Pathogenic variants identified using whole-exome sequencing in Chinese patients with primary ciliary dyskinesia

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Abstract
The genetic factors contributing to primary ciliary dyskinesia (PCD), a rare autosomal recessive disorder, remain elusive for ~20%–35% of patients with complex and abnormal clinical phenotypes. Our study aimed to identify causative variants of PCD-associated pathogenic candidate genes using whole-exome sequencing (WES). All patients were diagnosed with PCD based on clinical phenotype or transmission electron microscopy images of cilia. WES and bioinformatic analysis were then conducted on patients with PCD. Identified candidate variants were validated by Sanger sequencing. Pathogenicity of candidate variants was then evaluated using in silico software and the American College of Medical Genetics and Genomics (ACMG) database. In total, 13 rare variants were identified in patients with PCD, among which were three homozygous causative variants (including one splicing variant) in the PCD-associated genes CCDC40 and DNAI1. Moreover, two stop-gain heterozygous variants of DNAAF3 and DNAH1 were classified as pathogenic variants based on the ACMG criteria. This study identified novel potential pathogenic genetic factors associated with PCD. Noteworthy, the patients with PCD carried multiple rare causative gene variants, thereby suggesting that known causative genes along with other functional genes should be considered for such heterogeneous genetic disorders.

KEYWORDS
DNAAF3, DNAI1, pathogenic, primary ciliary dyskinesia, whole-exome sequencing

1 | INTRODUCTION

Primary ciliary dyskinesia (PCD) is an autosomal recessive disorder characterized by respiratory distress, tympanitis, sinusitis, and bronchiectasis (Horani & Ferkol, 2018). Kartagener syndrome (MIM# 244400) is a subtype of PCD exhibiting the situs inversus phenotype. The estimated prevalence of PCD ranges from 1 in 10,000 to 1 in 15,000 among Europeans (Bush et al., 2007). PCD is diagnosed by measuring nasal nitric oxide level and through brush biopsy (Lucas et al., 2016).

Moreover, transmission electron microscopy (TEM) can be used to visualize the nasal mucosa cilia ultrastructure and achieve a definitive diagnosis. Given the main feature of this disorder (ciliary dyskinesia), male patients with PCD commonly present with infertility due to abnormally immotile or dyskinetic sperm flagella (Liu et al., 2020).

Genetic screenings have improved our understanding of the pathogenesis of inherited PCD (Khalid et al., 2019). Previous genetic and functional studies revealed a series of PCD-associated genes causing congenital ultrastructural abnormalities that resulted in dysfunctional immotile cilia (Lucas et al., 2020). Inherited genetic variations are present in ~60% of PCD cases, indicating a high genetic heterogeneity of...
this disorder (Lucas et al., 2016). Pathogenic variants in inner dynein arm (IDA) and outer dynein arm (ODA) complex-coding genes cause ciliary abnormalities, but some patients with confirmed PCD clinical features also show normal ciliary motility and ultrastructure (Wallmeier et al., 2014). Interestingly, some studies have reported that PCD with heterotaxy and airway ciliary dysfunction only involves heterozygous variants in PCD-associated genes (Wilson et al., 2009). Additionally, an autosomal dominant mutation of FOXI1 has been reported to cause a distinct motile ciliopathy related to defective ciliogenesis, with a clinical phenotype similar to that of PCD (Wallmeier et al., 2019). Taken together, these findings suggest the potential involvement of unknown genes and mechanisms in pathologies associated with ciliary function.

Next generation sequencing has advanced the identification of candidate causative genes in PCD (Fassad et al., 2020). In this study, we aimed to identify pathogenic gene variants of sporadic PCD in Chinese patients, thereby allowing to identify genetic risk factors associated with PCD that could be relevant for the diagnosis and counseling of patients with this disorder.

2 | MATERIALS AND METHODS

2.1 | Subjects and PCD diagnosis

Five patients with PCD, together with control individuals, were enrolled in the study from 2008 to 2020. High-resolution computed tomography (CT), magnetic resonance (MR) imaging, and TEM examinations were conducted to investigate the clinical phenotype of PCD, including chronic sinusitis, bronchiectasis, situs inversus, and ciliary ultrastructural defects. Patients were diagnosed with PCD according to the classic characterization and guidelines (Shapiro et al., 2018). The control subjects included in the study were the unrelated healthy individuals, and healthy parents of the patients. Ethical approval was obtained from the ethics committee of the Shenzhen People’s Hospital, as per the ethical standards recommended in the 1964 Declaration of Helsinki. Informed consent was obtained from the subjects or parents in the study.

2.2 | Identification of variants by whole exome sequencing (WES) and bioinformatic analysis

An enriched library targeting the exome of all subjects was sequenced using the NovaSeq platform (Illumina, San Diego, CA, USA) by Fulgent Technology Inc (Fuzhou, China). Exome capture was performed using the Agilent SureSelect V6 Capture Kit (Agilent, CA, USA), according to the manufacturer’s protocols. The sequencing coverage of captured libraries was more than 100x. Variants revealed using WES data were analyzed using the Genome Analysis Toolkit (hg19) and Annovar (Wang et al., 2010). Subsequently, all variants were filtered based on minor allele frequency (MAF < 0.1%), using the following databases: 1000 Genomes and gnomAD (1000 Genomes Project Consortium et al., 2015; Karczewski et al., 2020). Sorting Intolerant From Tolerant, MutationTaster, Polymorphism Phenotyping v2 (PolyPhen-2), and Combined Annotation Dependent Depletion tools were used to assess the pathogenicity of the identified variants. Furthermore, an in-house filtering pipeline was used to identify candidate variants.

2.3 | Pathogenicity assessment and validation of candidate variants in PCD-associated genes

Gene functional and frequency-based filtering analysis were conducted for all variants. In total, 46 pathogenic genes highly associated with PCD were selected as candidate gene variants (Table S1), as described previously (Yue et al., 2019). Gene function- and frequency-based advanced filtering analyzes were conducted for candidate genes variants. Variant pathogenicity assessment was conducted using American College of Medical Genetics and Genomics (ACMG) database (Richards et al., 2015) and various bioinformatics programs, including Sift, Polyphen-2, and MutationTaster. According to the prediction tools, the prediction results were classified as tolerated (T), benign (B), possibly damaging (P), polymorphism (N), and deleterious (D). The classification and interpretation of the variants were performed following the ACMG guidelines. Identified candidate causative variants were validated in patients with PCD and control subjects using the Sanger sequencing platform. Chromas software was used to analyze the sequencing results (Technelysium Pty Ltd., South Brisbane, Australia).

2.4 | Polymerase chain reaction (PCR) and sequencing confirmation of the CCDC40 splicing variant

Total RNA was extracted from whole blood of the patients with PCD and reverse-transcribed using the RR036A kit (TAKARA Bio, Kusatsu, Japan). Primers for CCDC40 exons were designed using the IDT PrimerQuest tool (Owczarz et al., 2008). cDNA segments transcribed from CCDC40 exons were amplified using standard PCR. The primer sets for exon 13–14 (primer 4) and exon 14–15 (primer 6) transcripts were as follows: F-5’ GGACCAGGCAGCTGAAATTG 3’; and R-5’ CTGTCGCTTGAGGACCAGTC 3’, respectively. Transcript products caused by the splicing variant in KT8 were confirmed by Sanger sequencing and agarose gel electrophoresis.

3 | RESULTS

3.1 | Demographics and clinical phenotype of patients with PCD

The patients were diagnosed with PCD using typical methods, as described in the Materials and Methods section, and all patients had clinical history of recurrent cough and expectoration for several years (Table 1). TEM examination showed the presence of ODA defects and microtubular disorganization of the cilia for patients of KT8 (Figure 1a).
and KT7 (Figures 1b and S1). Chest CT or MR imaging revealed bronchiectasis in all patients. Moreover, situs inversus was identified in four patients (Figure 1c,d), leading to the diagnosis of Kartagener syndrome (MIM# 244400), and a subtype of PCD. None of the PCD patients’ parents or siblings presented clinical PCD phenotypes.

### 3.2 Identification of rare variants by bioinformatic analysis

To identify candidate PCD-causative gene variants in the five Chinese patients with PCD, the identified variants were filtered to obtain rare genetic alterations based on function and frequency (Table S2). In total, 13 variants were identified in candidate genes with MAF < 1% in the East Asian population, according to the 1000 Genomes, ExAC, and gnomAD databases (Table 2). Three novel variants were identified in three genes that were not recorded in the gnomAD and dbSNP databases. One homozygous DNAI1 variant and one frameshift insertion homozygous CCDC40 variant identified in KT9 and KT10, respectively, were considered to be potential PCD-contributing factors (Table 2). No rare heterozygous genetic alterations were found besides the identified two potential pathogenic heterozygous stop-gain variants in KT7 and KT8, indicating that the genetic defects of these patients were not caused by compound heterozygosity. No rare pathogenic variants were identified in KT6, except for a likely benign rare variant in DNAH1.

| PCD ID | Age | Gender | Recurrent cough | Situs inversus | Bronchiectasis | Bronchitis |
|--------|-----|--------|-----------------|----------------|----------------|------------|
| KT6    | 30  | Female | Yes             | Yes            | Yes            | Yes        |
| KT7    | 32  | Female | Yes             | Yes            | Yes            | Yes        |
| KT8    | 48  | Male   | Yes             | Yes            | Yes            | Yes        |
| KT9    | 48  | Female | Yes             | Yes            | Yes            | No         |
| KT10   | 12  | Male   | Yes             | No             | Yes            | Yes        |

### TABLE 1 Clinical characteristics of PCD patients

**FIGURE 1** PCD diagnosis using traditional methods. TEM examination showed ODA structural defects in the cilia of patients KT8 (a) and microtubular disorganization of KT7 (b, arrows). The arrows represent the ODAs defects and microtubular disorganization. Chest images showed situs inversus (liver) in patient KT9 (c) and bronchiectasis in patient KT10 (d).
| Gene   | Variants and protein alteration (hg19) | KS carriers | Variant type | gnomAD_EAS | dbSNP150 | ACMG classification | Pathogenicity | Zygosity | Pathogenic |
|--------|---------------------------------------|-------------|--------------|------------|----------|---------------------|---------------|----------|------------|
| DNAH11 | NM_001277115:c.8023 A > G:p.I2675V    | KT6         | Missense     | 0.0023     | rs72657364 | PM1 + PP5 + BS1 + BP4 | Likely Benign  | T/B/N    |            |
| DNAH1  | NM_015512:c.4179 C > A:p.N1393K       | KT7         | Nonsense     | 5.6E-05    | NA       | PVS1 + PM2 + PP3    | Pathogenic    | T/NA/A   |            |
| DNAH1  | NM_015512:c.3537 C > G:p.Y1179X       | KT8         | Nonsense     | NA         | NA       | VUS                 | Heterozygous  | T/NA/A   |            |
| DNAH6  | NM_001370:c.4615 C > G:p.Q1539E       | KT8         | Missense     | 0.0036     | rs571512486 | PM1                | VUS           | D/P/N    |            |
| DNAH6  | NM_001083908:c.1349 G > T:p.S450I     | KT8         | Missense     | 0.0017     | rs18938951 | BP4                | VUS           | T/B/N    |            |
| CCDC40 | NM_001243342:c.2236-2delA: p.?        | KT8         | Splicing     | 5.5E-05    | NA       | NA                  | Likely Pathogenic | D/D/D    |            |
| DNAH9  | NM_001372:c.2525 G > A:p.R435P        | KT9         | Missense     | 5.4E-05    | rs556781332 | NA                | VUS           | T/B/D    |            |
| ZMYND10| NM_001308379:c.79 A > G:p.M27V        | KT9         | Missense     | 0.0019     | rs587680539 | PM2               | VUS           | T/B/N    |            |
| CCDC40 | NM_001243342:c.994dupT:p.Y332fs       | KT10        | Frameshift   | NA         | NA       | NA                  | Pathogenic    | Homozygous | NA         |
| DNAH11 | NM_001277115:c.5651 A > G: p.H1884R  | KT10        | Missense     | 0.0002     | rs748483534 | PM1 + PM2          | VUS           | T/B/D    |            |
| DNAH1  | NM_015512:c.11650 C > T:p.R3884C      | KT10        | Missense     | 5.5E-05    | rs369367984 | PM1               | VUS           | T/B/D    |            |

Abbreviations: EAS, East Asian population (gnomad211_exome); NA, not available; VUS, variant of uncertain significance.

*Abbreviation was adapted from the ACMG Standards and Guidelines.*
3.3 ACMG interpretation and pathogenicity assessment of candidate variants

Candidate variants were assessed using in silico tools and the ACMG guidelines (Table 2). All potential causative novel variants were validated by Sanger sequencing (Figure 2a and S1C). The frameshift insertion in CCDC40 (c.994dupT) was identified as a pathogenic homozygous variant causative of PCD in KT10. A homozygous DNAI1 variant was found in KT9, without additional rare pathogenic genetic variants. The change in protein structure predicted using the SDM (http://marid.bioc.cam.ac.uk/sdm2/) software indicated that the mutation could reduce the protein structure stability (data not shown). Thus, the DNAI1 variant could be the potential cause of PCD, even though it was considered as a variant of uncertain significance (VUS) according to the ACMG criteria. Further evidence is needed to confirm the association between this homozygous variation in DNAI1 and the observed PCD phenotype. Two stop-gain heterozygous variants of DNAF3 and DNAH1 were identified in KT7 and KT8. TEM examination confirmed the presence of cilia ODA and IDA defects in these patients, which was consistent with the identified variants (Figure 1).

The ACMG criteria classified these DNAF3 and DNAH1 stop-gain heterozygous variants as pathogenic, with reliable evidence of pathogenicity (PV1). No other rare variants of these two genes were identified in the KT7 and KT8, indicating that these two PCD cases were not only caused by heterozygous but also may by transheterozygous gene interactions.

**FIGURE 2** (a) Validation of causative candidate variants in healthy control and parents by sanger sequencing. The variant positions are indicated with arrows. (b) Splicing deletion (c.2236-2delA) identified in the genome of KT8 (arrows). (c) Agarose gel electrophoresis confirmed the CCDC40 splicing variant caused the absence of exon 13–14 transcript product (primer 4) in KT8. Primer 6 represents the exon 14–15 transcript product amplification. (d) Without the splicing variant, cDNA sequencing of a healthy control (con) showed a normal sequence between exon 13 and exon 14 of the transcript.
3.4 Confirmation of pathogenicity of the CCDC40 splicing variant

The identified homozygous deletion c.2236-2delA in CCDC40 (NM_017950) of KT8 was found to alter the nucleotide sequence at the splicing site near exon 14. Genomic DNA sequencing revealed the splicing deletion (del A allele) in the genome of KT8 (Figure 2b). To confirm whether this variation could cause abnormal mRNA transcription, cDNA PCR and sequencing were conducted using samples from KT8 and a healthy control. Agarose gel electrophoresis results of the PCR products showed that a portion of the cDNA between exons 13 and 14 (primer 4) was lost in KT8 compared with the normal PCR products of the healthy control (Figure 2c). cDNA-based PCR product (primer 4) sequencing showed the normal sequence of CCDC40 cDNA in healthy control (Figure 2d). These results indicated that the splicing deletion variant of CCDC40 (c.2236-2delA) resulted in the production of a shorter mRNA transcript in the variant carrier KT8. Together with the clinical phenotype resulting from the CCDC40 defect, these findings suggest that the CCDC40 splicing variant c.2236-2delA is likely to be pathogenic.

Two rare variants were predicted as likely benign, and the others were classified as VUS (Table 2). These variants were not considered to be associated with PCD based on the ACMG guidelines. In summary, the five rare variants in known PCD-causative genes were considered to potentially cause PCD based on the ACMG guidelines and clinical phenotype of the cohorts (Table 2).

4 DISCUSSION

In the present study, WES was conducted to identify candidate PCD-causative pathogenic gene variants in five patients. Five causative variations in CCDC40, DNAH1, DNAF3, and DNAI1 were considered pathogenic. Three homozygous variants, including a splicing variant, were identified in CCDC40 and DNAI1. Further analyzes showed that the splicing variant of CCDC40 could result in the production of a shortened protein, which may contribute to PCD pathogenesis. Two stop-gain heterozygous variants of DNAF3 and DNAH1 were classified as pathogenic variants according to the ACMG criteria. It is possible that some unidentified pathogenic factors, in regards with the two heterozygous variants, may contribute to the pathogenesis of the PCD carriers. The two heterozygous nonsense mutations were likely to be pathogenic since they are presenting PCD-causative genes, prediction showed pathogenicity, and appeared with abnormal cilia structure. Overall, in this study, we identified five potential genetic factors involved in PCD pathogenesis.

To date, strong functional evidence has demonstrated the contribution of over 50 genes to PCD pathogenesis, most of which mainly cause structural abnormalities of motile cilia, such as complete or partial deletion of IDAs or ODAs (Lucas et al., 2020). Defects in CCDC40 are responsible for IDA defects and microtubular disorganization (Becker-Heck et al., 2011). Herein, two homozygous variants of CCDC40 were identified and the carrier showed the classic PCD phenotype involving situs inversus and bronchiectasis. TEM examination further revealed that the abnormal cilia structure in agreement with the previously described effects of CCDC40 defects. Moreover, a splicing deletion in CCDC40 was also found to potentially produce a shorter mRNA transcript. Based on this evidence, the splicing deletion variant of CCDC40 may likely be a PCD-causative variant. Nevertheless, the exact function and mechanisms of the CCDC40 splicing variant in PCD pathogenesis warrants further investigations.

One novel PCD-causative homozygous variant was identified in DNAI1. ACMG predicted this variant to be a VUS, but the variant was predicted to be pathogenic by all in silico tools. DNAI1 is an axonemal dynein intermediate gene that encodes the intermediate chain of ODAs (Zariwala et al., 2006). Two large cohort studies showed that around 4%–10% of PCD was caused by a DNAI1 genetic defect (Zariwala et al., 2006; Zietkiewicz et al., 2010). Another study showed that compound heterozygous defects in DNAI1 contributed to PCD without situs inversus. (Guichard et al., 2001). Our reported case KT9 carried a homozygous variant and presented situs inversus. The carrier of the DNAI1 variant exhibited the classic PCD phenotype and had no other rare gene variants; hence, this DNAI1 variant was recognized as the main potential genetic factor contributing to PCD in this patient. However, appropriate functional studies would be helpful to further confirm the pathogenicity of this DNAI1 variant.

A rare stop-gain heterozygous variant of DNAF3 was also identified in a patient who did not harbor any other pathogenic variants. DNAF3 pathogenic variants were reported to cause ODA and IDA defects in cilia. Functional analysis in zebrafish showed that DNAF3 plays a role in the dynein assembly process (Mitchison et al., 2012). Further studies have provided strong evidence that mutations in DNAF3 can cause situs inversus and dynein arms defects (Guan et al., 2020; Guo et al., 2019). One rare VUS variants of DNAH1 was also identified, but whether the observed transheterozygous interactions between the variant could play a role in PCD remains to be confirmed. Mutations in DNAH1 lead to dysfunction of the cilia, causing male infertility (Ben Khelifa et al., 2014). DNAH1 genetic defects in families and sporadic subjects presented PCD clinical phenotypes (Imtiaz et al., 2015; Yue et al., 2019). In addition, one rare VUS variants of DNAH11 were also identified. Based on previous study, DNAH11 variants were significantly associated with heterotaxy (Liu et al., 2019). Identification of ciliary structural defects is helpful to confirm the association between a gene pathogenic variant and PCD phenotype. Unfortunately, some patients in our study refused to undergo TEM examination.

As PCD is an autosomal recessive disorder, homozygous or compound heterozygous variants are commonly considered contributing genetic factors. However, the present and previous studies demonstrated that heterozygous pathogenic variants are frequent in patients with PCD (Yue et al., 2019), especially frameshift insertions or deletions and stop-gain or -loss variants. Nevertheless, none of the other candidate pathogenic variants was co-present. Interestingly, the inheritance model of the candidate variants was very similar to that for autosomal dominant disorders. Some studies have shown that
autosomal dominant variants can also cause defects in ciliogenesis with a clinical phenotype similar to that of PCD (Wallmeier et al., 2019). However, further investigation is required to determine whether these heterozygous variants can cause PCD on their own. The main limitation of our study is that the co-segregation analysis of the PCD relatives was not conducted. In silico prediction, ACMG criteria, and variants carrier clinical phenotype have provided reliable evidence that these variants are involved in the pathogenesis of PCD. Additional evidence is required to confirm the pathogenicity of various types of genetic variants in PCD, given the heterogeneous nature of this genetic disorder.

5 | CONCLUSION

In conclusion, five candidate PCD-causative gene variants in CCDC40, DNAH1, DNAAF3, and DNA1 were identified. Moreover, the collected data demonstrates that heterozygous pathogenic variants alone are frequent in patients with PCD and that rare variants can occur in several causative genes in the same PCD patient. Thus, this genetic disorder may be caused by heterozygous and transheterozygous variants. Confirmation of the causative genetic factors and clinical ciliary defect type in PCD are important steps toward developing personalized clinical diagnosis and genetic counseling strategies.

AUTHOR CONTRIBUTIONS

Yongjian Yue, Rongchang Chen, and Yingyun Fu prepared the project proposal and study design. Yongjian Yue and Lipeng Chen conducted bioinformatics and statistical analysis of sequencing data. Qijun Huang and Fang Yuan conducted sample collection and Sanger sequencing validation. Shengguo Liu, Xiangxia Zhang, and Yutian Ye conducted clinical diagnosis of PCD. All the authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Genome Sequence Archive at https://bigd.big.ac.cn/gsa-human/browse/HRA001494, reference number HRA001494.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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