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Mutations in *CFAP47*, a previously reported MMAF causative gene, also contribute to the respiratory defects in patients with PCD

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Abstract

Background: Primary ciliary dyskinesia (PCD) is a genetic ciliopathy characterized by dysfunction of motile cilia. Currently, approximately 50 causative genes accounting for 60%–70% of all PCD cases have been identified in PCD-affected individuals, but the etiology in approximately 30%–40% of PCD cases remains unknown.

Methods: We analyzed the clinical and genetic data of two PCD individuals who were suspected of having PCD. Whole-exome sequencing and Sanger sequencing were performed to identify and verify the variants in *CFAP47*. We also evaluated the expression of CFAP47 by real-time quantitative PCR and immunofluorescence. Transmission electron microscopy in respiratory epithelial cells was also conducted to analyze ciliary function.

Results: Two hemizygous missense variants of X-linked *CFAP47* in two unrelated PCD individuals were identified. The expression of CFAP47 in two PCD individuals was significantly reduced in vivo and in vitro assays. A reduction in the amount of epithelial ciliary cells and basal bodies from PCD individuals was also observed.

Conclusions: We describe two hemizygous missense variants of X-linked *CFAP47* in two unrelated PCD individuals and prove *CFAP47* variants are related to a reduced number of epithelial ciliary cells. Therefore, we suggest that *CFAP47* should be known as a novel pathogenic gene of human PCD.

Haijun Ge and Wangji Zhou contributed equally to this work.

1 | INTRODUCTION

Primary ciliary dyskinesia (PCD, MIM 244400) is a rare genetic disease, characterized by abnormal ultrastructures and/or dysfunction of motile cilia. PCD affects approximately 1/10,000–1/20,000 individuals, but the actual prevalence is probably underestimated (Niziolek et al., 2022). PCD patients in the neonatal period have a wet cough caused by recurrent infections of the upper and lower respiratory tract and frequently exhibit sinusitis, otitis media, and hearing defects. Approximately 50% of patients exhibit situs inversus resulting from embryo nodal cilia dysfunction. Many male PCD patients manifest infertility, associated with asthenoteratozoospermia, which is often seen in patients with multiple morphological abnormalities of the sperm flagella (MMAF) (Fassad et al., 2020).

The specific phenotypes or severity of PCD symptoms depend upon the extent of the alterations of cilia/flagella structure and function, which is directly or indirectly caused by the mutated genes. To date, approximately 50 genes have been reported to be involved in PCD, although they are estimated to account for only 60%–70% of PCD cases (Niziolek et al., 2022). There are still many individuals with PCD for whom no known causative genes were identified, indicating the involvement of other potential novel pathogenic genes. A large proportion of related proteins encoded by these genes are responsible for the highly conserved "9 + 2" arrangement composed of nine microtubule doublets surrounding a central pair apparatus. The outer microtubule doublets are attached to outer and inner dynein arms (ODAs and IDAs), which are essential for the motor force and regulation of the beating frequency of motile cilia and flagella (Guo et al., 2020). In recent years, genes such as *CCNO* and *FOXJ1* that do not encode microtubule structural proteins have been shown to be critical regulators of human multiciliated cells (MMCs) differentiation. Similarly, *NEK10* is a serine/threonine kinase that regulates ciliated cells (Boon et al., 2014; Chivukula et al., 2020).

Cilia- and flagella-associated proteins (CFAPs), such as CFAP221, CFAP53, CFAP54, CFAP300, CFAP43, CFAP44, CFAP300 and CFAP251, are linked to flagellum biogenesis and morphogenesis (Wang et al., 2021; Wu et al., 2021). Previous studies indicated that mutations in *CFAP221*, encoding proteins of the central pair apparatus, caused PCD in humans (Bustamante-Marin et al., 2020), and mice carrying mutations in *Cfap221* and *Cfap54* typically exhibited PCD-associated phenotypes of hydrocephalus, mucociliary clearance defects in the upper airway and abnormal spermatogenesis (McKenzie & Lee, 2020). In addition, CFAP300 interaction with cytoplasmic ODA/IDA assembly factor DNAAF2 is essential for dynein arm assembly and *CFAP300* genetic defects cause immotility of cilia (Fassad et al., 2018; Höben et al., 2018). More recently, studies have reported that hemizygous mutations of X-linked *CFAP47* can induce MMAF and asthenoteratozoospermia in three Chinese male infertile patients, whose sperm presented with reduced motility and abnormal flagellar morphology (Liu et al., 2021). However, no publication has shown that *CFAP47* mutations can lead to the dysfunction of motile cilia and PCD.

In this study, we performed WES and genetic analysis of two unrelated Han Chinese patients with typical PCD phenotypes and identified two hemizygous missense variants of *CFAP47*. We also confirmed the pathogenic effects of these variants in HEK293T cells in vitro and in the spermatozoa of PCD individuals by real-time quantitative PCR (qPCR) and immunofluorescence. In particular, we found that the *CFAP47* mutation may be responsible for the defects of motile cilia from bronchial tissues of proband of family 1 (P1) by transmission electron microscopy (TEM). Altogether, our results indicate that *CFAP47* hemizygous variants can result in PCD phenotypes both in lung and in sperm and that this gene is a novel PCD pathogenic gene.

2 | MATERIALS AND METHODS

2.1 | Subjects

Two unrelated Han Chinese patients were diagnosed or suspected with PCD based on one of the following criteria at Peking Union Medical College Hospital (PUMCH): (1) The ultrastructural defect of the respiratory cilia was recognized by TEM; (2) Biallelic pathogenic or likely pathogenic variants of one of known PCD-associated genes were detected; and (3) At least 2 of the following 4 clinical features for PCD were present together with low nasal nitric oxide (nNO) level: unexplained neonatal respiratory distress in full-term infant; year-round daily cough beginning before 6 months of age; year-round daily nasal congestion beginning before 6 months of age; and organ

laterality defect. (4) Patients with Kartagener syndrome (Shoemark et al., 2019).

2.2 | Whole-exome sequencing (WES) and filtering

Blood samples from two probands were collected, and genomic DNA was extracted using a DNA extraction kit (Qiagen). Genomic DNA was then used for WES performed by the Novogene Bioinformatics Institute. Whole exomes were captured with the Agilent SureSelect Human All ExonV5 kit (Agilent) and sequenced on the Illumina HiSeq2500 platform.

The candidate pathogenic variants in the VCF file were screened using the following criteria: (1) Variants within upstream/downstream, intergenic, intronic, ncRNA and UTR regions and synonymous mutations were excluded; (2) Variants with a minor allele frequency below 1% as reported by public databases (1000 Genomes Project, Exome Aggregation Consortium, and East Asians in gnomAD) were screened out; (3) Candidates with only one heterozygous variant were excluded according to autosomal recessive inheritance, except hemizygous variants in the sex chromosomes; (4) The candidates should be highly expressed in ciliated tissues and organs (lung, brain, and testis); 5. The effect of candidates on protein function was deleterious as predicted by bioinformatic programs (SIFT, PROVEAN, Mutation Taster, CADD).

2.3 | Sanger sequencing and bioinformatic analysis

For validation of *CFAP47* variants, specific PCR primers were designed to target the region of the variants in the candidate gene using Primer Premier 5, and the sequences of the primers are listed in Table S1. The potential effect of variants on protein function was evaluated by InterVar.

2.4 | Reverse-transcription PCR (RT-PCR)

Total RNA was extracted from isolated tissues in an adult male mouse of C57BL/6 by the traditional extraction method with TRIzol® reagent (Ambion), and the quality of extracted total RNA was assessed by 1.5% agarose gel electrophoresis and quantified by spectrophotometry. Approximately 1.0 μg of RNA was reverse transcribed into cDNA with PrimeScript™ RT Master Mix (No.RR036A) in

each sample. The sequences of the RT-PCR primers are listed in Table S1.

2.5 | Real-time quantitative PCR (qPCR)

Plasmids containing the complementary DNA (cDNA) sequence of the longest open reading frame (ORF) of *CFAP47* were purchased from Youbio Biological Technology. Subsequently, the expression vectors of the wild-type and mutated plasmids were transfected into human embryonic kidney cells (HEK293T) using Lipofectamine™ LTX Reagent with PLUS™ Reagent (A12621), and the cells were cultured for 48 h. Total RNA was extracted from HEK293T cells and was reverse transcribed into cDNA as described previously. Relative mRNA levels of *CFAP47* were investigated with qPCR by using a Hieff® qPCR SYBR Green Master Mix kit (Cat:1120ES03). Relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method, and *GAPDH* was used as a reference control in parallel. The sequences of the qPCR primers are listed in Table S1.

2.6 | Immunofluorescence analysis (IF)

Spermatozoa were obtained from PCD patients and controls. Then, the sperm samples were washed in phosphate-buffered saline (PBS) three times (5 min each) and fixed in 4% paraformaldehyde for 30 min at room temperature on adhesion microscope slides. The fixed sperm cells were permeabilized with 0.5% Triton X-100 for 15 min and blocked with 1% bovine serum albumin in PBS (blocking solution) for 30 min. The primary antibodies were incubated overnight at 4°C, and the appropriate secondary antibodies were incubated for 1 h at room temperature (Table S2). After washing with PBS three times (5 min each), nuclei were stained using mounting medium and antifading (with DAPI) (Solarbio; S2110). The immunofluorescence images were acquired using the UltraVIEW VoX 3D live cell imaging system.

2.7 | Transmission electron microscopy (TEM)

Fresh bronchial mucosas were obtained by bronchoscopy from PCD individuals. The samples were immersed in a fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde/PBS mixed solution). After postfixation in osmium tetroxide for an hour, progressive dehydration with graded ethanol, and being embedded in Epon 812, the samples

were sliced into 600 nm sections and stained with tolonium chloride to localize the target structure under light microscopy. Selected regional sample sections were stained with uranyl acetate and lead citrate and photographed via TEM with an acceleration voltage of 80 kV. The images were captured using a VELETA CCD camera (OSIS).

3 | RESULTS

3.1 | Clinical features

The P1 was a 37-year-old man who came to PUMCH in 2020 due to recurrent productive cough for 28 years and shortness of breath for 20 years. He had otitis media in 1993 at the age of 10. He was diagnosed with chronic sinusitis and underwent nasal polypectomy in 2012. He was diagnosed with bronchiectasis in 2017 by chest CT after a slight hemoptysis episode. He had tuberculosis at the age of 7 and was cured by anti-tuberculosis therapy. He had two naturally conceived healthy children. Pulmonary function tests showed very severe obstructive ventilation dysfunction with forced expiratory volume in 1 s (FEV_1) 16.9% of the predicted value, forced vital capacity (FVC) 35.3% of the predicted value, and FEV_1/FVC ratio 42.9% (Table S3). He had a negative bronchodilation test. nNO is far below the value of 25.8 nL/min. His arterial blood gas analysis showed type II respiratory failure with PaO_2 56 mmHg and $PaCO_2$ 57 mmHg. The culture result of bronchoalveolar lavage fluid was *Moraxella catarrhalis*. Bronchiectasis secondary to infection, autoimmune disease, and allergic bronchial-pulmonary aspergillosis (ABPA) were excluded based on bronchiectasis screening tests. Electron microscopy of bronchial mucosal biopsy showed that the number of cilia was significantly reduced, the cilia were short and basal bodies were of disarrangement, and there was no obvious abnormality in the structure of dynein arms. PCD was diagnosed based on his respiratory symptoms and electron microscope findings.

The proband of family 2 (P2) came to PUMCH due to recurrent productive cough for nearly 40 years in 2014. He had chronic sinusitis and otitis media as a teenager. He was finally diagnosed with bronchiectasis in 2010. This patient had intermittent hemoptysis and underwent selective bronchial artery embolization due to massive hemoptysis in 2015. In 2005, he successfully received intracytoplasmic sperm injection (ICSI) for asthenospermia at PUMCH. According to birth records, the patient was delivered via full-term natural labor and had no history of neonatal pneumonia or asphyxia. But he had persistent productive cough and nasal congestion since his infant time. Pulmonary function tests

showed slight obstructive ventilation dysfunction with FEV_1 84.4% of the predicted value, FVC 104.9% of the predicted value, and FEV_1/FVC ratio 66.56% (Table S3). He had negative bronchodilation test. Sputum culture yielded no pathogens. Bronchiectasis secondary to infection, autoimmune disease, and ABPA were excluded based on bronchiectasis screening tests. Combining all respiratory symptoms including bronchiectasis, otitis media, sinusitis with infertility, PCD was highly suspected.

3.2 | Identification of rare and hemizygous *CFAP47* variants in PCD

We performed WES on two individuals diagnosed or suspected with PCD based on clinical criteria of PCD. After a strict filtration process as described in the Materials and Methods, we identified two hemizygous missense variants (NM_001304548, c.4316A > T: p.Asn1439Ile, and c.8786C > A: p.Pro2929Gln) of X-linked *CFAP47* in these two patients. Two variants of *CFAP47* were verified through Sanger sequencing (Figure 1b). Unfortunately, co-segregation analysis was not conducted because family members were unavailable. The frequency of two hemizygous variants of *CFAP47* was absent or very low in the human population genome datasets, including 1000 Genomes Project, Exome Aggregation Consortium, and East Asians in gnomAD, and the pathogenicity of hemizygous missense variants of *CFAP47* was evaluated to be pathogenic by InterVar (Table 1).

CFAP47 is located on human chromosome Xp21.1 and contains 3187 amino acid (aa) residues, and the structural and functional domain is calponin homology (CH) located in residues 1746 aa–1869 aa. The missense variant of p.Asn1439Ile is close to the N-termination, while p.Pro2929Gln is near the C-termination, and the p.Asn1439Ile residue is highly conserved among different species of vertebrate mammals according to MultAlin (Figure S1). To assess the potential effects of *CFAP47* variants on the spatial structure of the CFAP47 protein, both wild-type and mutant three-dimensional (3D) structural homology models were generated using I-TASSER. In the 3D structural model of I1439, the reduced numbers of β-sheets in the bridge section may slightly destabilize the structure of CFAP47, and the decreased numbers of hydrogen bonds around mutant residue Ile1439 might lead to a loss of interactions with other proteins. Additionally, the substitution of Pro2929Gln may disturb the unique rigid backbone conformation by spatially reducing the number of α-helices near this residue based on the 3D structural model of CFAP47 (Figure S1).
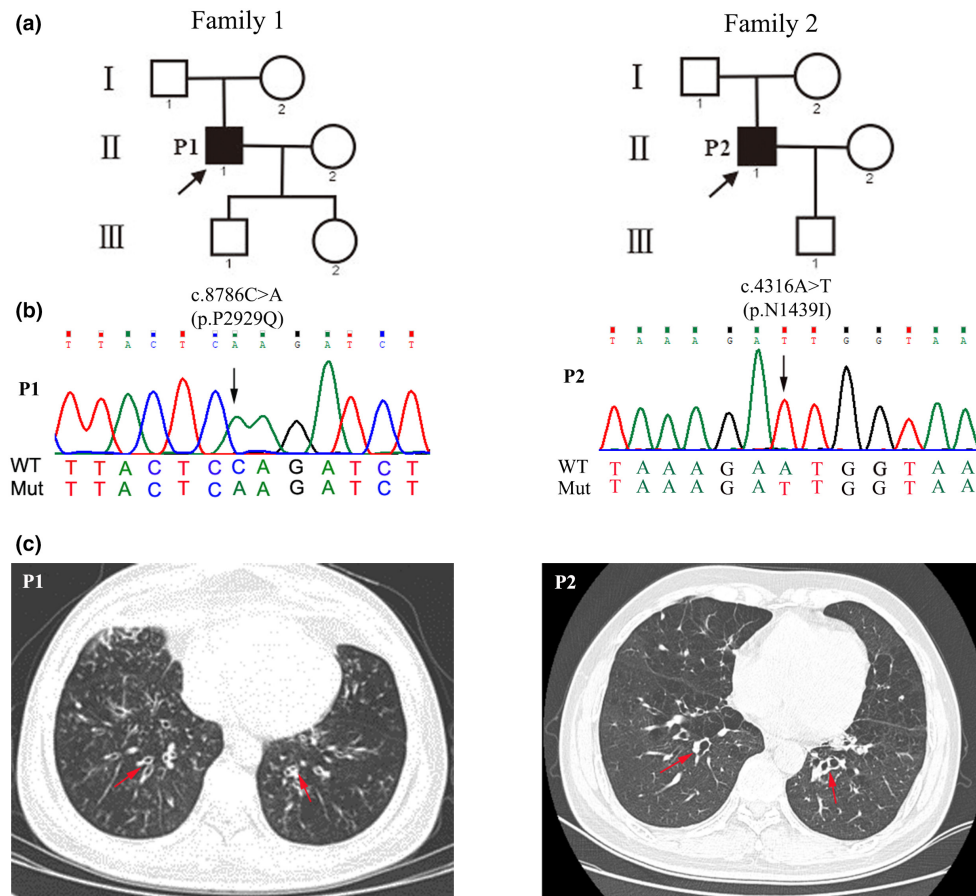
(a)

(b)

c.8786C>A
(p.P2929Q)

c.4316A>T
(p.N1439I)

P1

WT T T A C T C C A G A T C T
Mut T T A C T C A A G A T C T

P2

WT T A A A G A A T G G T A A
Mut T A A A G A T T G G T A A

(c)

FIGURE 1 Two X-linked *CFAP47* hemizygous variants were identified in two independent PCD individuals. (a) The families with the probands carrying *CFAP47* missense variants inherited from mothers, respectively. Black arrow, proband; black filled squares indicate the probands of family 1 (P1) and family 2 (P2). (b) Sanger sequencing results of the two variants carried by P1 and P2 validated the results from WES. The variant positions are indicated by black arrows. (c) Thoracic computed tomography showed bilateral lower lung predominant bronchiectasis and typical imaging manifestations like signet ring sign (red arrows).

TABLE 1 Hemizygous deleterious *CFAP47* variants identified in two unrelated Chinese PCD-affected individuals.

CFAP47 variants	M1	M2
cDNA alteration	c.C8786A	c.4316A > T
Protein alteration	p.Pro2929Gln	p.Asn1439Ile
Variant allele	Hemizygous	Hemizygous
Variant type	Missense	Missense
1000 Genomes Project	Absent	Absent
Exome Aggregation Consortium	Absent	Absent
East Asians in gnomAD	0.001	Absent
InterVar	Pathogenic	Pathogenic

Note. Genomic databases coordinate in GRCh37/hg19.

3.3 | High Cfap47 expression in ciliated tissues of adult mice

CFAP47, defined as a cilia and flagella associated protein, is presumably highly expressed in ciliary tissues and organs such as the lung, brain, and testis. RT-PCR was performed to investigate *Cfap47* mRNA expression in various tissues of adult mice. Specific bands were detected in the lung, testis, trachea, and brain (Figure 2).

3.4 | Significantly decreased CFAP47 in patient spermatozoa and in HEK293T cells

To investigate the functional effects of the identified *CFAP47* variants in our study, we analyzed CFAP47 expression by qPCR and IF on spermatozoa from P1 and a control individual. The qPCR assays showed significantly reduced expression of *CFAP47* mRNA in the spermatozoa from P1, which harbors the c.8786C > A

(p.P2929Q) of *CFAP47*. As shown in Figure 3, CFAP47 is localized at the junction of the spermatozoa head and flagella in a control individual. Consistently,
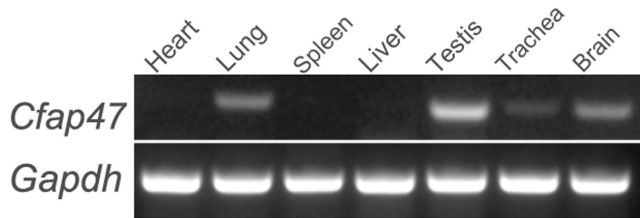

FIGURE 2 Expressions of *Cfap47* were investigated by RT-PCR in various tissues from adult male C57BL/6 mouse; *Gapdh* was used as a control.

the CFAP47 signal was absent in mutated spermatozoa of P1 (Figure 3). To further validate the pathogenicity of the c.4316A > T (p.N1439I) of *CFAP47*, we constructed two expression plasmids pcDNA3.1-3xFlag-CFAP47-WT and pcDNA3.1-3xFlag-CFAP47-c.4316A > T, and transfected wild-type and mutant vectors into HEK293T cells. The qPCR assays indicated that *CFAP47* mRNA was significantly reduced in the mutant vector pcDNA3.1-3xFlag-CFAP47-c.4316A > T, compared with the wild-type plasmid (Figure 3). These results suggest that *CFAP47* variants of c.4316A > T (p.N1439I) and c.8786C > A (p.P2929Q) both caused the extremely reduced expression of CFAP47 in sperm or in HEK293T cells.
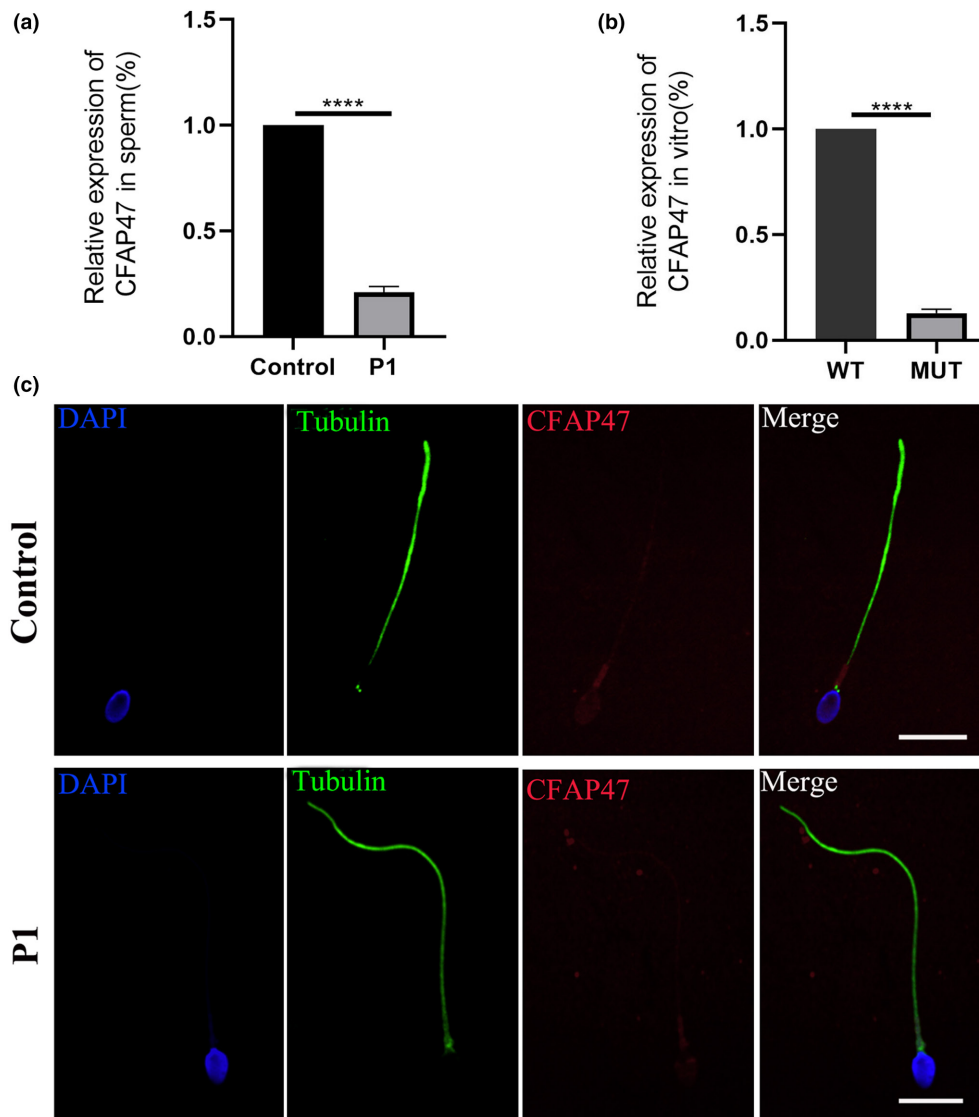

FIGURE 3 Expression analysis of CFAP47 in sperm of P1 and in cell line in vitro. (a) The abundance of *CFAP47* mRNA extracted from sperm was decreased as shown by qPCR analysis of P1, as compared to a control male. (b) The RNA was extracted from HEK293T cells transfected with wild-type and mutant plasmids of the *CFAP47* variant identified in P2. The expression of CPAP47 was reduced in the mutant plasmid-transfected HEK293T cells. *T* tests were used as appropriate (***$p < 0.001$). (c) Immunofluorescence analysis showed CFAP47 is located to junction of head and flagellum in sperm of control but absent in sperm of P1. Acetylated α-tubulin (in green) localizes to the entire length of the ciliary axoneme. DAPI (in blue) as a nuclear marker. Scale bars: 5 mm.

3.5 | *CFAP47* variants reduced the length and number of respiratory cilia

To explore the potential functional effects of the *CFAP47* variants in respiratory cilia, we performed TEM analyses on bronchial tissues from P1 and a control individual. TEM showed that the respiratory cilia appeared stubby and were reduced in length and number in P1 without altering the complete structure of "9 + 2" axoneme (Figure S1), compared to the control. Furthermore, together with the overall reduced number of basal bodies, they also disarranged in diverse orientations and were mislocated in the cytoplasm (Figure 4). Thus, the bronchiectasis phenotype of P1 was probably associated with the dysfunction of mucociliary clearance caused by the reduced number and length of respiratory cilia.

4 | DISCUSSION

PCD is a clinically and genetically heterogeneous disease caused by dysfunction of motile cilia in mucociliary clearance and abnormal flagella in sperm movement. At present, more than 50 causative genes have been identified in PCD, and most of the associated genes are predominantly inherited in an autosomal recessive mode, with the two exceptions that *PIH1D3* and *OFD1* are X-linked inherited (Bukowy-Bieryllo et al., 2019; Olcese et al., 2017). In this study, we reported two hemizygous missense variants c.4316A > T (p.N1439I) and c.8786C > A (p.P2929Q) of X-linked *CFAP47* in two unrelated Chinese Han PCD-suspected individuals, who presented typical PCD symptoms. Both patients present with PCD/PCD like symptoms such as recurrent wet cough as well as sinusitis and otitis media since early childhood. According to the 2018 American Thoracic Society clinical practice guideline for PCD (Feller-Kopman et al., 2018), combined with the low nNO (below the diagnostic cutoff of 77 nL/min), the diagnosis of PCD in P1 was definite. TEM results showed abnormal cilia number and morphology, which further confirmed the diagnosis. As for P2, due to the COVID-2019 pandemic, this patient was not available for nNO testing and no bronchial mucosa was collected. But considering the patient's typical respiratory symptoms with infertility, we consider the patient highly likely to have PCD. Two identified variants of *CFAP47* were absent or present in low frequency in the general population databases, and they were predicted to be deleterious by multiple bioinformatic tools. Moreover, *Cfap47* from adult mice was highly expressed in ciliary tissues and organs. The missense variant c.4316A > T in P2 reduced the expression of *CFAP47* mRNA in vitro, and sperm cells from P1 carrying the c.8786C > A variant
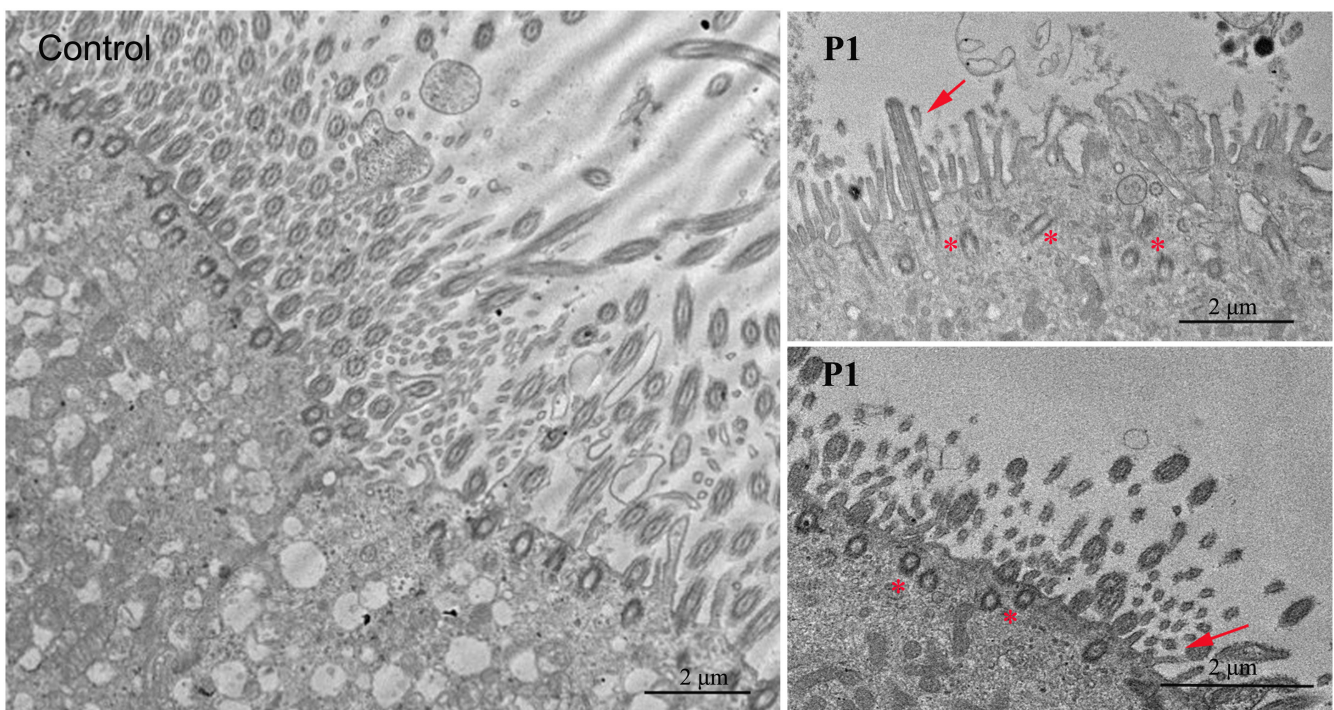

FIGURE 4 Transmission electron microscopy of ciliated respiratory epithelium from a healthy control subject and P1. The control showed normal longitudinal section and complete arrangements of the basal bodies of the respiratory epithelium. While the amount and the length of cilia is reduced (red arrows) in P1. Cilia-anchoring basal bodies in the cytoplasm displayed disorganized arrangements, and some basal bodies plunged into cytoplasm deeply (red asterisks) in P1.

displayed reduced *CFAP47* expression by both qPCR and IF. These results collectively confirm the pathogenicity of two hemizygous *CFAP47* variants in two independent PCD patients.

In this study, our patients with hemizygous missense variants of *CFAP47* showed combined classic PCD phenotypes of bronchiectasis, otitis media, and sinusitis. And the nNO value is only 25.8 nL/min in P1. Other than classic respiratory symptoms of PCD, P2 also manifested infertility and received ICSI treatment leading to successful fertilization, while P1 had normal functional sperm. The difference of clinical features among patients with PCD is not uncommon. PCD individuals who carry various associated genes or even the same pathogenic variant might exhibit different PCD-related phenotypes, especially differing in situs inversus, rhinosinusitis, and hydrocephalus (Bustamante-Marin et al., 2019). Biallelic mutations in *RSPH1* were reported in 16 PCD individuals who manifested wide-ranging PCD features with different numerical nNO values, with or without rhinosinusitis, otitis media, and infertility. Similarly, sixteen PCD individuals with the CCDC103 p.His154Pro variant displayed distinct phenotypes of nNO values, situs inversus, and ciliary beating frequency and pattern (Knowles et al., 2014; Shoemark et al., 2018). And the variant [c.921 + 3_6delAAGT] in *RSPH4A* has been reported as a common cause of PCD in the Hispanic population. These patients harboring [c.921 + 3_6delAAGT] of *RSPH4A* also manifested various clinical PCD phenotypes (De Jesus-Rojas et al., 2021; De Jesus-Rojas et al., 2022; de Jesús-Rojas et al., 2023). These observations indicate that PCD individuals can present independent and heterogeneous symptoms caused by different genetic variants, even the same variant.

Several MMAF causative genes were confirmed to result in PCD. Ting Guo et al. reported a *BRWD1* variant related to MMAF and PCD/PCD-like symptoms in humans and discussed that the MMAF pathogenic genes *DNAH1*, *SPEF2*, and *CFAP65* can cause PCD phenotypes ranging from low-noise PCD manifestations to typical PCD symptoms (Guo et al., 2021). *DNAH1* variants initially had been reported to cause asthenozoospermia without any PCD or PCD-like symptoms. The authors later on suggested that DNAH1 function in motile cilia had also been damaged but was possibly compensated by other DNAH protein family members (Ben Khelifa et al., 2014), indicating that some PCD individuals with a weak or nonclassic PCD phenotype may be diagnosed MMAF only.

As for *CFAP47*, Liu et al. (2021) reported hemizygous missense variants of *CFAP47* caused MMAF and asthenozoospermia phenotypes in three unrelated men, and

they concluded *CFAP47* was a novel pathogenic gene for MMAF. In our study, we also collected two unrelated PCD-affected individuals harboring two new hemizygous missense variants of *CFAP47*, but the inconsistency is that the two cases in our study present typical manifestations of PCD, such as sino-pulmonary manifestations including rhinosinusitis, otitis media, bronchiectasis, and low level of nNO and asthenoteratozoospermia. Furthermore, RT-PCR assays in the study of Chunyu Liu et al. using various tissues of adult C57BL/6N mice indicated the preferential expression of mouse Cfap47 in the testis. However, as shown in Figure 2, the same primers used in our RT-PCR analysis revealed that Cfap47 in adult C57BL/6N mice is primarily expressed in the testis, trachea, brain, and lung. As a supportive evidence of the pathogenicity of two missense variants in *CFAP47*, qPCR assays also showed significantly reduced expression of *CFAP47* mRNA either in vivo (sperm) or in vitro (HEK293T cells). In addition, Chunyu Liu et al. conducted co-immunoprecipitation assays to confirm that CFAP47 has a functional link with CFAP65 and reported that CFAP65 deficiency caused severe asthenoteratozoospermia. It is known that some MMAF individuals harboring *CFAP65* variants also exhibited weaker typical PCD symptoms (Wang et al., 2019). Therefore, together with our results, we suggest that the effect of missense variants in *CFAP47* is not limited to reducing sperm motility and abnormal flagella resulting in MMAF, but also plays an important role in the respiratory defects in the patients with PCD.

5 | CONCLUSIONS

In conclusion, we report two hemizygous *CFAP47* variants in two independent PCD-affected individuals. Our study proves that *CFAP47* variants cause reduced numbers of cilia and misaligned basal bodies. The findings have important clinical implications for facilitating PCD diagnosis and genetic counseling. Overall, we suggest that *CFAP47* should be known as a novel pathogenic gene of PCD.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

INSTITUTIONAL REVIEW BOARD STATEMENT AND ETHICS STATEMENT
All methods carried out in this study were approved by the Institutional Review Board committee at PUMCH. All procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975 (as revised in 2000).

INFORMED CONSENT STATEMENT
Informed consent was obtained from all patients for which identifying information is included in this article.

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REFERENCES
Ben Khelifa, M., Coutton, C., Zouari, R., Karaouzène, T., Rendu, J., Bidart, M., Yassine, S., Pierre, V., Delaroche, J., Hennebicq, S., Grunwald, D., Escalier, D., Pernet-Gallay, K., Jouk, P. S., Thierry-Mieg, N., Touré, A., Arnoult, C., & Ray, P. F. (2014). Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella. *American Journal of Human Genetics*, *94*(1), 95–104. https://doi.org/10.1016/j.ajhg.2013.11.017

Boon, M., Wallmeier, J., Ma, L., Loges, N. T., Jaspers, M., Olbrich, H., Dougherty, G. W., Raidt, J., Werner, C., Amirav, I., Hevroni, A., Abitbul, R., Avital, A., Soferman, R., Wessels, M., O'Callaghan, C., Chung, E. M. K., Rutman, A., Hirst, R. A., ... Omran, H. (2014). MCIDAS mutations result in a mucociliary clearance disorder with reduced generation of multiple motile cilia.

Nature Communications, *5*, 4418. https://doi.org/10.1038/ncomms5418

Bukowy-Bieryllo, Z., Rabiasz, A., Dabrowski, M., Pogorzelski, A., Wojda, A., Dmenska, H., Grzela, K., Sroczynski, J., Witt, M., & Zietkiewicz, E. (2019). Truncating mutations in exons 20 and 21 of OFD1 can cause primary ciliary dyskinesia without associated syndromic symptoms. *Journal of Medical Genetics*, *56*(11), 769–777. https://doi.org/10.1136/jmedgenet-2018-105918

Bustamante-Marin, X. M., Shapiro, A., Sears, P. R., Charng, W. L., Conrad, D. F., Leigh, M. W., Knowles, M. R., Ostrowski, L. E., & Zariwala, M. A. (2020). Identification of genetic variants in CFAP221 as a cause of primary ciliary dyskinesia. *Journal of Human Genetics*, *65*(2), 175–180. https://doi.org/10.1038/s10038-019-0686-1

Bustamante-Marin, X. M., Yin, W. N., Sears, P. R., Werner, M. E., Brotslaw, E. J., Mitchell, B. J., Jania, C. M., Zeman, K. L., Rogers, T. D., Herring, L. E., Refabért, L., Thomas, L., Amselem, S., Escudier, E., Legendre, M., Grubb, B. R., Knowles, M. R., Zariwala, M. A., & Ostrowski, L. E. (2019). Lack of GAS2L2 causes PCD by impairing cilia orientation and Mucociliary clearance. *American Journal of Human Genetics*, *104*(2), 229–245. https://doi.org/10.1016/j.ajhg.2018.12.009

Chivukula, R. R., Montoro, D. T., Leung, H. M., Yang, J., Shamseldin, H. E., Taylor, M. S., Dougherty, G. W., Zariwala, M. A., Carson, J., Daniels, M. L. A., Sears, P. R., Black, K. E., Hariri, L. P., Almogarri, I., Frenkel, E. M., Vinarsky, V., Omran, H., Knowles, M. R., Tearney, G. J., ... Sabatini, D. M. (2020). A human ciliopathy reveals essential functions for NEK10 in airway mucociliary clearance. *Nature Medicine*, *26*(2), 244–251. https://doi.org/10.1038/s41591-019-0730-x

de Jesús-Rojas, W., Meléndez-Montañez, J., Muñiz-Hernández, J., Marra-Nazario, A., Alvarado-Huerta, F., Santos-López, A., Ramos-Benitez, M. J., & Mosquera, R. A. (2023). The RSPH4A gene in primary ciliary dyskinesia. *International Journal of Molecular Sciences*, *24*(3). https://doi.org/10.3390/ijms24031936

De Jesus-Rojas, W., Muniz-Hernandez, J., Alvarado-Huerta, F., Melendez-Montanez, J. M., Santos-Lopez, A. J., & Mosquera, R. A. (2022). The genetics of primary ciliary dyskinesia in Puerto Rico. *Diagnostics (Basel)*, *12*(5). https://doi.org/10.3390/diagnostics12051127

De Jesus-Rojas, W., Reyes-De Jesus, D., & Mosquera, R. A. (2021). Primary ciliary dyskinesia diagnostic challenges: Understanding the clinical phenotype of the Puerto Rican RSPH4A founder mutation. *Diagnostics (Basel)*, *11*(2). https://doi.org/10.3390/diagnostics11020281

Fassad, M. R., Patel, M. P., Shoemark, A., Cullup, T., Hayward, J., Dixon, M., Rogers, A. V., Ollosson, S., Jackson, C., Goggin, P., Hirst, R. A., Rutman, A., Thompson, J., Jenkins, L., Aurora, P., Moya, E., Chetcuti, P., O'Callaghan, C., Morris-Rosendahl, D. J., ... Mitchison, H. M. (2020). Clinical utility of NGS diagnosis and disease stratification in a multiethnic primary ciliary dyskinesia cohort. *Journal of Medical Genetics*, *57*(5), 322–330. https://doi.org/10.1136/jmedgenet-2019-106501

Fassad, M. R., Shoemark, A., le Borgne, P., Koll, F., Patel, M., Dixon, M., Hayward, J., Richardson, C., Frost, E., Jenkins, L., Cullup, T., Chung, E. M. K., Lemullois, M., Aubusson-Fleury, A., Hogg, C., Mitchell, D. R., Tassin, A. M., & Mitchison, H. M. (2018).

C11orf70 mutations disrupting the Intraflagellar transport-dependent assembly of multiple axonemal Dyneins cause primary ciliary dyskinesia. _American Journal of Human Genetics_, _102_(5), 956–972. https://doi.org/10.1016/j.ajhg.2018.03.024

Feller-Kopman, D. J., Reddy, C. B., DeCamp, M. M., Diekemper, R. L., Gould, M. K., Henry, T., Iyer, N. P., Gary Lee, Y. C., Lewis, S. Z., Maskell, N. A., Rahman, N. M., Sterman, D. H., Wahidi, M. W., & Balekian, A. A. (2018). Management of Malignant Pleural Effusions. An official ATS/STS/STR clinical practice guideline. _American Journal of Respiratory and Critical Care Medicine_, _198_(7), 839–849. https://doi.org/10.1164/rccm.201807-1415ST

Guo, T., Tu, C. F., Yang, D. H., Ding, S. Z., Lei, C., Wang, R. C., Liu, L., Kang, X., Shen, X. Q., Yang, Y. F., Tan, Z. P., Tan, Y. Q., & Luo, H. (2021). Bi-allelic BRWD1 variants cause male infertility with asthenoteratozoospermia and likely primary ciliary dyskinesia. _Human Genetics_, _140_, 761–773. https://doi.org/10.1007/s00439-020-02241-4

Guo, Z., Chen, W., Wang, L., & Qian, L. (2020). Clinical and genetic Spectrum of children with primary ciliary dyskinesia in China. _The Journal of Pediatrics_, _225_, 157–165.e5. https://doi.org/10.1016/j.jpeds.2020.05.052

Höben, I. M., Hjeij, R., Olbrich, H., Dougherty, G. W., Nöthe-Menchen, T., Aprea, I., Frank, D., Pennekamp, P., Dworniczak, B., Wallmeier, J., Raidt, J., Nielsen, K. G., Philipsen, M. C., Santamaria, F., Venditto, L., Amirav, I., Mussaffi, H., Prenzel, F., Wu, K., ... Omran, H. (2018). Mutations in C11orf70 cause primary ciliary dyskinesia with randomization of left/right body asymmetry due to defects of outer and inner dynein arms. _American Journal of Human Genetics_, _102_(5), 973–984. https://doi.org/10.1016/j.ajhg.2018.03.025

Knowles, M. R., Ostrowski, L. E., Leigh, M. W., Sears, P. R., Davis, S. D., Wolf, W. E., Hazucha, M. J., Carson, J. L., Olivier, K. N., Sagel, S. D., Rosenfeld, M., Ferkol, T. W., Dell, S. D., Milla, C. E., Randell, S. H., Yin, W., Sannuti, A., Metjian, H. M., Noone, P. G., ... Zariwala, M. A. (2014). Mutations in RSPH1 cause primary ciliary dyskinesia with a unique clinical and ciliary phenotype. _American Journal of Respiratory and Critical Care Medicine_, _189_(6), 707–717. https://doi.org/10.1164/rccm.201311-2047OC

Liu, C., Tu, C., Wang, L., Wu, H., Houston, B. J., Mastrorosa, F. K., Zhang, W., Shen, Y., Wang, J., Tian, S., Meng, L., Cong, J., Yang, S., Jiang, Y., Tang, S., Zeng, Y., Lv, M., Lin, G., Li, J., ... Zhang, F. (2021). Deleterious variants in X-linked CFAP47 induce asthenoteratozoospermia and primary male infertility. _American Journal of Human Genetics_, _108_(2), 309–323. https://doi.org/10.1016/j.ajhg.2021.01.002

McKenzie, C. W., & Lee, L. (2020). Genetic interaction between central pair apparatus genes CFAP221, CFAP54, and SPEF2 in mouse models of primary ciliary dyskinesia. _Scientific Reports_, _10_(1), 12337. https://doi.org/10.1038/s41598-020-69359-3

Niziolek, M., Bicka, M., Osinka, A., Samsel, Z., Sekretarska, J., Poprzeczko, M., Bazan, R., Fabczak, H., Joachimiak, E., & Wloga, D. (2022). PCD genes-from patients to model organisms and Back to humans. _International Journal of Molecular Sciences_, _23_(3). https://doi.org/10.3390/ijms23031749

Olcese, C., Patel, M. P., Shoemark, A., Kiviluoto, S., Legendre, M., Williams, H. J., Vaughan, C. K., Hayward, J., Goldenberg, A.,

Emes, R. D., Munye, M. M., Dyer, L., Cahill, T., Bevillard, J., Gehrig, C., Guipponi, M., Chantot, S., Duquesnoy, P., Thomas, L., ... Mitchison, H. M. (2017). X-linked primary ciliary dyskinesia due to mutations in the cytoplasmic axonemal dynein assembly factor PIH1D3. _Nature Communications_, _8_, 14279. https://doi.org/10.1038/ncomms14279

Shoemark, A., Dell, S., Shapiro, A., & Lucas, J. S. (2019). ERS and ATS diagnostic guidelines for primary ciliary dyskinesia: Similarities and differences in approach to diagnosis. _The European Respiratory Journal_, _54_(3), 1901066. https://doi.org/10.1183/13993003.01066-2019

Shoemark, A., Moya, E., Hirst, R. A., Patel, M. P., Robson, E. A., Hayward, J., Scully, J., Fassad, M. R., Lamb, W., Schmidts, M., Dixon, M., Patel-King, R. S., Rogers, A. V., Rutman, A., Jackson, C. L., Goggin, P., Rubbo, B., Ollosson, S., Carr, S., ... Mitchison, H. M. (2018). High prevalence of CCDC103 p.His154Pro mutation causing primary ciliary dyskinesia disrupts protein oligomerisation and is associated with normal diagnostic investigations. _Thorax_, _73_(2), 157–166. https://doi.org/10.1136/thoraxjnl-2017-209999

Wang, J., Zhang, C., Tang, H., Zheng, A., Li, H., Yang, S., & Xiang, J. (2021). Successful results of intracytoplasmic sperm injection of a Chinese patient with multiple morphological abnormalities of sperm flagella caused by a novel splicing mutation in CFAP251. _Frontiers in Genetics_, _12_, 783790. https://doi.org/10.3389/fgene.2021.783790

Wang, W., Tu, C., Nie, H., Meng, L., Li, Y., Yuan, S., Zhang, Q., du, J., Wang, J., Gong, F., Fan, L., Lu, G. X., Lin, G., & Tan, Y. Q. (2019). Biallelic mutations in CFAP65 lead to severe asthenoteratospermia due to acrosome hypoplasia and flagellum malformations. _Journal of Medical Genetics_, _56_(11), 750–757. https://doi.org/10.1136/jmedgenet-2019-106031

Wu, B., Yu, X., Liu, C., Wang, L., Huang, T., Lu, G., Chen, Z. J., Li, W., & Liu, H. (2021). Essential role of CFAP53 in sperm flagellum biogenesis. _Frontiers in Cell and Development Biology_, _9_, 676910. https://doi.org/10.3389/fcell.2021.676910

SUPPORTING INFORMATION

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