Cytoplasmic "ciliary inclusions" in isolation are not sufficient for the diagnosis of primary ciliary dyskinesia

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Abstract

Background: The diagnosis of primary ciliary dyskinesia (PCD) is difficult and requires a combination of clinical features, nasal nitric oxide testing, cilia ultrastructural analysis by electron microscopy (EM), and genetics. A recently described cytoplasmic ultrastructural change termed "ciliary inclusions" was reported to be diagnostic of PCD; however, no supporting evidence of PCD was provided. In this study, we sought to confirm, or refute, the diagnosis of PCD in subjects with "ciliary inclusions" on EM.

Methods: Six subjects from five families with previous lab reports of "ciliary inclusions" on EMs of ciliated cells were identified and evaluated at a Genetic Disorders of Mucociliary Clearance Consortium site. We performed a detailed clinical history, nasal nitric oxide measurement, genetic testing including whole-exome sequencing (WES), and when possible, repeat ciliary EM study.

Results: Only one of six subjects had multiple and persistent clinical features congruent with PCD. No subject had situs inversus. Only one of six subjects had a very low nasal nitric oxide level. No "ciliary inclusions" were found in three subjects who had a repeat ciliary EM, and ciliary axonemal ultrastructures were normal. Genetic testing, including WES, was negative for PCD-causing genes, and for pathogenic variants in gene pathways that might cause "ciliary inclusions," such as ciliary biogenesis.

Conclusion: "Ciliary Inclusions", in isolation, are not sufficient to diagnosis PCD. If seen, additional studies should be done to pursue an accurate diagnosis.

KEYWORDS
cilia EM, ciliary inclusions, primary ciliary dyskinesia

1 | INTRODUCTION

Kartagener et al[1] in 1933 described the classic clinical triad of bronchiectasis, chronic sinusitis, and situs inversus. In the 1970s, immotile cilia and ciliary electron microscopy (EM) defects associated with ciliary dysfunction were found to be the underlying cause of defective mucociliary clearance and chronic sinopulmonary infections in patients with Kartagener syndrome.[3] Eventually, it was recognized that abnormal cilia movement, not simply immotile cilia, can cause disease; hence, the current name primary ciliary dyskinesia (PCD).[4]

Ciliary ultrastructural EM changes have traditionally been used as the "gold standard" for diagnosis of PCD, but we now recognize limitations to this approach.[5-7] Changes in cilia ultrastructure can be

nonspecific, reflecting air pollutants and smoke exposure, or infection and inflammation.[8,9] Some patients phenotypically have PCD, but normal cilia ultrastructure, for example, patients with mutations in *DNAH11*, a PCD-causing gene associated with a normal cilia EM.[5] There have also been instances where cilia ultrastructural defects have been initially thought to cause PCD, and later were shown to be nonspecific, such as missing inner dynein arms in isolation, or misalignment of the central pairs.[6,10-12]

The current sensitivity of cilia EM for the diagnosis of PCD is ~70%.[7] Assessment of ciliary waveforms using high-speed video microscopy for the diagnosis of PCD is difficult to replicate, requires a high level of skill to perform, and is not universally accepted.[13-15] Recently, PCD diagnostic guidelines have stressed the use of commercially available genetic test panels due to clinical expertise needed for ciliary EM interpretation.[16] Due to the complexities of PCD diagnosis, a combination of clinical features, genetics, nasal nitric oxide testing (nNO), and cilia EM are required for confident diagnosis across the spectrum of PCD.

A recent manuscript reported an EM finding of "ciliary inclusions" in the cytoplasm of ciliated cells as diagnostic of PCD.[17] These inclusions were reported to reflect cilia in the cytoplasm of airway epithelial cells, because of an inability to reach the cell surface. Although this EM finding was interpreted to be diagnostic of PCD, there was no supporting evidence to support a diagnosis of PCD in the reported cases, including no phenotype, ciliary axonemal defect, clinical nNO, or genetic testing provided to support a diagnosis of PCD. In this study, we sought to confirm, or refute, the diagnosis of PCD in subjects with cytoplasmic "ciliary inclusions" on EM.

2 | MATERIALS AND METHODS

Six subjects (three from the original report) were identified who had "ciliary inclusions" reported on at least one respiratory cilia biopsy. These subjects were evaluated at a Genetic Disorders of Mucociliary Clearance Consortium (GDMCC) center at the University of North Carolina (n = 4) or Children's Hospital Colorado (n = 2). Subjects underwent standard collection of medical history as it pertains to PCD, which included: neonatal history; cough and sputum production; pulmonary infections; nasal congestion and sinusitis; surgical history; antibiotic usage; and any other pertinent medical conditions. Nasal NO was measured using a chemiluminescence analyzer (ECO PHYSICS AG, Duernten, Switzerland) using a previously described technique.[18] If possible, plateau measurements were used for nNO; however, due to the young age of many of the subjects, tidal breathing measurements were obtained when necessary. Nasal scrape biopsy for repeat ciliary EM analysis used the previously described GDMCC technique.[5,19-21] At least 25 ciliated cells were examined for ciliary inclusions.

Finally, blood (proband) and/or a buccal swab (family members) was obtained for genetic analysis. Initially, a PCD gene panel of either 30 (subject #1, 2, 4, 6) or 34 (#3) PCD-associated genes was performed by Invitae (https://www.invitae.com/en/). The Invitae panel consists of sequencing and analysis of coding regions and splice junctions, as well as exon-level deletion/duplication analysis using next-generation sequencing. Whole-exome sequencing (WES) and data analysis were then performed for two subjects (#2 and 3) at the Yale Center for Mendelian Genomics, or at the McDonnell Genome Institute in St. Louis on four subjects (#1, 2, 4, and 6) using previously described methods.[22] Finally, manual review of WES data was also performed for all currently known PCD-associated genes (E-Table 1).

Informed consent was obtained from the subject's parents and the study was approved by the University of North Carolina at Chapel Hill and the University of Colorado Institutional Review Boards.

3 | RESULTS

Three subjects from the original manuscript were further evaluated (#1-3 in Table 1), and three additional subjects reported to have "ciliary inclusions" (#4-6 in Table 1) were evaluated at a GDMCC site. Clinical characteristics, including PCD-related medical history, and diagnostic studies are summarized in Table 1. The majority of subjects had at least one clinical feature congruent with PCD, including year-round nasal congestion that started under 6 months of age.[23] No subject had a laterality defect or bronchiectasis. One subject (#2) had three clinical characteristics associated with, but not diagnostic of, PCD.[23] Two other subjects (#1 and 6) had two clinical features consistent with PCD when evaluated at age 3 years, but these were not present when re-evaluated at 5 years of age. Nasal nitric oxide testing was performed at least once on all six subjects (Table 1). One subject (#2) had an abnormal nNO (plateau) value below 77 nL/min,[17] but all other subjects had normal values for age.

Subjects in the original paper (#1-3) in Table 1 were reported to have normal ciliary axonemal structure.[17] A repeat nasal ciliary EM was performed at a GDMCC site on three subjects (#3-5). No "ciliary inclusions" were found on repeat ciliary ultrastructural analysis (Figure 1), and the ciliary axonemal structure was normal.

All unrelated subjects underwent genetic testing (n = 5). No known mutations or pathogenic variants were found in 30 known PCD causing genes (Table 1). All subjects were also negative for pathogenic variants in *CCNO* and *MCIDAS*, recently described genetic causes of PCD associated with a decreased number of cilia and retained basal bodies and rootlets in the cytoplasm, but normal ciliary axonemal structure.[24,25] Finally, WES to identify novel PCD or "ciliary inclusion" disease-causing genes was also negative. Subject #3 had a large deletion of chromosome 22 on the microarray, a known cause of Phelan-McDermid syndrome,[26] but this deletion did not include any known or candidate PCD-causing genes.

TABLE 1 Demographics, clinical characteristics, and diagnostic testing results of subjects with "ciliary inclusions"

Subject #	Sex	Age at ciliary inclusion report	Age at 1st GDMCC visit	Symptoms at 1st GDMCC visit	Nasal NO at 1st GDMCC visit[a]	GDMCC repeat EM	Age at 2nd GDMCC visit	Symptoms at 2nd GDMCC visit	Nasal NO at 2nd GDMCC visit	Bronchiectasis	Genetic results	Diagnosis of PCD confirmed
1	M	11 mo	3 y	Cough, Nasal congestion	50 (plateau)	N/D	5 y	None	469 (plateau)	N	Negative 30 PCD gene panel,[b] negative *CCNO/MCIDAS*, negative WES[c]	No
2	F	5 y	8 y	Cough, Nasal congestion, NRDS	14 (plateau)	N/D	N/D	N/D	N/D	N	Negative 30 PCD gene panel, negative *CCNO/MCIDAS*, negative WES[c,d]	No[e]
3	F	13 mo	4.9 y	NRDS	202 (tidal)	Normal	N/D	N/D	N/D	N	Negative 34 PCD gene panel,[b] negative *CCNO/MCIDAS*, negative WES[d], Phelan-McDermid syndrome[f]	No
4[g]	M	3 y	3 y	Nasal congestion	143 (tidal)	Normal	N/D	N/D	N/D	N	Negative 30 PCD gene panel,[b] negative *CCNO/MCIDAS*, negative WES[c]	No
5[g]	M	11 mo	14 mo	Nasal congestion	50 (tidal)	Normal	N/D	N/D	N/D	N	NA	No
6	F	3 y	3 y	Cough, Nasal congestion	120 (tidal)	N/D	5 y	None	272 (plateau)	N	Negative 30 PCD gene panel,[b] negative *CCNO/MCIDAS*, negative WES[c]	No

Abbreviations: EM, electron microscopy; GDMCC, genetic disorders of mucociliary clearance consortium site; NO, nitric oxide; NRDS, neonatal respiratory distress syndrome; N/D, not done; PCD, primary ciliary dyskinesia; WES, whole-exome sequencing.

[a]Values are given in nL/min. Normal values for plateau measurement in children older than 5 years of age are 77 nL/min. Tidal values vary with age; however, a value of 50 nL/min or greater in a child under 1 year of age or older would be normal.

[b]PCD genetic panel performed by Invitae (San Francisco, CA). A full list of genes tested is available in Table S1.

[c]WES performed by McDonnell Genome Institute at Washington University (St Louis, MO).

[d]WES performed by Yale University (New Haven, CT).

[e]Although clinical phenotype and low nasal NO on one occasion are congruent with PCD, we were unable to get a repeat nasal NO measurement, and ciliary EM and genetic testing (including WES) were negative for PCD.

[f]Phelan-McDermid syndrome was diagnosed based on microarray showing a large deletion on chromosome 22 (arr 22q 13.31q 13.33(4426896S 5122a252) × 1) which included *SHANK3*, which would lead to Phelan-McDermid syndrome, but does not include any known or candidate PCD-causing genes.

[g]Subjects are sibling pairs, therefore only one was tested for a genetic cause of PCD.

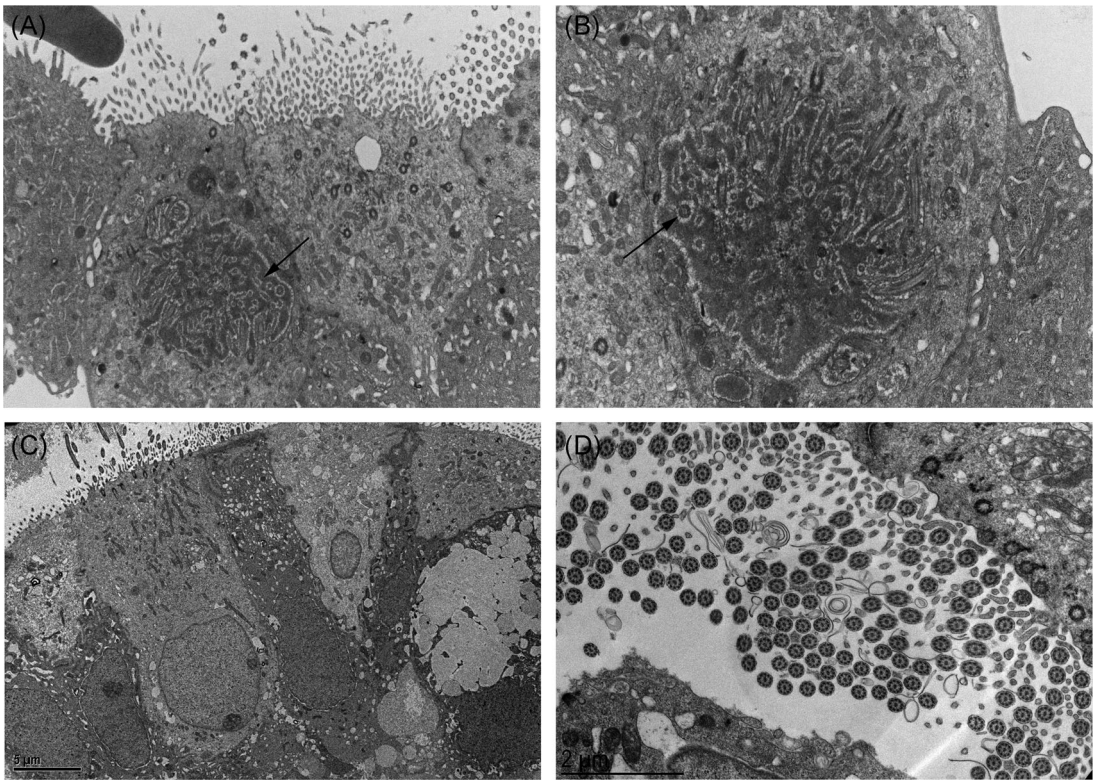

FIGURE 1 Selected EM images from subject #4 (Table 1). Images (A, B) are from the original tracheal mucosal biopsy read as "ciliary inclusion" disease by the authors of the Wartchow et al[17] "ciliary inclusions" manuscript. Image (A) demonstrates a ciliary "inclusion" (black arrow) with a vesicular inclusion containing proteinaceous material. Image (B) is a higher power image of a ciliary "inclusion" which, per the Wartchow et al's lab, demonstrates disorganized cilia within the "inclusion" (black arrow). Images (C, D) are from a repeat nasal cilia biopsy done at a Genetic Disorders of Mucociliary Clearance Consortium site and processed and analyzed at the University of North Carolina. Image (C) shows a respiratory epithelial cell on repeat biopsy on subject #4 without "ciliary inclusions", and none were seen in any of the greater than 25 cells that were examined. Note that the cell surface, basal bodies, and cell nucleus are included in a single cell. Image (D) shows a normal number of cilia present on the repeat biopsy of subject #4 (Table 1)

4 | DISCUSSION

The diagnosis of PCD is complex, and no single test is sensitive or specific enough to be considered a gold standard for establishing a diagnosis. Therefore, diagnosis requires patients to have compatible clinical features of PCD along with a combination of either low nNO testing on more than one occasion, and/or positive genetic testing, and/or positive findings on ciliary EM.[16,23,27] Recently published diagnostic guidelines from the American Thoracic Society and the PCD Foundation have emphasized genetic testing over EM, as EM is prone to errors in both processing and interpretation.[16,27] Regardless of what testing is done first, patients must have a thorough clinical evaluation for PCD before testing, and all testing must be interpreted carefully.

A recent manuscript reported a new EM finding of "ciliary inclusions" in the cytoplasm of ciliated cells that was interpreted as being diagnostic of PCD in six subjects. Unfortunately, no phenotypic information or other diagnostic testing results (nNO; ciliary axonemal defect; genetics) were provided to support the diagnosis of PCD. Therefore, we sought to confirm, or refute, the diagnosis of PCD in subjects with cytoplasmic "ciliary inclusions" on EM. Our studies show that at least two of the subjects (#1 and #3 for Table 1) from that original report do not have PCD, based on clinical phenotype and normal lab studies of ciliary ultrastructure, nNO, and genetic testing. Further, repeat EM studies of nasal ciliated cells in subject #3, who had Phelan-McDermid syndrome, did not show "ciliary inclusions." We also studied an additional three subjects that had been diagnosed with PCD on the basis of EM findings, "ciliary inclusions," but our studies did not support the diagnosis of PCD, based on clinical phenotype and lab studies, including normal nNO, normal cilia EM, and negative genetic testing, including WES.

The failure to demonstrate "ciliary inclusions" on repeat cilia EM in three subjects provides further evidence against "ciliary inclusions" being PCD-causing. All known causes of PCD that lead to ultrastructural changes in cilia are consistently present on repeat respiratory epithelial tissue samples, which reflects genomic mutations, and not secondary changes where variable findings are more common.[28] We sought to identify a genetic cause for "ciliary inclusions" or PCD, but were unable to identify one, either in PCD-causing genes or in ciliary biogenesis genes that might cause "ciliary inclusions."

The most likely etiology of "ciliary inclusions" in the cytoplasm of ciliated cells is that they reflect technical artifacts during cellular processing and/or EM imaging. Epithelial cells are not received in an EM lab in well-aligned rows but are instead in clumps with cells facing

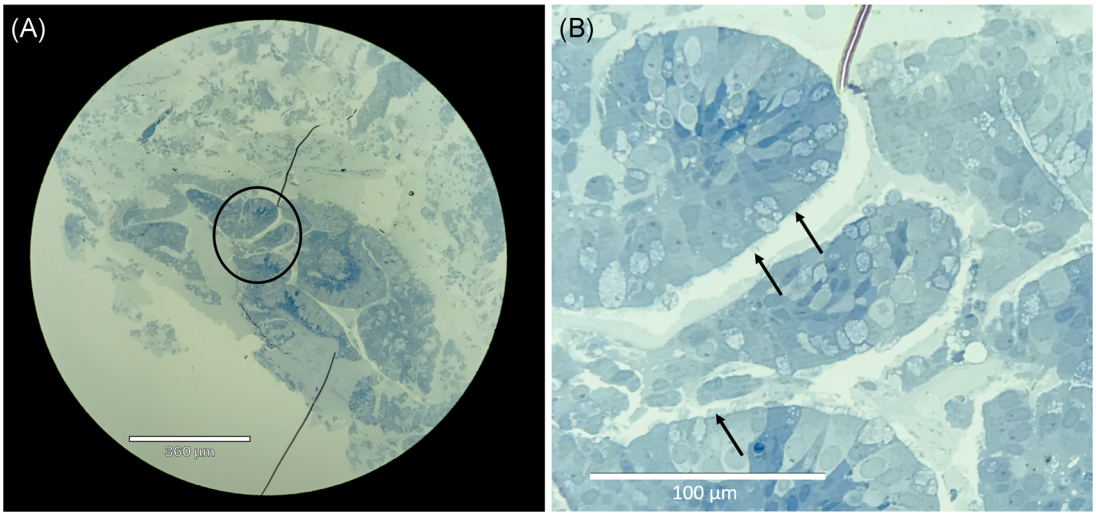

FIGURE 2 Richardson stained nasal biopsy from subject 3 (Table 1). A, ×10 magnification shows multiple groups of ciliated cells. B, magnification of circled area from (A) demonstrates cilia at the surface of the cells in multiple directions (arrows)

multiple directions (Figure 2). These cells are also not perfectly symmetric shapes, but rather complex three-dimensional shapes and the cell surface is not smooth, but rather has multiple invaginations. When cells are cut tangentially, the resulting image could give the appearance of a cilia within the cytoplasm of the cell. Importantly, if the image does not include the cell surface, basal bodies, and nucleus in the same image, it cannot reliably be interpreted as a defect and could be the result of technical artifact (Figure 1). While the original paper claims to show entire ciliated epithelial cells, close inspection shows that none of the cells in the figures contain cilia, an undisrupted cell surface, basal bodies, and a nucleus in the same image.

For any new ciliary EM finding in subjects suspected of having PCD, confirmatory testing must be done before adopting it for general use for diagnostic purposes, because misdiagnosis of PCD can lead to significant consequences. Patients and their families can experience unnecessary anxiety at a new diagnosis, and delay of the proper diagnosis may delay the initiation of appropriate therapy.[27] An incorrect diagnosis of PCD can be a significant cause of harm to patients and should be avoided.

In conclusion, we were unable to confirm a diagnosis of PCD in any of the six patients in our study, although we were not able to rule out PCD in one of the subjects (#2, Table 1). Therefore, "ciliary inclusions" in isolation are not a hallmark of PCD, and patients with "ciliary inclusions" alone should not be diagnosed with PCD. If "ciliary inclusions" are seen on ciliary EM, other causes for the patient's symptoms should be investigated.

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SUPPORTING INFORMATION

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