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Identification of Genetic Variants in *CFAP221* as a Cause of Primary Ciliary Dyskinesia

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Abstract

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Contributors:

XBM and LEO wrote the manuscript and MRK and MAZ contributed to the manuscript. XBM, MRK, MAZ and LEO conceived the experiments, and designed strategies. XBM cultured the HNE cells, and performed experiments, and MAZ performed genotyping and segregation analysis. XBM, LEO, PS, and MAZ analyzed and interpreted data. AS, MWL, MAZ and MRK, identified the patient and performed exome sequencing. DFC and WC performed the whole exome sequencing analysis, AS performed nasal scrape, and PS, performed waveform analysis. All authors reviewed the manuscript.

Patient consent: Obtained.

Ethics approval: Provenance and peer review not commissioned; externally peer reviewed.

Web resources:

PSAP, <https://github.com/awilfert/PSAP-pipeline>

OMIM, <http://www.omim.org>

GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>

Ensembl, http://grch37.ensembl.org/Homo_sapiens/Info/Index

1000Genome, <http://www.internationalgenome.org/>

gnomAD, <http://gnomad.broadinstitute.org/>

Uniprot, <https://www.uniprot.org/>

dbSNP, <https://www.ncbi.nlm.nih.gov/snp>

Supplementary information is available at the Journal of Human Genetics' website.

Conflict of interest

The authors declare no conflicts of interest

Primary ciliary dyskinesia (PCD) is a rare disorder that affects the biogenesis or function of motile cilia resulting in chronic airway disease. PCD is genetically and phenotypically heterogeneous, with causative mutations identified in over 40 genes; however, the genetic basis of many cases is unknown.

Using whole exome sequencing, we identified three affected siblings with clinical symptoms of PCD but normal ciliary structure, carrying compound heterozygous loss-of-function variants in *CFAP221*. Computational analysis suggests that these variants are the most damaging alleles shared by all three siblings. Nasal epithelial cells from one of the subjects demonstrated slightly reduced beat frequency (16.5 Hz vs 17.7 Hz, $p=0.16$); however, waveform analysis revealed that the *CFAP221* defective cilia beat in an aberrant circular pattern.

These results show that genetic variants in *CFAP221* cause PCD and that *CFAP221* should be considered a candidate gene in cases where PCD is suspected but cilia structure and beat frequency appear normal.

Keywords

Primary ciliary dyskinesia; PCD; CFAP221; PCDP1; ciliary waveform

Introduction

Primary ciliary dyskinesia (PCD) is a rare genetic disease (MIM: 244400), caused by genetic lesions that impair the function of the motile cilia that line the airways, thereby reducing mucociliary clearance and resulting in chronic respiratory infections. Currently there are no specific treatments available for PCD, and early diagnosis is crucial to ensure proper disease management and minimize loss of lung function¹. Advances in whole exome sequencing and analysis have contributed to the identification of PCD-causing mutations in >40 genes². However, the underlying genetic cause is still unknown in ~30% of PCD cases, and in subjects with no obvious defects in ciliary structure or beat frequency, establishing a firm diagnosis remains challenging.

Material and Methods

Subjects

Individuals included in the study had a clinical diagnosis of PCD confirmed by standard clinical diagnostic criteria. For studies of affected individuals and their families, a signed and informed consent was obtained from all participants. All protocols involving human studies were approved by University of North Carolina Medical School and/or McGill University Health Centre Research Institute Institutional Review Boards.

Genetic Analysis

Identification of *CFAP221* variants was performed by whole exome sequence analysis using the population sampling probability (PSAP) framework as previously described³, as detailed in Supplementary Information.

Airway epithelial cell cultures

Human nasal epithelial (HNE) cells from proband 2-I and controls were obtained as previously described⁴. The nasal cells were expanded as conditionally reprogrammed cells (CRC)⁵ and cultured as previously described⁶. *Human bronchial epithelial (HBE) cells* were cultured at the air/liquid interphase (ALI) as previously described⁷. These protocols have been approved by the University of North Carolina (UNC) Medical School Institutional Review Board.

Reverse transcription polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed using standard protocols (Takara Bio USA, Inc.), as detailed in Supplementary Information.

Waveform analysis and ciliary beat frequency measurements

To evaluate the waveform and beat direction, high resolution videos of ciliated cells were recorded and analyzed as described in Supplementary Information.

Results and Discussion

We identified three French-Canadian siblings from family UNC-1200 (Figure 1A) with phenotypes suggestive of PCD (Supplementary Table 1), including neonatal respiratory distress, chronic sinus disease, recurrent otitis media, and bronchiectasis in two individuals. None of the subjects had *situs inversus*. Sweat chloride testing and immunology investigations were normal in all three siblings. The rate of nasal nitric oxide production (nNO) was normal (122, 171, 189 nL/min; cut-off of 77 nL/min⁸), and examination of ciliary axonemes by TEM did not reveal obvious ultrastructural defects (Figure 1B).

We carried out whole exome sequencing in one proband (2-II) and analyzed the resulting genotype data using a statistical framework for assessing the significance of variants from $n=1$ cases of rare genetic disease³. This identified compound heterozygous variants in cilia and flagella associated protein 221 (*CFAP221*; [GenBank: NM_001271049.2, Chr.2q14.2] previously known as *PCDPI*) as the most damaging variants in the genome of 2-II and most plausible cause of a rare recessive disorder (Table 1^{9, 10} and Figure 1C). Segregation analysis confirmed that both variants were present in all three affected siblings and parents were carriers for each variant (Supplementary Figure 1). A frameshift variant in exon 22 [c.2303_2307delTAGAA p.Leu768HisFs*5] is predicted to result in a premature termination codon. The other variant (c.2318+1G>A) disrupts a canonical splice donor site in intron 22 that causes aberrant splicing (Figure 1C)⁹. RT-PCR analysis of exon 22, using primers spanning exon 19–23, of control human nasal epithelial (HNE) cells amplified a 539 base pair product (Figure 1C). In HNE cells from subject 2-I we confirmed the five base pair deletion (r.2303_2307del) resulting in a premature stop codon (Figure 1C). Analysis of the intron 22 splice site variant revealed the in frame deletion of exon 22 (r.2226_2318del). This is predicted to result in deletion of 31 amino acid residues (p.Cys743_Arg773del) (Figure 1C).

We examined the expression and localization of *CFAP221* in normal human bronchial epithelial (HBE) cells (Supplementary Figure 2) cultured at an air/liquid interface (ALI)⁷. RT-PCR demonstrated that the expression of *CFAP221* increased during ciliated cell differentiation (Supplementary Figure 2A). Western blotting and immunofluorescence showed that CFAP221 is not expressed in undifferentiated airway cells, and is localized primarily to the axoneme in differentiated ciliated cells (Supplementary Figure 2B and 2C).

To investigate the impact of the genetic variants in *CFAP221* on ciliary function, a nasal biopsy from subject 2-I was expanded as conditionally reprogrammed cells¹¹, cultured at the ALI to induce differentiation, and examined for *CFAP221* expression (Supplementary Figure 3A). The localization of CFAP221 in ciliated cells was evaluated by immunofluorescence (Supplementary Figure 3B). In contrast to control cells, cells from the PCD subject showed no specific staining. The absence of CFAP221 in ciliated cells from subject 2-I probably results from the non-sense mediated decay of aberrant transcripts (Figure 1). Immunofluorescent staining for other ciliary proteins, RSPH1 and ERICH3 showed a similar pattern in the 2-I cells, as compared to control cells (Supplementary Figure 3C). This result agrees with the observation of normal ciliary ultrastructure by TEM (Figure 1B).

After differentiation of cells from subject 2-I, we observed vigorous ciliary activity, with a beat frequency that was only slightly reduced compared to parallel control cultures (16.5 Hz vs 17.7 Hz, $p=0.16$, Figure 2A). However, waveform analysis in the xy direction (top down) revealed that the *CFAP221*-deficient cilia beat in a slightly circular pattern, compared to the linear, back and forth motion of control cilia (Figure 2B, 2C and Supplementary Videos 1-4). This difference, when measured as the maximum deviation from a linear beat, was highly significant (control= $0.27 \pm 0.06 \mu\text{m}$; 2-II= $0.92 \pm 0.5 \mu\text{m}$; $p<0.0015$, $n=24$ cilia for each group).

In a mouse model, mutation of *Pcdp1* was reported to cause symptoms of PCD, including mucus accumulation in the nasal cavity, male infertility, and hydrocephalus¹². Ciliary ultrastructure was reported as normal, however ciliary beat frequency (CBF) was reduced approximately 25% in tracheal samples of the mutant mice. In *Chlamydomonas* flagella, the orthologue of *CFAP221*, (FAP221) has been shown to be a component of the central pair projection C1d and to bind calcium¹³. Further, the absence of the C1d complex results in a significant impairment of mobility, including a reduced beat frequency and altered waveform. These results suggest that CFAP221 might play an important role in calcium-mediated regulation of dynein activity. This is an interesting hypothesis to explore in deciphering the underlying mechanism by which genetic variants of *CFAP221* are a likely cause of PCD in humans.

Subjects with genetic variants in *CFAP221* exhibited typical symptoms of classical PCD, but ciliary axonemes appeared normal by TEM. PCD causing mutations in other genes have also been associated with apparently normal ciliary ultrastructure¹⁴, including *DNAH11*¹⁵⁻¹⁷ (MIM:603339) and *HYDIN*^{15, 18} (MIM: 610812). A more accurate analysis of the axoneme structure of *CFAP221* deficient cells could be achievable by cryo-electron microscopy. This could confirm a central pair defect, as previously shown in *Chlamydomonas*¹³. A central

pair defect is consistent with a ciliary beat in a circular motion, similar to the pattern of ciliary motion in PCD-subjects carrying genetic variants in *RSPH1*¹⁹ (MIM: 609314). In addition, the nNO levels were normal in all affected individuals, and the CBF was only slightly reduced; thus, it is clear that the diagnosis of PCD remains challenging, and a single test cannot be relied upon to detect all cases.

Our results demonstrate for the first time that genetic variants that affect the central pair protein CFAP221 cause PCD in humans. Our results suggest that in subjects with normal ultrastructure and normal or only slightly reduced CBF, *CFAP221* should be considered as a candidate gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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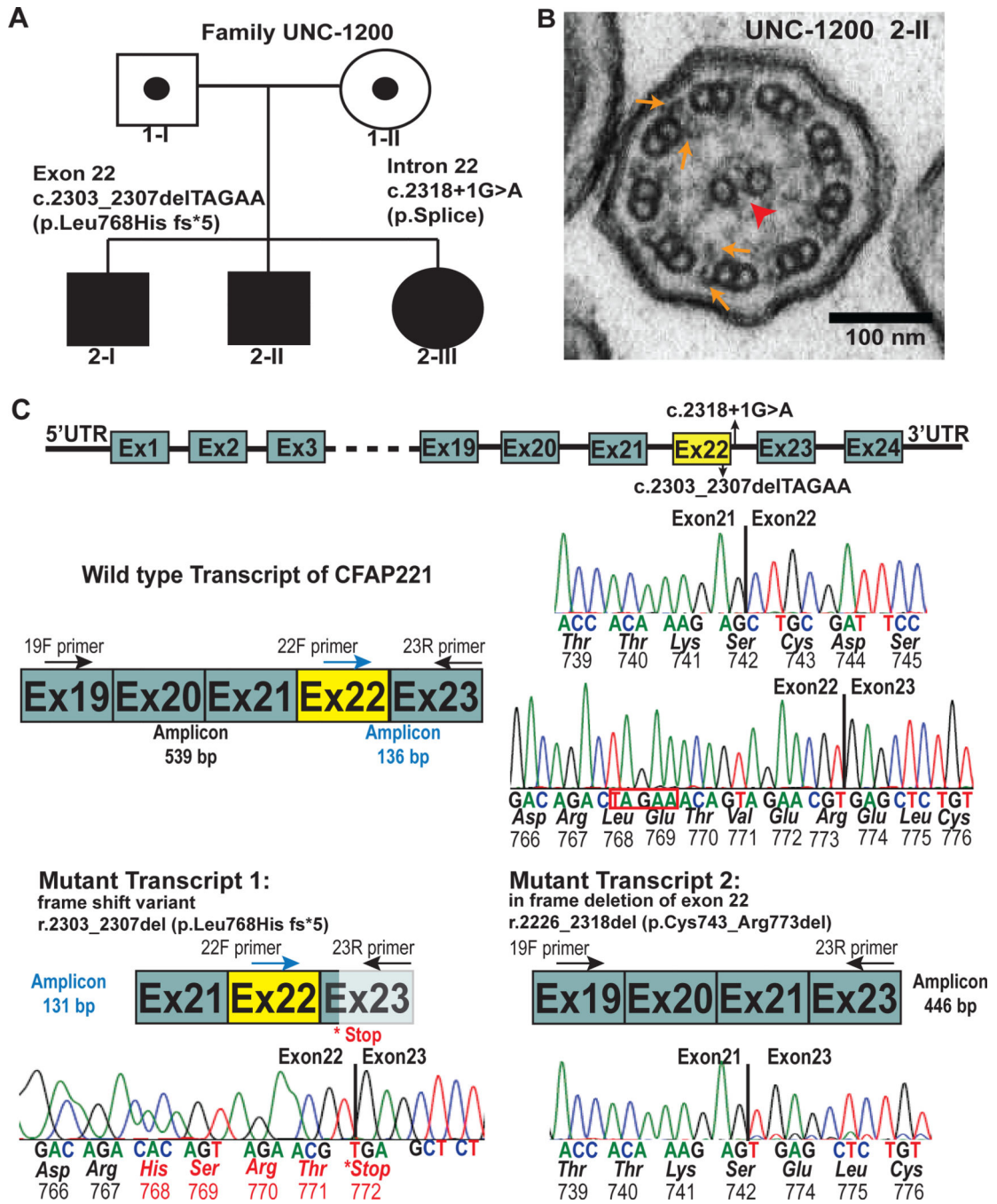


Figure 1: CFAP221 variants in PCD subjects.

(A) Segregation analysis of *CFAP221* genetic variants found in family UNC-1200 [c.2303_2307delTAGAA p.Leu768Hiffs*5] and c.2318+1G>A p. splice. Filled symbols indicate PCD affected individuals.

(B) Transmission electron micrograph of axonemal cross sections of nasal epithelium from proband 2-II showing the central pair (arrowhead) surrounded by nine microtubule doublets. Outer dynein arms and inner dynein arms (arrows) project from each doublet normally.

(C) Genomic organization of *CFAP221* and sequencing of transcript variants. *(top)* Schematic representation of the genomic localization of the genetic variants in *CFAP221* found in the PCD subjects. Solid box designates exons, horizontal lines designates introns. *(middle)* Schematic showing a wild type transcript region of *CFAP221* and its corresponding sequencing analysis. *(bottom)* Reverse-transcriptase polymerase chain reaction with primers 19F or 22F or 23R (see Supplementary Table 3 for primer sequences) showed two major transcripts. Transcript 1 shows the deletion of 5 bases (highlighted in red box, above), leading to a frame shift of the sequence, resulting in a premature stop codon. Transcript 2 shows the in frame deletion of exon 22. Schematic of wild type and mutant transcripts and the corresponding electropherograms with exact location of the deletions are shown. Primers are designated as F (forward) and R (reverse) in their corresponding cDNA locations. Exon-exon junctions are shown by the vertical solid lines. Base sequence, amino acid sequence, and codon numbers are shown.

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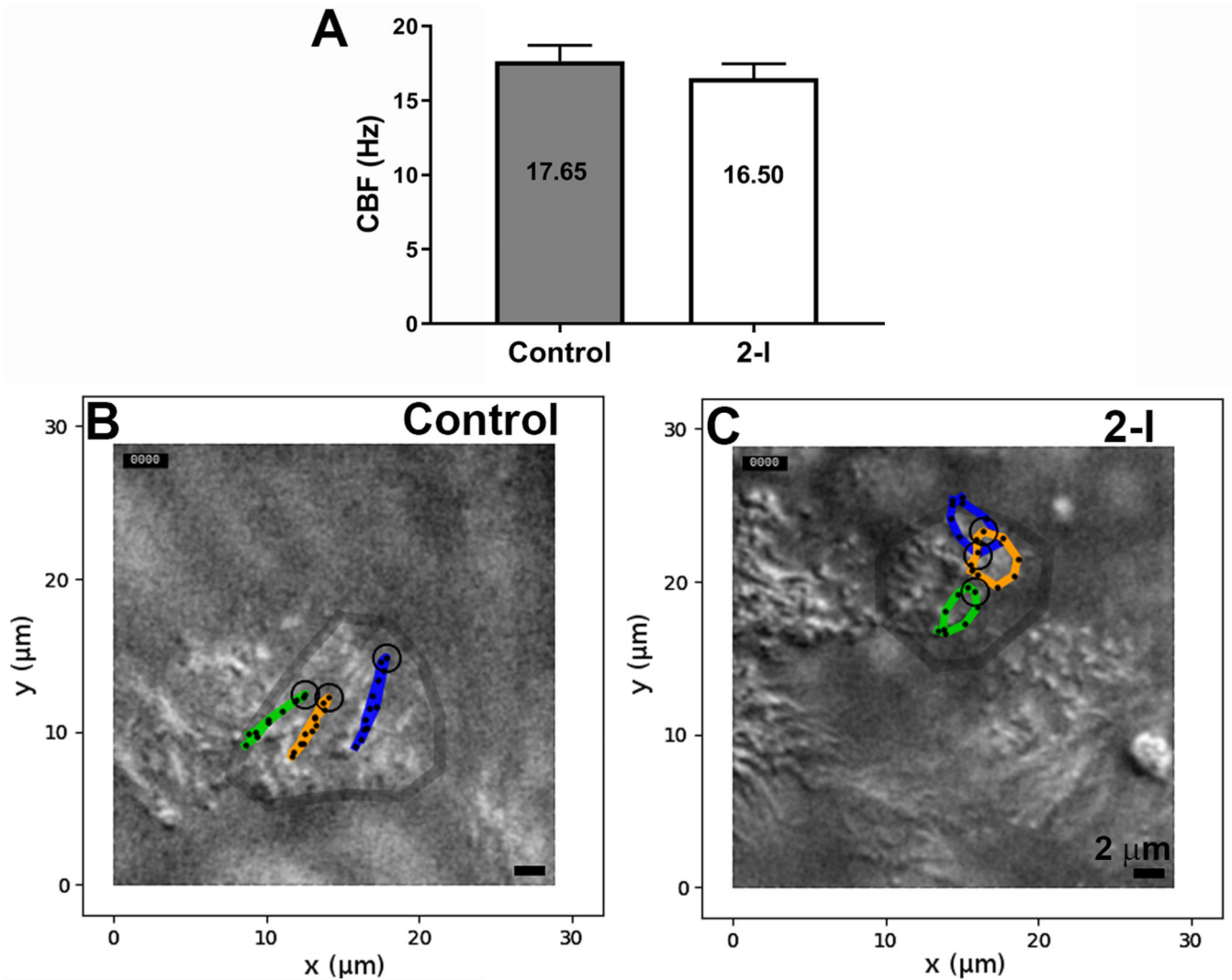


Figure 2: Genetic defects in *CFAP221* cause defective ciliary waveform.

(A) The CBF in *CFAP221* deficient cells (2-II) was slightly reduced compared to control (n=6 cultures from each).

(B and C) Two-dimensional plot representing the patterns of the ciliary beat. The tips of eight cilia were traced manually in three ciliated cells from each genotype (n = 24 each). Deviation from linearity was measured as the furthest ciliary displacement from the linear axis as defined by the end-effective and end-recovery positions.

Table 1.

List of the top genes identified in 2-II using PSAP analysis. Three genes in the entire exome contained two damaging variants after filtering.

Gene	Transcript	Position (hg19)	Type	AAChange	Alt Read	Total Read	gnomAD Freq	CADD	PSAP pvalue
PCDPI	ENST00000413369.3	2:120404611_TAGAA>-	frameshift deletion	L768fs	13	29	NA	35	1.00E-06
PCDPI	ENST00000413369.3	2:120404627_G>A	splicing	NA	9	20	0	24.3	1.00E-06
NEDD4 ¹	ENST00000338963.2	15:56208554_->TA	frameshift insertion	N159fs	36	82	NA	38	1.00E-06
NEDD4 ¹	ENST00000338963.2	15:56208558_C>A	nonsynonymous SNV	D158Y	39	85	NA	26.4	1.00E-06
URB1 ²	ENST00000382751.3	21:33735605_A>G	nonsynonymous SNV	S457P	36	72	0.0001	26.6	0.000135
URB1 ²	ENST00000382751.3	21:33719498_G>A	nonsynonymous SNV	P1212L	62	110	0.0004	13.69	0.000135

¹Review of the exome sequencing data revealed that both NEDD4 variants were in cis; hence the variants in NEDD4 were excluded as a cause of PCD.

²URB1 is involved in the pre-rRNA processing pathway¹⁰ and is ubiquitously expressed. The two missense variants in URB1 are classified as variants of unknown significance, based on ACMG/AMP guidelines⁹.