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Interpreting Variants of Uncertain Significance in PCD: Abnormal Splicing Caused by a Missense Variant of *DNAAF3*

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Keywords: *DNAAF3* | minigene assay | molecular diagnosis | primary ciliary dyskinesia (PCD) | variants of uncertain significance (VUS)

ABSTRACT

Background: Primary ciliary dyskinesia (PCD) is a rare autosomal recessive disorder characterized by dysfunction of motile cilia. While approximately 50 genes have been identified, around 25% of PCD patients remain genetically unexplained; elucidating the pathogenicity of specific variants remains a challenge.

Methods: Whole exome sequencing (WES) and Sanger sequencing were conducted to identify potential pathogenic variants of PCD. Minigene assays were performed to evaluate the pathogenicity of variants. Transmission electron microscopy (TEM) and high-speed video analysis (HSVA) were conducted to analyze the function of cilia in respiratory epithelial cells.

Results: We identified two variants of *DNAAF3*: c.557G>A, p.G186E in exon 5, and c.1364G>A, p.G455D at the terminal nucleotide of exon 10 in a 16-year-old male patient. Through a minigene assay, we demonstrated that the c.1364G>A variant led to a four-nucleotide skipping. The cilia in epithelial ciliary cells of the proband were almost immotile. The absence of outer dynein arms and inner dynein arms was also observed.

Conclusions: Our study identified two compound heterozygous variants of *DNAAF3*, a pathogenic gene for PCD, and proved that a novel missense variant c.1364G>A affects splicing. Our findings not only expanded the spectrum of mutations in the *DNAAF3* gene but also highlighted the importance of investigating variants of uncertain significance (VUS) for comprehensive genetic diagnoses.

Haixia Zheng and Chongsheng Cheng contributed equally to this work.

1 | Introduction

Primary ciliary dyskinesia (PCD, MIM 244400) is an inherited disorder defined by aberrations in the structure and function of cilia, which typically follow a recessive inheritance pattern, occasionally linked to X-linked or dominant variants. Clinically, PCD is characterized by a spectrum of symptoms, including neonatal respiratory distress, chronic wet cough, recurrent chest infections, rhinosinusitis, otitis media, bronchiectasis, and a common incidence of infertility. Notably, approximately half of the individuals with PCD exhibit situs inversus, underscoring the disorder's impact on bodily organ arrangement (Wallmeier et al. 2020). Affecting an estimated 1 in 10,000 to 20,000 individuals worldwide, PCD is notable for its clinical and genetic diversity (Horani and Ferkol 2021).

PCD's pathogenic mechanisms predominantly involve defects in motile cilia, essential for mucociliary clearance and cellular signaling. These impairments are typically a consequence of mutations in genes coding for ciliary structural proteins, components of the dynein arms, or other regulatory molecules, resulting in compromised ciliary motility. The motile cilium's scaffold, known as the axoneme, consists of nine peripheral microtubule doublets encircling a central pair of microtubules, adorned with intricate multiprotein complexes (9 + 2 structure). Integral to this structure are the nexin-dynein regulatory complexes (N-DRC), radial spokes (RS1–RS3), and axonemal dynein arms. This assembly is characterized by a repeating 96-nm pattern, encompassing four Outer Dynein Arms (ODAs), seven Inner Dynein Arms (IDAs), three radial spokes, N-DRC, and other minor complexes (Fabczak and Osinka 2019; Horani and Ferkol 2021; Legendre, Zaragosi, and Mitchison 2021). Axonemal Dynein Assembly Factors (DNAAFs), such as CCDC103, CFAP298, DAW1, DNAAF1, DNAAF2, DNAAF3, DNAAF4, DNAAF5, DNAAF6, ZMYND10, DNAAF8, DNAAF9, DNAAF10, DNAAF11, LRRC56, SPAG1, PIH1D1, PIH1D2, (Braschi et al. 2022), play a pivotal role in the cytoplasmic preassembly of these dynein arms, which are subsequently transported to the cilia via intraflagellar transport (IFT) (Fabczak and Osinka 2019). The discovery of over 50 genes associated with PCD (Legendre, Zaragosi, and Mitchison 2021), beginning with *DNAI1* in 1999 (Pennarun et al. 1999), highlights the genetic complexity of this disorder.

Diagnosing PCD presents significant challenges due to its genetic heterogeneity. No single diagnostic test can conclusively confirm or exclude PCD, necessitating a multifaceted approach encompassing nasal nitric oxide (nNO) measurements, high-speed video microscopy analysis (HSVA), transmission electron microscopy (TEM), and immunofluorescence of ciliated cells (Lucas et al. 2017; Shapiro et al. 2018). The integration of genetic testing into diagnostic protocols, facilitated by advancements in next-generation sequencing, has been a significant stride. However, approximately 25% of PCD patients remain genetically unexplained (Lucas et al. 2020).

A critical obstacle in PCD diagnosis and management is the identification and interpretation of Variants of Uncertain Significance (VUS) within PCD-associated genes. Our study focused on a Chinese patient exhibiting characteristic PCD phenotypes, in whom we identified two compound heterozygous missense variants in the *DNAAF3* (MIM 614566). Employing a minigene assay in HEK293T cells, we demonstrated that one of these *DNAAF3* variants leads to altered splicing, resulting in a four-base skipping event in exon 10. This investigation reinforces the significance of deciphering VUS in genes implicated in PCD and emphasizing the potential of such findings in refining diagnostic and therapeutic approaches for PCD.

2 | Materials and Methods

2.1 | Patients and Clinical Materials

The proband was a 16-year-old Chinese male diagnosed with PCD according to the diagnostic algorithm (Shapiro et al. 2018) at Peking Union Medical College Hospital (PUMCH), with both parents unaffected. This patient's main symptoms were year-round, productive cough, nonseasonal rhinosinusitis, and discharge from the ear that began in early childhood. Imaging examination revealed situs inversus totalis, bronchiectasis predominantly in the left middle lobe, and sinusitis (Figure 1a–c). The CT scan of the paranasal sinuses showed thickening of the outer wall of the right maxillary sinus with unevenly increased density, suggesting a possible combination of fibrous dysplasia of bone. (Figure 1a) His nasal nitric oxide (nNO) was 7.2 nL/min, and during the 1-year follow-up when his respiratory symptoms were stable, the repeated nNO measurement was 19.3 nL/min, both significantly below the 77 nL/min threshold (Shapiro et al. 2020). The patient's baseline pulmonary function tests revealed a forced vital capacity (FVC) of 4.92 L (91% of predicted) and a forced expiratory volume in one second (FEV_1) of 3.81 L (86% of predicted). The FEV_1/FVC ratio was 0.77, indicating a normal PFT pattern (Ferraro et al. 2023). Blood samples from the proband were collected. All methods carried out in this study were approved by the Institutional Review Board committee at PUMCH (I-23PJ390). The informed consent form had been signed by the patient and his parents.

2.2 | Transmission Electron Microscope

Transmission electron microscopy (TEM) was employed to observe the microtubule structures of ciliated cells in the bronchial mucosa, which were obtained from the PCD individual through mucosal biopsy by bronchoscopy. The sample was submerged in a fixative solution comprising 2.5% glutaraldehyde in phosphate-buffered saline (PBS). After postfixation in osmium tetroxide for one hour and dehydration process using graded concentrations of ethanol, the sample was then embedded in Epon 812 and sliced into 600 nm sections. The target structure was localized under light microscopy. After being stained with uranyl acetate and lead citrate, the sample was photographed via TEM with an acceleration voltage of 80 kV. The images were captured using a VELETA CCD camera (OSIS).

2.3 | High-Speed Video Analysis of Beat Frequency of Nasal Respiratory Cilia

Nasal epithelial cells (NECs) were obtained from the inferior nasal turbinate using a cytobrush. Disaggregated tissue samples

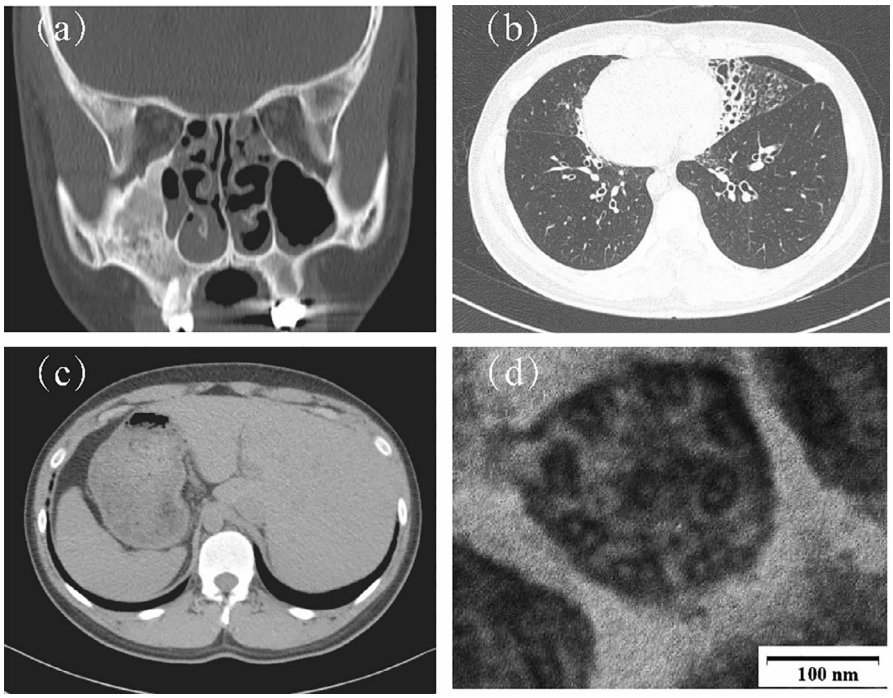

FIGURE 1 | Clinical investigation of the proband. (a) Reconstruction of the sinuses CT showed inflammation of maxillary sinus and ethmoid sinus on both sides. (b) HRCT of the chest showed inversion of the heart and the lung, and marked cystic bronchiectasis in the middle lobe of the left lung. (c) Abdominal CT revealed situs inversus totalis. (d) TEM showed the absence of ODAs and IDAs.

were stored in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), enriched with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Ciliary motion was visualized using a CellManipulator (Eching, Germany) attached to a Nikon microscope at room temperature within 1 h after brushing. More than 20 ciliated cells were observed, and five representative cells among them were video-recorded (Jackson and Bottier 2022).

2.4 | Whole Exome Sequencing, Sanger Sequencing, and Bioinformatic Analysis

Venous blood samples, anticoagulated with EDTA, were collected from the patient. Genomic DNA (gDNA) was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), following the provided protocol. The patient's gDNA underwent whole-exome sequencing (WES) using the Agilent SureSelect Human All Exon V6 (Agilent, USA) and sequenced on Illumina platforms (PE150 strategy) at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). Post-sequencing quality control involved aligning reads to the NCBI human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner (BWA) software to generate BAM files (Li and Durbin 2010). Variant calling for germline SNPs and InDels was performed using SAMtools (Li et al. 2009), with the following filter parameters: QUAL ≥ 20; DV ≥ 4; MQ ≥ 30. Copy Number Variations (CNVs) were detected using CoNIFER (Krumm et al. 2012), and variant annotation was executed by ANNOVAR (Wang, Li, and Hakonarson 2010), encompassing details on protein-coding changes, genomic regions, allele frequency, and deleterious predictions. Candidate variants were filtered against public databases (1000 Genomes, ExAC, gnomAD, and ESP) (Abecasis et al. 2012; Kobayashi et al. 2017; Pio et al. 2021), discarding those with minor allele frequency ≥ 0.01, synonymous variants, noncoding, and intronic variants in non-canonical splicing sites. The confirmed variants were further verified by Sanger sequencing using primers designed by Primer3 (http://primer3.ut.ee/):

DNAAF3-1267-F: 5′-ACTCACCGGGCTAATTCCAC -3′.
DNAAF3-1267-R: 5′-ATCTCTCTGGTTTTCCGCCC -3′.
DNAAF3-3628-F: 5′-GGGAACGATTCCCCCGTTT -3′.
DNAAF3-3628-R: 5′-CCCCAAATATTCTCTGTCTGCG -3′.

2.5 | TA Cloning

To confirm the c.557G>A and c.1364G>A variants in trans, the target fragment spanning from intron 4 to intron 10 was cloned into a pMD18-T vector (Takara, Japan). Transformation was conducted in Trans5α chemically competent cells (Transgene, Beijing), and colonies were isolated on LB plates containing appropriate antibiotics. Sanger sequencing confirmed that the two variants do not coexist on the same allele. Primer sequences used were:

DNAAF3-T-F: 5′-GCTGAGATTCAGCTCCAAATCC-3′.
DNAAF3-T-R: 5′-ACTCTAATTCCCTCTTCTAGGTGG-3′.

2.6 | Minigene Plasmid Construction

A minigene assay was employed to assess the pathogenicity of the c.1364G>A variant and determine the splicing capacity of the wild-type (WT) and mutant sequence. The assay used pCAS2

vectors with a multicloning site between two encoding exons (exons A and B), allowing insertion of the target sequence (ranging from intron 8 to exon 12) with 15-bp extensions complementary to the MluI site of the linearized vector. The construction was performed using the In-Fusion HD Cloning Kit (Takara, Japan), generating wild-type (c.1364G) and mutant (c.1364A) plasmids. Sanger sequencing confirmed the plasmid sequences. Primer sequences used were:

DNAAF3-MluI-F: GTTTGGAATCACGCGCGCTTGGTGGGAACGAC.
DNAAF3-MluI-R: TCAAAACAAGACGCGGTTGCTGGCTTCAAGAGGC.

2.7 | Transfection of Minigene Plasmid and cDNA Sequencing

HEK293T cells were cultured in 6-well plates (1 × 106 cells/well) and transfected with recombinant vectors using Lipofectamine 3000 (Life Technologies, CA, USA). After 48 h, cells were harvested, and RNA was extracted using TRIzol (Gibco-BRL, San Francisco, CA, USA). RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo, Lithuania), and reverse transcription was conducted using Prime Script RT Master Mix (Takara, Japan). RT-PCR was utilized to analyze the splicing outcomes, employing primers located between exons A and 9 and between exons B and 12:

pCAS2-RT-F: CTGCTGCTGGCTGGGACG.
pCAS2-RT-R: CACGTCCACCAGGTACCGG.

The RT-PCR products were analyzed via agarose gel electrophoresis, UV imaging, and Sanger sequencing.

2.8 | Pathogenicity Analysis

All variants are classified based on the ACMG-AMP guidelines. The criteria fall into two sets: pathogenic or likely pathogenic (P/LP) and benign or likely benign (B/LB), whereas "uncertain significance" is assigned to variants for which the criteria are contradictory or not met. Standardized evaluation incorporated diverse data types, including population frequency, in silico prediction, functional effects, segregation data, de novo data, allelic data and clinical relevance. There are a total of 28 criteria: the 16 criteria for the P/LP criterion are very strong (PVS1), strong (PS1–PS4), moderate (PM1–PM6), or supporting (PP1–PP5); whereas the 12 criteria for the B/LB criterion are standalone (BA1), strong (BS1–BS4), or supporting (BP1–BP7) (Li and Wang 2017).

3 | Results

3.1 | Transmission Electron Microscope

The cilia were analyzed by transmission electron microscopy (TEM). TEM revealed the absence of ODA and IDA of observable respiratory cilia (Figure 1d).

3.2 | High-Speed Video Analysis

High-speed video analysis (HSVA) was performed on nasal brushings from the affected individual, revealing that their cilia were almost immotile (see Video S1). In contrast, cilia from a control individual exhibited a normal beat frequency (Video S2).

3.3 | Variant Analysis

WES was conducted to identify potential pathogenic variants in the proband. The analysis generated 12.72 Gb of raw data. Post-filtering, 99.30% of the reads were effective, with 99.92% mapping to the human reference sequence. The average depth of sequencing in the target region was 144.00×, ensuring sufficient accuracy for variant calling, with 97.60% of the target regions covered > 10×. A total of 116,549 single nucleotide polymorphisms (SNPs) and 12,353 Insertions and Deletions (Indels) were identified. Variants with a minor allele frequency ≥ 1% in 1000 genomes, ExAC, and gnomAD were excluded. Given the autosomal recessive inheritance pattern of PCD, compound heterozygous variants in the *DNAAF3* gene (NM_001256714.1: c.557G>A, p.G186E, and c.1364G>A, p.G455D) were identified as potential disease-associated variants for the proband (Table 1). No other variants in known PCD-associated genes were detected. The confirmed variants were further verified by Sanger sequencing (Figure 2a). Due to the unavailability of parental samples, pedigree co-segregation analysis could not be conducted. However, TA cloning assay results suggested that the c.557G>A and c.1364G>A variants are in trans (Figure 2c,d).

3.4 | Functional Testing by Using Minigene Assay

The c.1364G>A variant was predicted to impact splicing. To investigate this, a minigene assay was performed. Agarose gel electrophoresis of the RT-PCR products revealed distinct band patterns for the wild-type and mutant plasmids. The transcript from the c.1364G (wild type) plasmid exhibited a band corresponding to approximately 354 bp. This band represents the wild-type transcript, including 15 nucleotides (nt) at the 5′ end of exon A from the pCAS2 plasmid, the sequence from exon 9 to exon 11, and 16 nt at the 5′ end of exon 12 from the inserted *DNAAF3* segment. In contrast, the transcript from the c.1364A (mutant type) plasmid showed a shorter band of approximately 350 bp, with Sanger sequencing indicating the skipping of four nucleotides at the end of exon 10 (Figure 3).

3.5 | Pathogenicity Analysis

After assessment of the ACMG/AMP guidelines (Table 1), the c.557G>A and c.1364G>A variants were found to be likely pathogenic.

4 | Discussion

Primary ciliary dyskinesia (PCD) is a highly heterogeneous recessive inherited disorder (Wallmeier et al. 2020). While the advent

TABLE 1 | Compound heterozygous *DNAAF3* variants identified in a Chinese PCD-affected individual.

***DNAAF3* variants**	**M1**	**M2**
cDNA alteration	c.557G>A	c.1364G>A
Protein alteration	p.G186E	p.G455D
Zygosity	compound heterozygous	compound heterozygous
Variant type	Missense	Missense
1000 Genomes Project	Absent	Absent
Exome Aggregation Consortium	Absent	Absent
East Asians in gnomAD	Absent	Absent
MutationTaster	Disease_causing(1.00)	Disease_causing(1.00)
SIFT	Damaging (0.0)	Tolerable (0.615)
SpliceAI	Benign (0)	Splice-Altering/strong (0.6)
phyloP	Conserved (4.244)	Conserved (4.025)
Initial ACMG criteria	PM1 PM2 PP3	PM2 PP3
Initial pathogenicity	Uncertain Significance	Uncertain Significance
Added ACMG criteria	PM3 PP4	PS3 PP4
Pathogenicity	Likely pathogenic	Likely pathogenic
Reference	Guo et al. (2020)	Present study
HGMD accession number	CM2011061	Present study

Note: Genomic databases coordinate in GRCh37/hg19. RefSeq reference transcript: NM_001256714.1 (*DNAAF3*).

of next-generation sequencing (NGS), particularly Whole-Exome Sequencing (WES), has significantly advanced our ability to identify genetic causes of PCD, approximately 25% of PCD patients still lack a precise genetic diagnosis (Gileles-Hillel et al. 2020; Guan et al. 2021). Multiple factors contribute to the low rate of PCD diagnosis, of which the following two are the most noteworthy. First, the technical limitations of WES may make it difficult to identify a pathogenic variant. Second, there may be insufficient information to interpret the pathogenicity of a variant (VUS) (Wojcik et al. 2023). WES only sequences exons, which constitute about 1%–2% of the human genome. Consequently, it may miss important genetic variations in non-coding regions, such as introns and regulatory areas. Additionally, WES has a limited detection scope and may fail to identify certain types of genetic variations, including large-scale chromosomal structural variations, copy number variations, and some deeply nested intronic variations. At the same time, insufficient interpretation of variants may also lead to missed pathogenic variants.

In our study, we performed genetic analysis of a PCD patient presenting with situs inversus sinusitis, bronchiectasis, childhood recurrent pneumonia, and with the nNO reduction. WES revealed a compound heterozygous c.557G>A, p.G186E in exon 5, and c.1364G>A, p.G455D at the terminal nucleotide of exon 10 of the *DNAAF3* gene. Both missense variants were initially assessed as VUS. However, the variation in the last base of the exon is likely to affect splicing; the c.1364G>A variants would be further validated by functional experiments. In addition, the pathogenicity of the c.1364G>A variants also provided evidence of PM3 for the c.557G>A.

Comparing these findings with previously reported results, patient NO.10 reported by Guan et al. (2021) carried both a missense variant and a frameshift variant in the *DNAAF3* gene, exhibiting a similar phenotype to our patient, characterized by the absence of inner and outer dynein arms. Furthermore, patients P3 and P16 reported by Guo et al. (2020) also carried compound heterozygous variants in *DNAAF3*, including a missense variant and a frameshift variant. Their clinical phenotypes were consistent with those observed in our patient, showing reduced nasal nitric oxide, nearly immotile cilia, and the absence of inner and outer dynein arms. These findings highlight the recurrent nature of these genetic variants and their associated clinical manifestations.

Previous studies have shed light on the significant role of splicing mutations, accounting for approximately 62% of all pathogenic single nucleotide variants (SNVs) (López-Bigas et al. 2005). The majority of mutation analyses, both in research and clinical settings, are conducted primarily or exclusively at the genomic DNA level. Current bioinformatics filtering strategies and clinical interpretation guidelines often focus predominantly on the pathogenicity at the amino acid level. This approach can lead to the early-stage filtering out of synonymous variants and deep intronic mutations, even though these variants may have significant effects on splicing. Meanwhile, the pathogenic potential of some missense variants that may impact splicing is often underestimated. Validation of these variants is based on RNA-seq and function tests in vitro and in vivo. In this study, minigene was used for functional validation in vitro. A comprehensive approach that combines genomic DNA analysis with

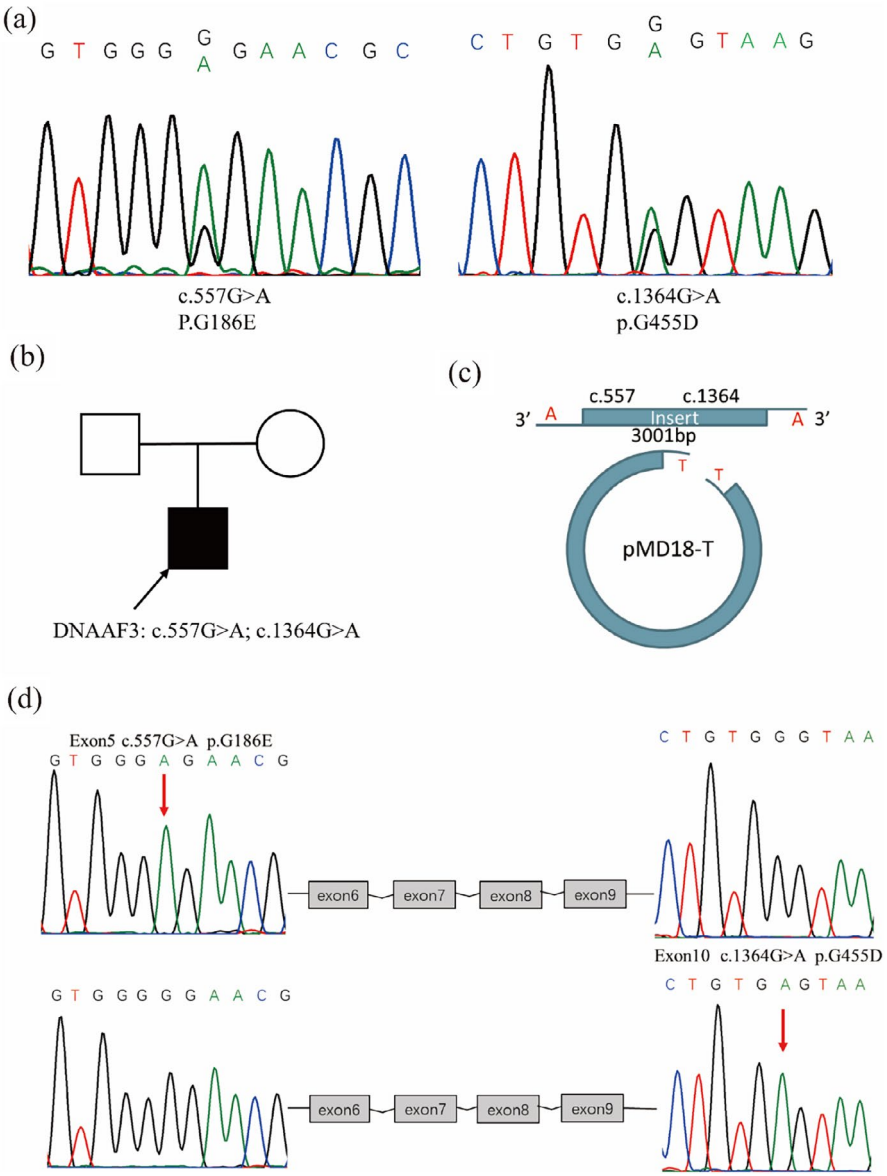

FIGURE 2 | Pedigree and sequence analysis by TA Cloning. (a) Sanger sequencing revealed two Missense Variants. (b) Pedigree and genotype. Squares represent male members, while circles represent female members. Unfilled symbols indicate unaffected individuals and the filled symbol denotes the affected individual (proband), highlighted with an arrow. (c) Vector Insertion of *DNAAF3* Gene: This panel shows the *DNAAF3* gene segment, including c.557 and c.1364 variants, extracted from the proband's genomic DNA (gDNA) and subsequently cloned into the pMD18-T vector. (d) Verification of Variants through Sanger Sequencing: Sanger sequencing results of the recombinant vectors are presented, confirming the presence of two distinct transcripts—one including the c.557 variant and the other containing the c.1364 variant.

RNA sequencing can provide a more complete understanding of the impact of genetic variants, including those affecting splicing (Licatalosi and Darnell 2010; Maddirevula et al. 2020). This approach is essential not only for accurate diagnosis but also for the identification of potential therapeutic targets.

Patient-derived RNA analysis is paramount for identifying splicing aberrations in PCD. The findings of Wai suggest that RNA analysis of blood samples can identify diagnostically important splicing abnormalities and clarify the functional effects of a significant proportion of VUSs (Wai et al. 2020). In Vicente A. Yepez's study, using the utilization of skin biopsies, an average of 12,500 genes were detected per sample, encompassing approximately 60% of all disease-related genes, a significantly higher coverage compared to whole blood analysis. Cumulative evidence from WES and RNA-seq contributed to the genetic diagnosis in 16% of patients where WES alone was inconclusive. (Yépez et al. 2022) However, this is challenging due to the limited expression of PCD genes in blood and the difficulty in obtaining adequate patient tissue samples.

Minigene assays are a valuable alternative for mutation screening, especially when patient samples are unavailable. The minigene assay is based on transient transfection of cells with a vector containing a genomic region of interest cloned between two constitutive exons. Cloning can be accomplished by the use of restriction enzymes or by site-specific recombination using Gateway cloning. They offer insights into how specific variants may affect

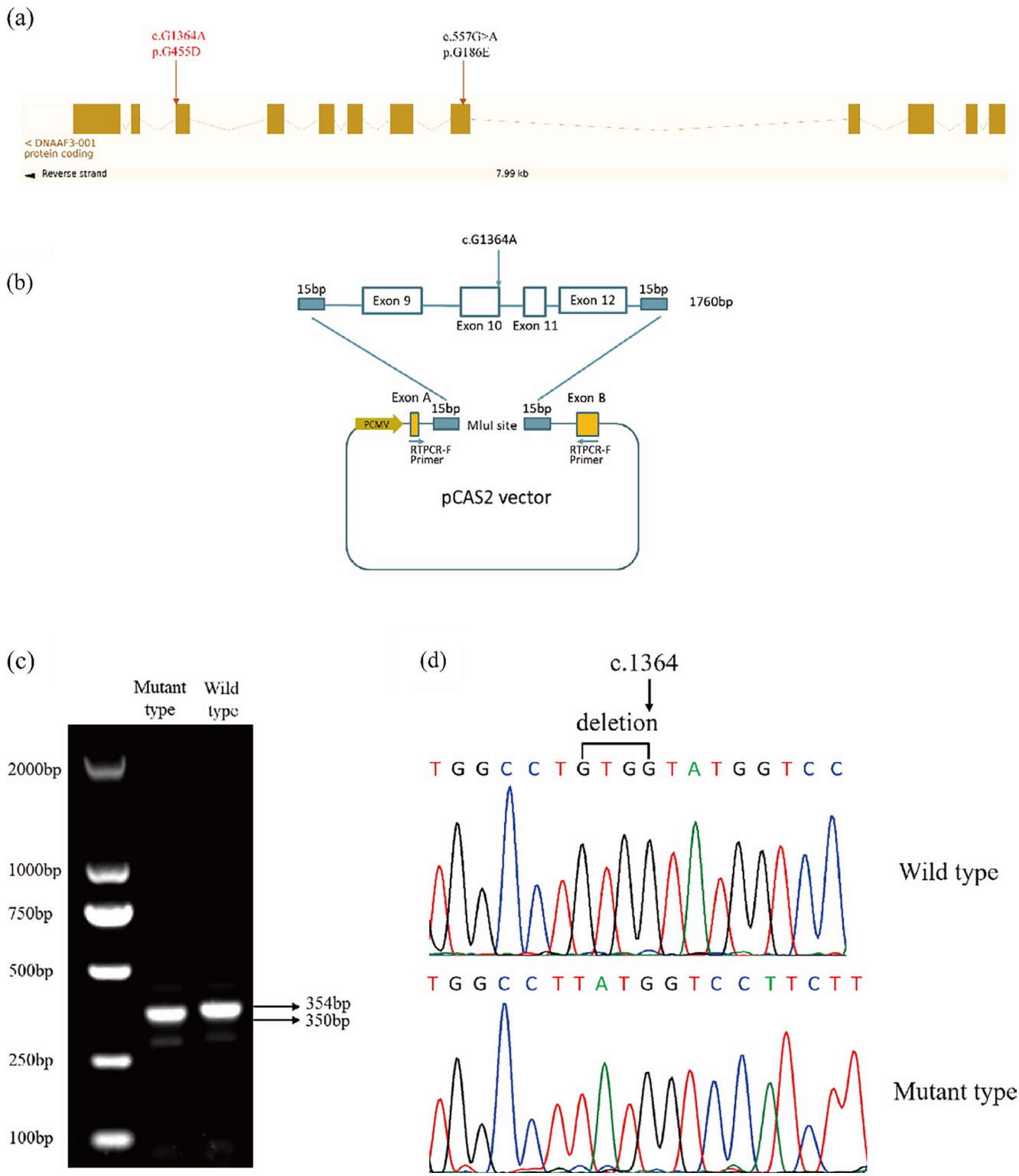

FIGURE 3 | Identification of a Splicing Variant in *DNAAF3* (a) Gene Structure and Variant Location: *DNAAF3* (NM_001256714.1) consists of 12 exons. The novel *DNAAF3* variant, highlighted in red, is situated at the last nucleotide of exon 10 (c.1364G>A), and is predicted to affect splicing. Previously reported variants in *DNAAF3* are indicated in black. (b) Minigene plasmid construction. A fragment of genomic DNA including 1760bp was amplified. Two types of target sequences encompassing two variants in exon10 were designated as c.1364G (wild type) and c.1364A (mutant type). The amplicons were cloned into the pCAS2 vector, which has a PCMV promoter (orange) and two exons (exons A and B, yellow). Two blue boxes on the pCAS2 vector and target sequences represent two 15 bp fragments complementary to MluI ends, which help clone the target sequence into the pCAS2 vector. (c, d) Analysis of Splicing Patterns through Electrophoresis and Sanger Sequencing: (c) Electrophoretic Comparison of Wild Type and Mutant Transcripts: The gel electrophoresis image compares the transcripts from the c.1364G (wild type) and c.1364A (mutant type) plasmids. The band corresponding to the wild-type transcript appears larger than that of the mutant type. (d) Mutant Transcript Analysis: Sanger sequencing reveals that the mutant transcript exhibits a splicing aberration, characterized by the skipping of four nucleotides at the end of exon 10.

splicing (Bueno-Martínez et al. 2022; Putscher et al. 2021). The experimental outcomes from our study using minigene assay provided compelling evidence that variants once deemed as having uncertain clinical significance can, in fact, have profound effects on gene function. The c.1364G>A variant, leading to the skipping of four nucleotides at the end of exon 10, highlighted the nuanced ways in which exonic variants can disrupt normal splicing processes. This alteration in splicing not only contributed to the PCD phenotype but also stressed the importance of considering non-canonical effects of exonic variants.

However, these assays may not fully replicate the complex in vivo splicing environment, leading to potential misinterpretations (Putscher et al. 2021). Base editing technology presents a promising avenue for mutation screening and validation in PCD research. Base editors enable precise, targeted modifications of DNA bases without introducing double-stranded breaks, offering a more accurate approach for studying the functional impact of specific mutations. This technology could be pivotal in overcoming some of the limitations with minigene assays by allowing for direct, targeted modification of genes within their natural genomic context (Komor et al. 2016). Kweon et al.'s study uses CRISPR-based base editing to evaluate the functional impacts of various BRCA1 gene variants, aiding in understanding their role in cancer (Kweon et al. 2020). Li et al.'s paper employs a large-scale CRISPR-based approach to investigate and characterize the pathogenicity of variants in the LDLR gene, which is crucial for understanding cholesterol metabolism disorders (Li et al. 2023). In light of these advancements, our future experiments plan to employ single-base editing techniques to further explore and validate the functional consequences of the c.1364G>A and the c.557G>A variant or even other PCD-related variants. By precisely targeting and modifying specific DNA bases, we aim to gain deeper insights into the pathogenic mechanisms of the variants, particularly those affecting splicing, within their authentic genomic environment. This approach promises to enhance the accuracy of our mutation analysis, bridging the gap between in vitro assays and in vivo biological relevance.

5 | Conclusion

In conclusion, we performed a study on the splicing effects of identified exonic variants in the *DNAAF3* gene. Accordingly, we determined the pathogenicity of the c.1364G>A and the c.557G>A variant. Furthermore, our study not only contributes to the expanding genetic landscape of PCD but also highlights the importance of assessing the splicing effects of DNA variants and emphasizes the critical role of functional genomics in interpreting VUS. These efforts are vital for enhancing our understanding of the disease, improving diagnostic accuracy, and paving the way for novel therapeutic strategies while acknowledging and addressing the limitations of current sequencing technologies. Looking forward, base editing technology presents an exciting opportunity for future mutation screening and validation in PCD research.

Author Contributions

Yaping Liu, Xinlun Tian, Kai-Feng Xu and Xue Zhang designed the study and reviewed the original version of the manuscript. Haixia Zheng and Chongsheng Cheng performed the experiments and wrote the first draft of the manuscript. Miao He and Yixuan Li helped in data collection and conception. Wangji Zhou, Jinrong Dai, and Ting Zhang recorded the clinic information. All authors read and approved the final manuscript.

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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).

Consent

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

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.