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Research Article

Keywords: primary ciliary dyskinesia, DNAAF3, DNAI1, whole-exome sequencing

Posted Date: February 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-156279/v1>

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

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Abstract

Background: Primary ciliary dyskinesia (PCD) is an autosomal recessive disorder. The genetic factors contributing to PCD pathogenesis remain elusive for approximately 20–35% of patients with complex and abnormal clinical phenotypes. Our study aimed to identify causative variants of sporadic PCD genes using whole-exome sequencing (WES).

Result: All patients were diagnosed with PCD based on clinical phenotype or transmission electron microscopy (TEM) images of cilia. WES and bioinformatic analysis were then conducted for 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, 15 rare variants were identified in five patients with PCD. Five new variants of *CCDC40*, *DNAH1*, *DNAAF3*, and *DNAI1* were considered causative variants and included one splicing and three homozygous variants.

Conclusion: Our study demonstrated that patients with PCD carry rare causative variants of multiple genes. Our findings indicated that not only known causative genes but also other functional genes should be considered for heterogeneous genetic disorders.

Keywords: primary ciliary dyskinesia, *DNAAF3*, *DNAI1*, whole-exome sequencing

Background

Primary ciliary dyskinesia (PCD) is an autosomal recessive disorder characterized by respiratory distress, tympanitis, sinusitis, and bronchiectasis [1]. 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 [2]. PCD is diagnosed by measuring nasal nitric oxide and through brush biopsy [3]. Transmission electron microscopy (TEM) is used to obtain a definitive diagnosis by visualizing the ultrastructure of cilia. Male PCD patients present with infertility due to dysfunctional immotile or dyskinetic sperm flagella [4].

Genetic screening has improved our understanding of the pathogenesis of inherited PCD [5]. Previous genetic and functional studies revealed a series of PCD-associated genes causing congenital ultrastructural abnormalities resulting in dysfunctional immotile cilia [6]. Inherited genetic variations are present in approximately 60% of PCD cases, indicating a high genetic heterogeneity of this disorder [3]. Mutations 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 [7]. Interestingly, some studies have reported that PCD with heterotaxy and airway ciliary dysfunction only involves

heterozygous mutations in PCD-associated genes [8]. Another study showed that an autosomal dominant mutation in *FOXJ1* can also cause a distinct motile ciliopathy related to defective ciliogenesis, presenting a similar clinical phenotype as that of PCD [9]. 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 [10]. Here, we aimed to identify pathogenic gene variants for sporadic PCD in Chinese patients. Genetic testing combined with TEM and clinical feature analysis have greatly improved the definitive diagnosis of and identification of genetic defects associated with PCD. These tools will allow us to identify risk factors for siblings of individuals with PCD, facilitating the genetic diagnosis of and counseling for this disorder.

Methods

Subjects and PCD diagnosis

Five PCD patients, together with control individuals, were enrolled in the study from 2008 to 2020. Patients were diagnosed with PCD according to the classic characterization and guidelines [11]. The control subjects included in the study were the healthy siblings or parents of patients and unrelated healthy individuals. Ethical

approval was obtained from the ethics committee of the Shenzhen People's Hospital (Guangdong, China), as per the ethical standards recommended in the 1964 Helsinki Declaration of Helsinki. Informed consent was obtained from each subjects enrolled in the study. High-resolution computed tomography (CT), magnetic resonance (MR) imaging, and TEM examination were conducted to investigate chronic sinusitis, bronchiectasis, situs inversus, and ciliary ultrastructural defects.

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

Genomes from all subjects were sequenced using the NovaSeq platform (Illumina). Variants revealed using WES data were analyzed using the Genome Analysis Toolkit and Annovar [12]. Subsequently, all variants were filtered based on minor allele frequency (MAF<0.1%), using the following databases: 1000 genomes, CG69, and gnomAD (<http://gnomad.broadinstitute.org/>). Sorting Intolerant From Tolerant, MutationTaster, Polymorphism Phenotyping v2 (PolyPhen-2), and Combined Annotation Dependent Depletion were used to assess the pathogenicity of variants. Further, an in-house filtering pipeline was used to identify candidate variants.

Pathogenicity assessment and validation of candidate variants

Gene function- and frequency-based filtering analyses were conducted for all variants.

In total, 46 pathogenic genes highly associated with PCD were selected as candidate gene variants, as described previously [13]. Variant pathogenicity assessment was conducted using bioinformatic software and ACMG [14]. The classification and interpretation of variants followed the ACMG guidelines. Identified candidate causative variants were validated in patients with PCD and control subjects using the Sanger sequencing platform. The Chromas software was used to analyze the sequencing results (Technelysium Pty Ltd.).

PCR and sequencing confirmation of the *CCDC40* splicing variant

Total RNA was extracted from whole blood of the PCD patients and reverse-transcribed using the RR036A kit (TAKARA, Japan). Primers for *CCDC40* exons were designed using the IDT PrimerQuest tool (<https://sg.idtdna.com/Primerquest/Home/Index>). cDNA segments transcribed from exon *CCDC40* mRNA were amplified using standard PCR. The primers for exon 13 to exon 14 transcripts were as follows: primer 4 F- 5' GGACCAGGACGTGAAGAAAG 3', and R- 5' CTGTGTCACCTTGACCATCTC; and for exon 14 to exon 15 transcripts: primer 6 F- 5' GATCGACGAGCACGATGG 3', and R- GAGCTTCTTCAGGTCGTTGT 3'. Transcript products of the splicing variant in patient KT8 were confirmed by Sanger sequencing and agarose gel electrophoresis.

Results

Demographics and clinical phenotypes of PCD patients

PCD patients were diagnosed using typical methods, as described in the Materials and Methods section. All patients with PCD had the clinical characteristics of recurrent cough and expectoration for several years (Table 1). Chest CT or MR imaging revealed bronchiectasis in all patients, and situs inversus in four patients (Fig. 1). The four subjects with situs inversus had kartagener syndrome. TEM examination showed the presence of ODA and IDA defects as well as microtubular disorganization of the cilia in KT7 and KT8 (Fig. 1).

Table 1. Clinical characteristics of PCD patients

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

Fig 1. PCD diagnosis using traditional methods. Chest images showed bronchiectasis and situs inversus in KT9 (A) and bronchiectasis in KT10 (B). TEM examination showed structural defects in KT7 (C) and KT8 (D) cilia, the arrows represent the ODA, IDA defects and microtubular disorganization.

Identification of rare variants by bioinformatic analysis

To identify candidate PCD-causative gene variants in the five Chinese PCD patients, we filtered variants to obtain rare ones based on function and frequency. In total, we

identified 15 variants of candidate genes with MAF<1% in the East Asian population from the 1000 genomes, ExAC, and gnomAD databases (Table 2). Six novel variants were identified for five genes that were not recorded in the gnomAD and dbSNP databases. We confirmed one homozygous variant of DNAI1 and two homozygous variants of CCDC40 in three patients with PCD to be likely genetic factors contributing to the disorder (Table 2). No other rare heterozygotes were found together with the identified potential pathogenic heterozygous variant in KT6 and KT7, indicating that the genetic defects of these patients were not caused by compound heterozygotes.

Table 2. The interpretation and pathogenicity assessment of candidate variants

C hr	Start	refGene	Alteration	PCD Carrier	Variant type	gnomAD EA S	ACMG Classification
chr7	2178 1653	DNA H11	NM_001277115:exon49 :c.A8023G:p.I2675V	KT6	Mis- sense	0.000 6	PM1+PP5+ BS1+BP4
chr19	5567 2134	DNA AF3	NM_001256714:exon9: c.G1126T:p.E376X	KT7	Stop- gain	NA	PVS1+PM 2+PP3
chr17	4008 6999	TTC 25	UNKNOWN	KT7	Mis- sense	NA	PM1+BP4
chr3	5239 2666	DNA H1	NM_015512:exon25:c. C4179A:p.N1393K	KT7	Mis- sense	NA	PM1+BP4
chr5	1391 1508	DNA H5	NM_001369:exon12:c. C1631T:p.T544I	KT8	Mis- sense	0.009 3	PM1+PP5+ BS1+BP4
chr3	5238 8915	DNA H1	NM_015512:exon21:c. C3537G:p.Y1179X	KT8	Stop- gain	NA	PVS1+PM 2+PP3
chr2	8486 1727	DNA H6	NM_001370:exon30:c. C4615G:p.Q1539E	KT8	Mis- sense	0.001 9	PM1
chr14	5010 0519	DNA AF2	NM_001083908:exon1:c. G1349T:p.S450I	KT8	Missen- se	0.000 6	BP4

chr	7805	CCD	NM_001243342:exon14:c.	KT8	Homozygote Splicing	NA	
17	9800	C40	2236-2A>-				
chr	3450	DNAI	NM_001281428:exon13:c.	KT9	Homozygote Missense	NA	PM1+PM2+PP3
9	6853	1	A1304C:p.H435P				
chr	1155	DNA	NM_001372:exon14:c.G2	KT9	Missense	NA	NA
17	6249	H9	525A:p.R842Q				
chr	5038	ZMY	NM_001308379:exon1:c.	KT9	Missense	0.0012	PM2
3	2932	ND10	A79G:p.M27V				
chr	7802	CCD	NM_001243342:exon7:c.9	KT10	Homozygote frameshift insertion	NA	
17	3917	C40	94dupT:p.Y332fs				
chr	2172	DNA	NM_001277115:exon33:c.	KT10	Missense	NA	PM1+PM2
7	6746	H11	A5651G:p.H1884R				
chr	5243	DNA	NM_015512:exon73:c.C1	KT10	Missense	NA	PM1
3	0923	H1	1650T:p.R3884C				

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172 ACMG guidelines based pathogenicity assessment of candidate variants

173 Candidate variants were assessed using *in silico* software and ACMG guidelines
174 (Table 2). All potential causative variants were validated by Sanger sequencing (Fig.
175 2). The frameshift insertion in *CCDC40* (c.994dupT) is a pathogenic homozygous
176 variation that was identified as the genetic cause of PCD in KT10. Another
177 homozygous variation in *DNAI1* was present in KT9 in the absence of other rare
178 pathogenic gene variants, the *DNAI1* variant could be the potential cause of PCD,
179 even though it is considered as a VUS according to the ACMG criteria. Further
180 evidence is needed to confirm the association between this homozygous variation in
181 *DNAI1* and the PCD phenotype. Two stop-gain heterozygous variants of *DNAAF3* and

DNAH1 were identified in KT7 and KT8. We did not find other rare variants of these two genes, indicating that PCD in the two cases was not only caused by heterozygous but also by trans-heterozygous gene interactions.

Fig 2. Validation of causative candidate variants by Sanger sequencing.

Confirmation of the pathogenicity of the *CCDC40* splicing variant

The homozygous deletion c.2236-2A>- in *CCDC40* (NM_017950) found in KT8 alters the nucleotide sequence at the splicing site near exon 14. To confirm whether this variation causes abnormal mRNA transcription, cDNA sequencing was conducted in KT8 and normal control. Genomic DNA sequencing revealed the splicing deletion in KT8 (Fig. 3A). Further PCR and sequencing results showed that the portion of the cDNA between exon 13 and exon 14 was lost in the variant containing the *CCDC40* splicing deletion in KT8 (Fig. 3C), while the portion of cDNA was normally present

in the control. cDNA-based PCR sequencing showed normal *CCDC40* cDNA products in the normal control (Fig. 3B). This indicated that the splicing deletion variant of *CCDC40* (c.2236-2A>-) resulted in the production of a shorter mRNA transcript compared with normal control. This finding, together with the clinical phenotype resulting from the *CCDC40* defect suggest that splicing variant c.2236-2A>- is likely to be pathogenic.

Fig 3. (A) Splicing deletion in the KT8 genomic DNA sequence. (B) cDNA sequencing of the control showed a normal sequence between exon 13 and exon 14 of the transcript. (C) Agarose gel electrophoresis showed the *CCDC40* cDNA PCR products was lost in KT8.

No rare pathogenic variants were identified in KT6, except for a likely benign rare variant in DNAH1. Two rare variants were predicted as likely benign, and the others were classified as VUS (Table 2). These variants were considered to not be associated with PCD based on the ACMG guidelines. In summary, five rare variants were

considered to potentially be causative for PCD based on ACMG guidelines (Table 2).

Discussion

In the present study, WES was conducted to identify candidate PCD-causative gene variants in five patients. Five causative variations were considered pathogenic in *CCDC40*, *DNAH1*, *DNAAF3*, and *DNAI1*. Three homozygous variants, including splicing variants, were identified for *CCDC40* and *DNAI1*. Further analyses showed that the splicing variant of *CCDC40* might result in the production of a shortened protein, which may contribute to PCD pathogenesis.

To date, there has been strong functional evidence for the contribution of over 50 genes to PCD pathogenesis, with the genes mainly causing structural abnormalities of motile cilia, such as complete or partial deletion of IDAs or ODAs [6]. Defects in *CCDC40* are responsible for IDA defects and microtubular disorganization [15]. Our study identified two homozygous variants of *CCDC40*. The carrier of these variants showed the classic PCD phenotype involving situs inversus and bronchiectasis. TEM examination demonstrated that the abnormal structure of cilia in the carrier was consistent with genetic defects in *CCDC40*. Our subsequent analysis confirmed that a splicing deletion in *CCDC40* could shorten its mRNA transcript. Based on this evidence, the splicing deletion variant of *CCDC40* may likely be a PCD-causative variant; The exact functions and mechanisms of the splicing variant of *CCDC40* in PCD pathogenesis need to be confirmed through further studies.

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* softwares. *DNAI1* encodes the intermediate chain of ODAs [16]. Mutations in *DNAI1* result in situs inversus and primary ciliary dyskinesia [17]. The carrier of the *DNAI1* variant exhibited the classic PCD phenotype and had no other rare gene variant. We speculated that the *DNAI1* variant is the main genetic factor contributing to PCD in this case. Therefore, appropriate functional studies would be helpful to confirm the pathogenicity of the *DNAI1* variant. We found a novel stop-gain heterozygous variant of *DNAAF3* but no other pathogenic variants in one of the assessed PCD patients. The mutation in *DNAAF3* causes ODA and IDA defects in cilia [18]. Two rare VUS mutations of *DNAH1* and *TTC25* were also identified, but whether the observed trans-heterozygous interactions between these mutations could play a role in PCD remains to be confirmed. Identification of ciliary structural defects is helpful to confirm the association between a gene mutation 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 mutations are commonly considered contributing genetic factors. However, our present and previous studies demonstrated that heterozygous pathogenic mutations are frequent in patients with PCD [13], especially frameshift insertions or deletions and stop-gain or -loss mutations. None of the other candidate pathogenic variants were present together. Interestingly, the inheritance model of the candidate variants is very

similar to that for autosomal dominant disorders. Some studies have shown that autosomal dominant variants can also cause defects in ciliogenesis with a similar clinical phenotype as that of PCD [9]. However, further investigation is required to determine whether these heterozygous variants can cause PCD on their own.

Conclusion

In conclusion, our study identified five candidate PCD-causative gene variants of *CCDC40*, *DNAH1*, *DNAAF3*, and *DNAI1*. More evidence is required to confirm the pathogenicity of various types of genetic variants in PCD, since PCD is a heterogeneous genetic disorders. Confirmation of the causative genetic factors and clinical ciliary defect type in PCD is an important step toward developing personalized clinical diagnosis and genetic counseling.

List of abbreviations

PCD: Primary ciliary dyskinesia; WES: whole-exome sequencing; TEM: transmission electron microscopy; CT: computed tomography; MR: magnetic resonance; ACMG: the American College of Medical Genetics and Genomics; IDA: inner dynein arm; ODA: outer dynein arm; MAF: minor allele frequency; VUS: variants of uncertain significance.

275 **Ethics approval and consent to participate**

276 The project was approved by the ethics committee of the Ethics Committee of
277 Shenzhen People's Hospital. All procedures performed in studies involving human
278 participants in accordance with the 1964 Declaration of Helsinki ethical standards.

279 **Consent for publication**

280 Written Informed consent for publication was obtained from all participants, all of the
281 co-author approved the publish of the article.

282 **Availability of data and materials**

283 The datasets used or analysed during the current study are available from the database
284 of GSA-Human (<https://bigd.big.ac.cn/>).

285 **Competing interests**

286 *The authors declare that the research was conducted in the absence of any commercial*
287 *or financial relationships that could be construed as a potential conflict of interest.*

288 **Funding**

289 The study was supported by the Guangdong Provincial Natural Science Foundation
290 (2018A030310674), the Guangdong Provincial Science and Technology Project
291 (2017A020214016), the Key Laboratory of Shenzhen Respiratory Diseases
292 (ZDSYS201504301616234), and Shenzhen Science and Technology Project
293 (JCYJ20170413093032806).

Authors' contributions

Yongjian Yue 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, Fang Yuan, Chunxian Liang, and Kaixue Zhuang conducted sample collection and Sanger sequencing validation. Qijun Huang, Xiangxia Zhang, and Yutian Ye conducted clinical diagnosis of PCD. Tao Liu, Jie Li and Rongchang Chen assisted with the prepared and revised manuscript. All the authors have read and approved the final manuscript.

Acknowledgments

Thanks to laboratory support from the Key Laboratory of Shenzhen Respiratory Diseases.

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Figure legends

Fig 1. PCD diagnosis using traditional methods. Chest images showed bronchiectasis and situs inversus in KT9 (A) and bronchiectasis in KT10 (B). TEM examination showed structural defects in KT7 (C) and KT8 (D) cilia, the arrows represent the ODA, IDA defects and microtubular disorganization.

Fig 2. Validation of causative candidate variants by Sanger sequencing.

Fig 3. (A) Splicing deletion in the KT8 genomic DNA sequence. (B) cDNA sequencing of the control showed a normal sequence between exon 13 and exon 14 of the transcript. (C) Agarose gel electrophoresis showed the *CCDC40* cDNA PCR products was lost in KT8.

Figures

Figure 1

PCD diagnosis using traditional methods. Chest images showed bronchiectasis and situs inversus in KT9 (A) and bronchiectasis in KT10 (B). TEM examination showed structural defects in KT7 (C) and KT8 (D) cilia, the arrows represent the ODA, IDA defects and microtubular disorganization.

Figure 2

Validation of causative candidate variants by Sanger sequencing.

Figure 3

(A) Splicing deletion in the KT8 genomic DNA sequence. (B) cDNA sequencing of the control showed a normal sequence between exon 13 and exon 14 of the transcript. (C) Agarose gel electrophoresis showed the CCDC40 cDNA PCR products was lost in KT8.