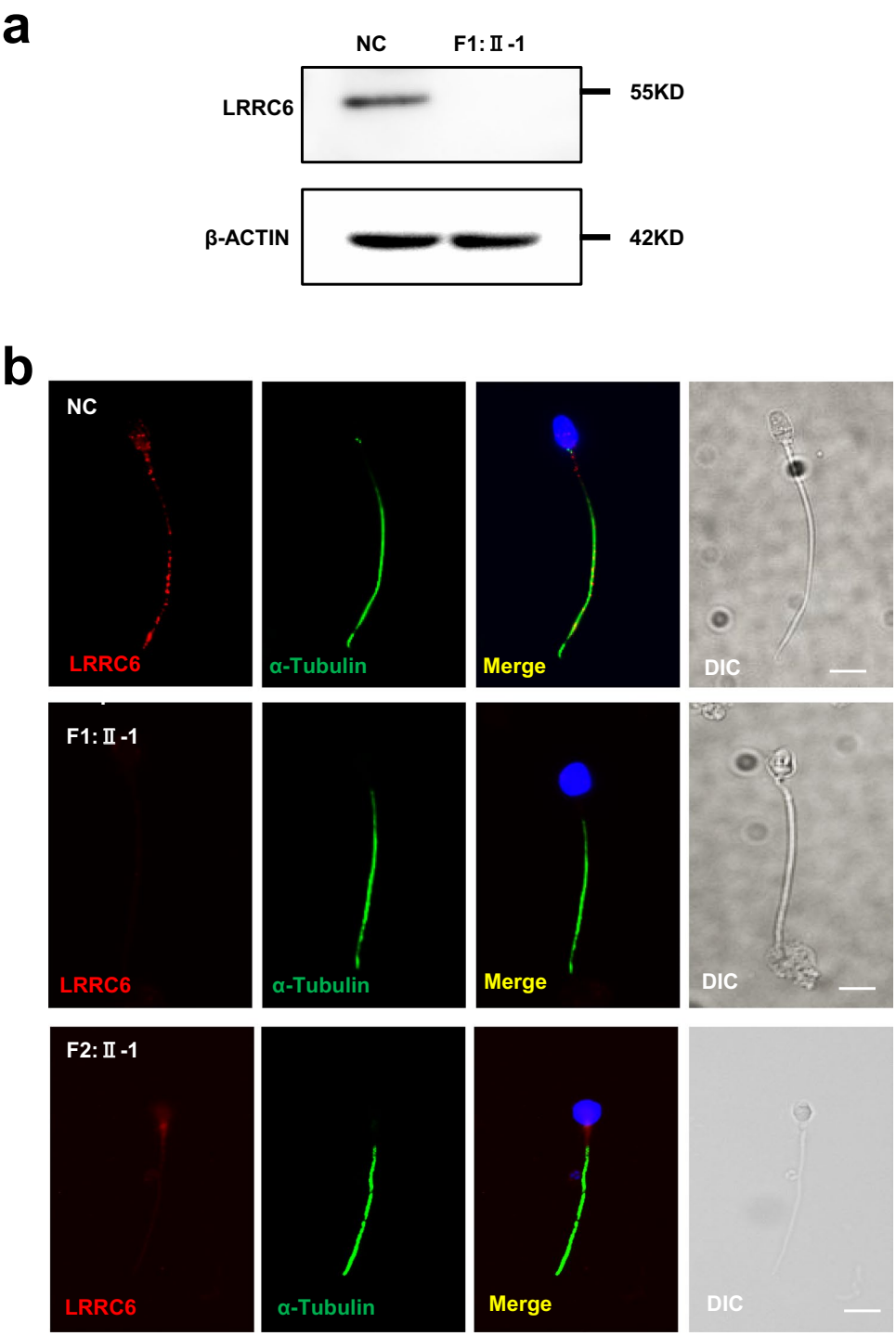


Fig. 2 The expression level of LRRC6 was significantly decreased in *LRRC6* mutant spermatozoa. **a** Western blotting analysis revealed that LRRC6 protein was absent in *LRRC6* mutant spermatozoa (F1: II-1). β-ACTIN was used as a loading control. *NC* normal control. **b** Immunofluorescence staining of *LRRC6* in *LRRC6* mutant spermatozoa (F1: II-1 and F2: II-1) and normal control. The LRRC6 protein (red) appeared as puncta on normal sperm flagella but absent or significantly decreased in *LRRC6* mutant spermatozoa. Anti-α-tubulin antibodies (green) were used as a loading control and DAPI (blue) was used as a nuclear marker. Scale bars: 5 μm; NC, normal control

Intracytoplasmic sperm injection outcome

Both couples selected ICSI after the *LRRC6* variant screening of the patient's partner. Thirteen oocytes were retrieved 36 h after hCG injection. Seven metaphase II oocytes were fertilized in two stimulated cycles. Seven blastocysts were formed by standard embryo culture and two were implanted (Table 1). For couple 2, fifteen oocytes were retrieved 36 h after hCG injection. Fifteen metaphase II oocytes were fertilized in three stimulated cycles. Thirteen blastocysts were formed by standard embryo culture and two were implanted (Table 1). After 14 days of embryos have been transferred into the two couples, pregnancy was identified by two hCG tests form peripheral blood. Furthermore, 35 days after embryos have been transferred, clinical pregnancy and the presence of a gestational sac and heartbeat was confirmed by ultrasound, and the both couples delivered a healthy baby, respectively.

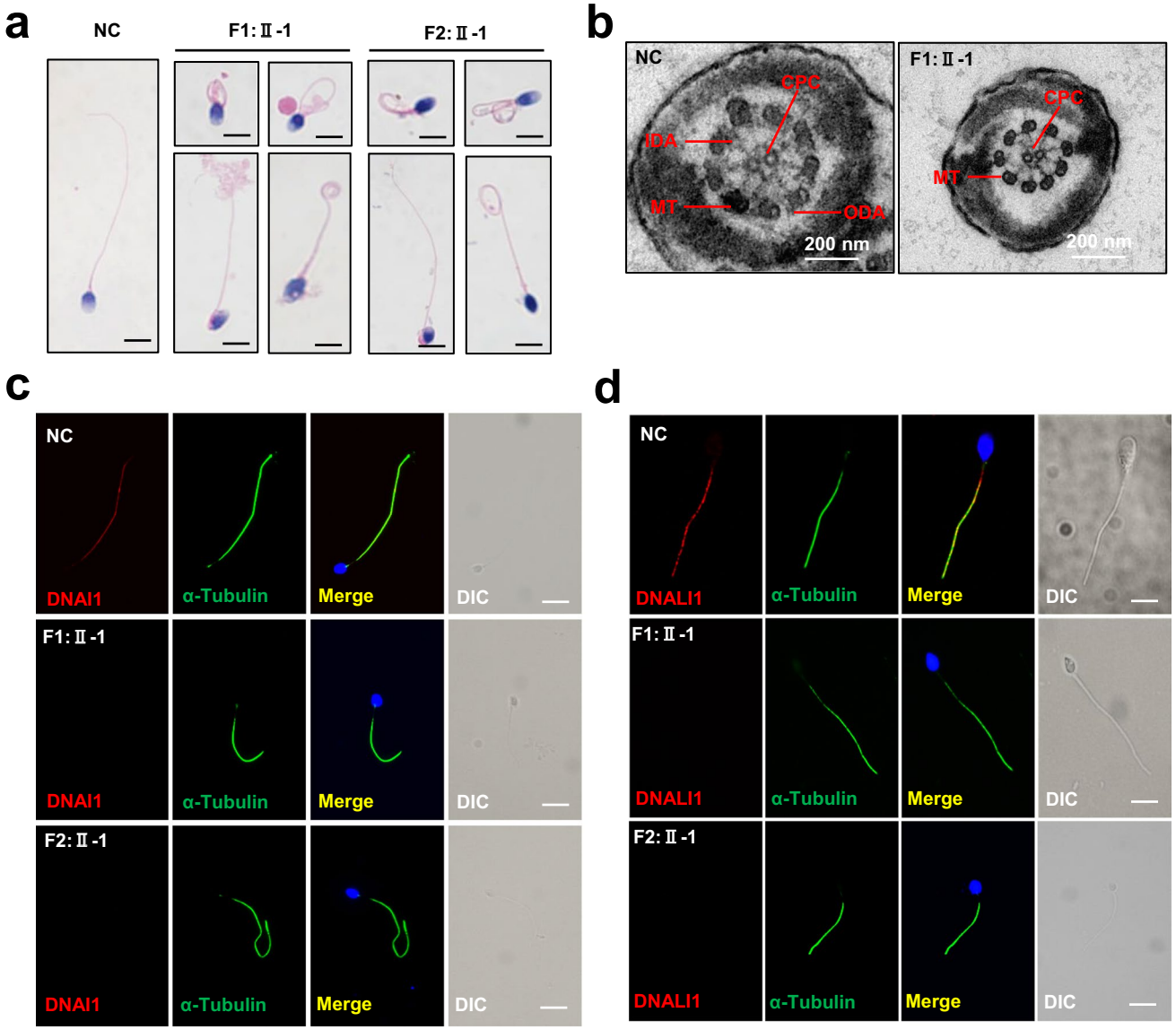


Fig. 3 Morphological ultrastructure defects in *LRRC6* mutant spermatozoa. **a** Hematoxylin and eosin staining revealing the abnormal flagella of patient 1 (F1: II-1) and patient 2 (F2: II-1). *NC* normal control. Scale bars: 10 μm. **b** Transmission electron microscope analysis revealing a lack of inner and outer dynein arms in the *LRRC6* mutant spermatozoa. *CPC* central pair complex, *MT* peripheral microtubule doublet, *IDA* inner dynein arms, *ODA* outer dynein arms, *NC* normal control; scale bars: 200 nm. **c**, **d** Immunofluorescence staining of spermatozoa from both patients (F1: II-1 and F2: II-1) and normal control with anti-DNAI1 antibodies (red) and anti-DNALI1 antibodies (red). Anti-α-tubulin antibodies (green) as a loading control and DAPI (blue) as a nuclear marker. Scale bars: 5 μm. *NC* normal control

Discussion

Herein, we identified two novel variants in *LRRC6* through WES in two Han Chinese patients suffering from PCD symptoms and male infertility. *LRRC6* is a known PCD pathogenic gene involving dynein arm pre-assembly in cilia [11, 16]. Moreover, all variants were completely absent or existed at very low allele frequencies in the public databases.

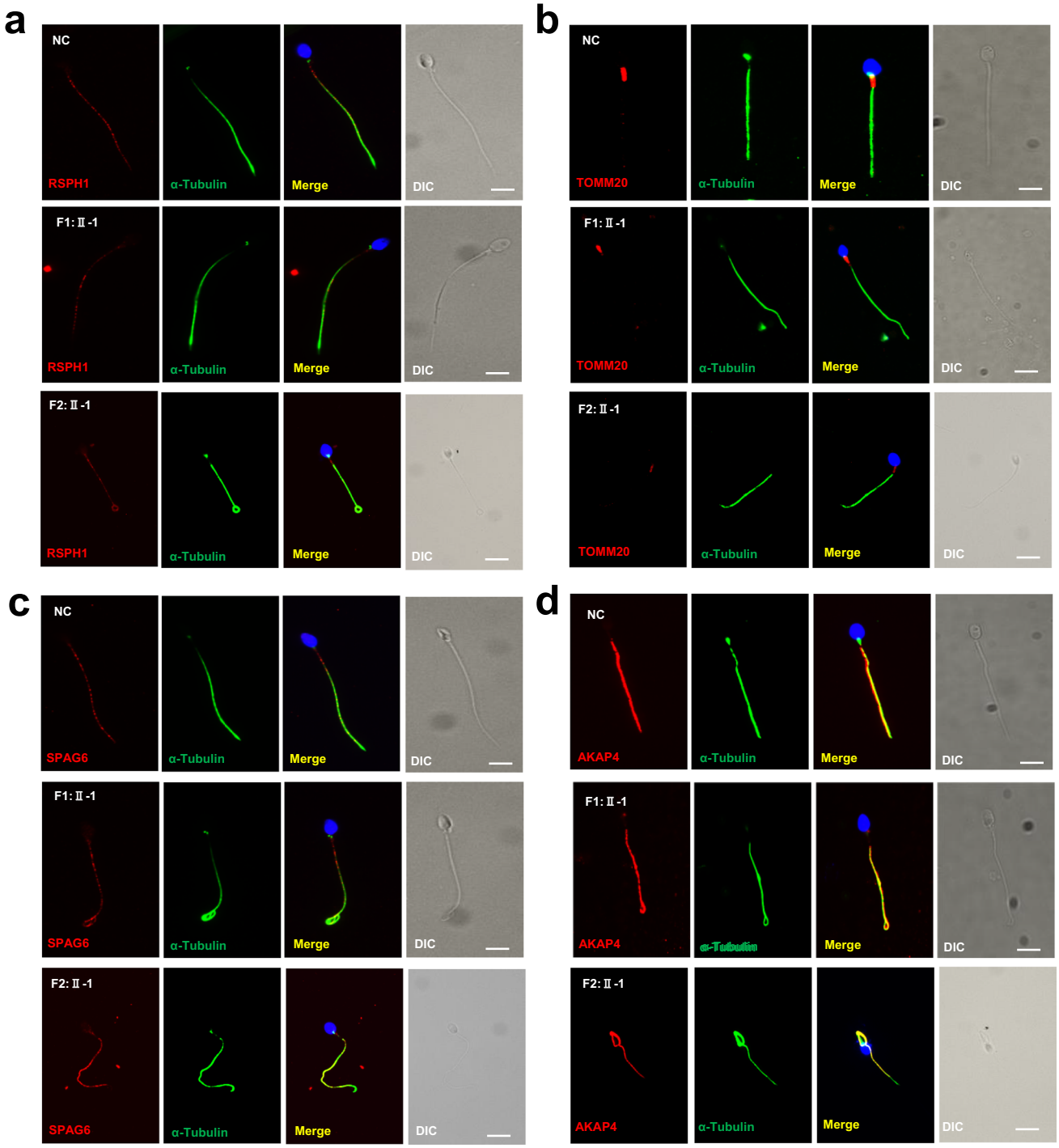


Fig. 4 No abnormalities were found in other flagellum components. Immunofluorescence staining with **a** anti-RSPH1, **b** anti-TOMM20, **c** anti-SPAG6, and **d** anti-AKAP4 antibodies (red) revealed *LRRC6* variants did not significantly impact the radial spokes, mitochondrial sheath, central microtubules, or fibrous sheath of the *LRRC6* mutant spermatozoa (F1: II-1 and F2: II-1). Anti-α-tubulin antibodies (green) were used as a loading control and DAPI (blue) was used as a nuclear marker. Scale bars: 5 μm; *NC* normal control

Western blotting and immunostaining analyses validated that *LRRC6*-mutant protein was degraded significantly. According to the American College of Medical Genetics and Genomics (ACMG) variant interpretation guidelines [34], these variants were likely pathogenic for this malady.

LRRC6 contains four leucine-rich repeat domains (LRRs), a LRRCT domain, a CS-like domain, and a coiled-coil domain predicted in Uniprot [11]. The CS-like domain is a critical HSP90 co-chaperone during the last steps of the HSP90 chaperone cycle, and cooperates with ZMYND10 and FKBP8 to mediate a key step in the pre-assembly pathway; specifically, maturation of axonemal dynein heavy chains [15, 35]. Two loss-of-function *LRRC6* variants (c.64dupT and c.538C > T) predicted to produce truncated proteins from the first LRRs and the coiled-coil domain were identified in our study, suggesting that LRRC6 may be involved in dynein arm assembly. In addition, the novel missense variant combined with other 27 reported variants in *LRRC6* are located on or before the CS-like domain indicating the critical role of the CS-like domain for pre-assembly of dynein arms [11, 16]. Considering that male infertility cannot be diagnosed until adulthood, previous studies have seldom focused on the morphological characteristics of the flagellum in PCD patients caused by *LRRC6* variants [11, 17], and only few PCD-related genes, such as *DRC1*, *DRC5*, and *RSPH4A*, have been reported to result in human male infertility[36–38]. According to a reported study, LRRC6 co-localizes with other DNAAF proteins and chaperones to form dynein axonemal particles in the cytoplasm of motile cilia [39]. In our study, the two patients with pathogenic *LRRC6* variants displayed male infertility and their spermatozoa were completely immotile with severely abnormal flagellar morphology and lacked inner and outer dynein arms, which consistent with the defects in mutated cilia [11]. We also identified a punctate pattern of LRRC6 protein along the sperm flagella and protein degradation in mutant sperm flagella. Our results showed a different location of the LRRC6 protein between sperm flagella and cilia, which indicated that *LRRC6* also plays a role in the intraflagellar transport of dynein arms during flagellar development. This may explain why all the male patients with *LRRC6* variants were infertile.

ICSI is the standard fertility treatment for males with asthenozoospermia [40]. ICSI using spermatozoa extracted from testis (TESE-ICSI) and ICSI with ejaculated spermatozoa (EJ-ICSI) are effective without adversely affecting embryonic development [34]. In this study, the couples chose EJ-ICSI and viable spermatozoa were identified by the hypo-osmotic swelling test. The pregnancy was successful and contributed to a healthy baby in each couple, consistent with the good prognosis of ICSI in previously reported *LRRC6*-mutant patients [14]. Interestingly, all the previously reported male PCD cases caused by *LRRC6* variants were infertile [13]. Therefore, ICSI is an effective way that can be used to treat male infertility caused by *LRRC6* variants and which may provide a guide for future treatment of male infertility caused by PCD.

In summary, we identified two novel pathogenic variants of *LRRC6* through WES, which extend our knowledge of the variant spectrum of the *LRRC6* gene. The protein degradation in sperm flagella caused by the *LRRC6* variants was also validated in both patients. Good ICSI outcomes obtained from *LRRC6* variant couples supporting a potential reproductive option for man harboring biallelic *LRRC6* variants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-022-02681-z.

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Author contribution Chaofeng Tu, Huan Zhang, and Huanzhu Li designed the study. Ying Wang, Lanlan Meng, and Chen Tan performed the variant analysis. Yunhao Li, Yong Li, Juan Du, Yue-Qiu Tan, and Hongchuan Nie carried out the evaluation of the pathogenicity of variations and spermatozoa functional analyses. Qianjun Zhang, Guangxiu Lu, and Ge Lin worked on the clinical study. Yunhao Li, Yong Li, Chaofeng Tu, Huan Zhang, and Huanzhu Li wrote the paper. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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