Primary ciliary dyskinesia with normal ultrastructure: three-dimensional tomography detects absence of DNAH11

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ABSTRACT  In primary ciliary dyskinesia (PCD), motile ciliary dysfunction arises from ciliary defects usually confirmed by transmission electron microscopy (TEM). In 30% of patients, such as those with DNAH11 mutations, apparently normal ultrastructure makes diagnosis difficult. Genetic analysis supports diagnosis, but may not identify definitive causal variants. Electron tomography, an extension of TEM, produces three-dimensional ultrastructural ciliary models with superior resolution to TEM. Our hypothesis is that tomography using existing patient samples will enable visualisation of DNAH11-associated ultrastructural defects. Dual axis tomograms from araldite-embedded nasal cilia were collected in 13 PCD patients with normal ultrastructure (DNAH11 n=7, HYDIN n=2, CCDC65 n=3 and DRC1 n=1) and six healthy controls, then analysed using IMOD and Chimera software.

DNAH11 protein is localised to the proximal ciliary region. Within this region, electron tomography indicated a deficiency of >25% of proximal outer dynein arm volume in all patients with DNAH11 mutations (n=7) compared to other patients with PCD and normal ultrastructure (n=6) and healthy controls (n=6). DNAH11 mutations cause a shared abnormality in ciliary ultrastructure previously undetectable by TEM. Advantageously, electron tomography can be used on existing diagnostic samples and establishes a structural abnormality where ultrastructural studies were previously normal.

This article has supplementary material available from erj.ersjournals.com

Received: Sept 08 2017 | Accepted after revision: Dec 10 2017

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Support statement: H.M. Mitchison is supported by the Great Ormond Street Hospital Children’s Charity, the UK PCD Family Support Group and the National Institute for Health Research (NIHR) Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London. A. Shoemark is funded by a postdoctoral research fellowship from the NIHR and Health Education England (HEE-HCS-P13-04-004), mentored by C. Hogg, H.M. Mitchison and A. Bush. A. Bush was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London. This report is independent research arising from a postdoctoral research fellowship supported by the NIHR and Health Education England. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. Funding information for this article has been deposited with the Crossref Funder Registry.

Conflict of interest: None declared.
**Methods**

**Patient selection**

Seven patients, aged 7–18 years, with known biallelic disease-causing mutations in *DNAH11* were selected. Details of subjects and a summary of their diagnostic investigations are shown in table 1. The study was approved by the London Bloomsbury research ethics committee under approval number 08/H0713/82.

Two comparator groups were used. Group one consisted of six healthy controls without PCD. Group two consisted of six subjects with a diagnosis of PCD and normal TEM, not carrying *DNAH11* mutations. These cases were due to biallelic mutations in *HYDIN* (n=2), *CCDC65* (n=3) and *DRC1* (n=1). The PCD comparator group was chosen because ultrastructure is often considered normal in these cases, but it is not anticipated nor has it ever been seen that the ODA is affected by mutations in these three genes [14–17]. These gene mutations are associated with isolated defects of the c2b central pair projection (*HYDIN*) and nexin–dynein regulatory complex (*CCDC65* and *DRC1*). A subject with a loss-of-function mutation in *DNAH5* with a known ODA defect by TEM was used as a negative control.

**Electron tomography sample preparation**

Ultrathin sections (~150 nm) were cut using an ultra-microtome from blocks of nasal brushings embedded in araldite. Sections were stained with 2% uranyl acetate and lead citrate and subsequently labelled with 10 nm gold fiducial markers.

The following areas (plane of sections are shown in figure 1c) were assessed using tomography: 1) longitudinal sections of the axoneme at the proximal portion of the cilium; 2) longitudinal sections of the axoneme at the distal portion of the cilium; and 3) transverse cross-sections, at least six per field of view, at the proximal portion of the axoneme (in the region of the microvilli).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at TEM years</th>
<th>Clinical signs and symptoms</th>
<th>nNO nL·min⁻¹ (normal &gt;77 nL·min⁻¹)</th>
<th>Light microscopy</th>
<th>TEM DA defects MTD defects</th>
<th>DNAH11 mutations</th>
<th>Genotype and predicted protein effect</th>
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<tr>
<td>#1</td>
<td>Male</td>
<td>18</td>
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<td>Normal DA 0% MTD 4%</td>
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<td>Recurrent chest infections/rhinitis</td>
<td>6</td>
<td>Static and hyperfrequent 22.6 Hz</td>
<td>Normal DA 0% MTD 5%</td>
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<td>14</td>
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<td>Normal DA 0% MTD 7%</td>
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<td>Slow with reduced bending 6.15 Hz</td>
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<td>[c.2832dup]; [c.13240dup] [p.Gln945Serfs<em>10]; [p.Thr4414Asnfs</em>34]</td>
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<td>Normal DA 0% MTD 4%</td>
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</table>

TEM: transmission electron microscopy; nNO: nasal nitric oxide; HSVM: high-speed video microscopy; CBF: ciliary beat frequency; DA: dynein arm; MTD: microtubular doublet; ND: not done, because dyskinesia frequency measurements were not taken. "\*: c.13531_13532insTTCCAGGCTGAAGA mutation (patient 1) has not been proven for pathogenicity.
Generation of electron tomograms

A JEOL 1400+ TEM (Welwyn Garden City, UK) was used to collect a series of images at different stage tilts. 4K × 4K digital images at ×20000 magnification were captured using JEOL tomography software and a digital camera (AMT 16X, Deben, Bury St Edmunds, UK). The software automatically adjusts stage position, focus and exposure. The specimen was tilted through angles of +65° to −65° and images acquired at increments of 1°. Following image generation the sample was manually rotated 90° and in the same region a second series of images was acquired. The two image series were aligned and combined to produce a dual axis reconstruction using IMOD software [18, 19].

Subtomographic averaging

Subtomographic averaging was conducted to enhance the ODA structure using particle estimation for electron tomography, part of the IMOD software package [19]. The regions chosen for averaging of longitudinal tomograms were at 96-nm intervals [20]. For transverse cross-sections the centre of the γ microtubule of each microtubule doublet (figure 1) was selected as the centre for averaging. For quality control, averages in which the inner dynein arm and radial spokes could be visualised were considered acceptable for further analysis.

Tomography data analysis

Imaging, modelling and quantification of the ODAs was performed using the UCSF Chimera programme developed by the Resource for Biocomputing, Visualisation, and Informatics at the University of California San Fransisco (supported by NIGMS P41-GM103311). The volume of averaged microtubule doublets and ODA was determined by a single individual blinded to details of the case using a bounding box as shown in figure 4c. From these measurements, the percentage of the microtubule doublet that the ODA occupied was calculated (online supplementary material).

Immunofluorescence

Immunofluorescence labelling was performed as previously described [3, 21]. Imaging was conducted using a confocal microscope (SP5; Leica, Milton Keynes, UK) (online supplementary material).

Results

DNAH11 longitudinal tomograms demonstrate a loss of volume from the ODA in the proximal cilia region

In light of evidence indicating different ODA types in the proximal and distal cilium [10] (figure 1c) we created averaged longitudinal tomograms from the proximal and distal regions of the ciliary axoneme from subjects with DNAH11 mutations. Tomograms obtained from the proximal region all showed a loss of ODA volume. A representative image of a 96-nm segment of the proximal ciliary microtubule containing four ODAs is shown in figure 2a, comparing subject #4 who carries two frameshift loss-of-function DNAH11 alleles to a healthy control. Quantification of arm volume in four DNAH11 subjects in comparison to controls without respiratory disease (n=3) shows that approximately two-thirds of the ODA volume is lost in individuals with DNAH11 defects (figure 2b) (p<0.01). Tomograms from the remaining three DNAH11 subjects and three controls were not collected due to the technical difficulty of longitudinal electron tomography.

The retained structures seen in the proximal ODA using tomography were investigated further. In the tomogram images, it appears that a heavy chain head is retained in each ODA, presumably devoid of DNAH11 but retaining DNAH5 protein as well as the light chain proteins associated with DNAH11 such as NME8 (figure 2b). This was confirmed by immunofluorescence antibody staining from two DNAH11-deficient subjects (DNAH11 #2 and #7) in whom the ODA heavy chain dynein DNAH5 (ODA head component) and axonemal light chain dynein NME8 (a component predicted to be closely associated with the ODA β heavy chain stalk) were both intact (figure 3b and d).

DNAH11 is localised to the proximal region of the cilium and tomograms of the distal region appear normal

No difference was seen in longitudinal tomograms from the distal region of the cilia in subjects with three DNAH11 mutations, compared to two control subjects (figure 2a and b). By immunofluorescence we confirmed a proximal axonemal localisation for DNAH11 in cilia from healthy controls with no protein present in the distal cilium (figure 3a and c).

In keeping with this finding, the ciliary beat pattern in DNAH11 subjects, as assessed by high-speed video microscopy demonstrated a higher incidence of dyskinesia in the proximal region of the cilia, compared to the distal region. In six out of seven samples there was reduced bending at the base of the cilium and preserved waveform at the tip (online supplementary video 1 and table 1) compared to healthy controls.
The incomplete beat in DNAH11 subjects results in an apparently hyperfrequent ciliary beat, as previously reported, presumably due to reduced bend and therefore a shorter effective and recovery stroke, resulting in more beats per second [8, 9]. We speculate that this results from a lack of DNAH11 in the proximal cilium while the DNAH9-containing top (distal) half of the cilium is still moving. Taken together, high-speed video microscopy, tomography and immunofluorescence studies confirm that DNAH11 is localised to the proximal portion of the cilium, and consequently all further tomograms were taken from this region.

Transverse tomograms consistently show a loss of ODA volume in DNAH11 subjects compared to controls

Longitudinal tomograms were found to have a high rate of technical failure (14 out of 26 tomograms) and consequently could not be used routinely. Transverse cross-sectional (plane shown in figure 1c) tomograms were trialled to produce a more robust methodology. All the transverse tomograms collected (n=19) contained dynein arms and could be analysed successfully. A reduction of volume of the ODA was identified in all seven DNAH11 subjects compared to healthy controls (figure 4a). The ODA proportion of the microtubular doublet (MTD) had a median (IQR) of 10.3% (9.3–10.5%) in DNAH11 subjects compared to 13.8% (12.9–14.4%) in healthy controls. Data from patients #2, #3 and #5 and the remaining controls could not be obtained despite multiple attempts, due to technical difficulties with the longitudinal tomography and averaging technique in plastic section. The chart shows a reduction in ODA volume in DNAH11 patients in the proximal region of the axoneme, but similar volumes between patients and controls in the distal region of the cilium.
Discussion

We show that DNAH11 mutations result in a characteristic abnormality of the ciliary ultrastructure detectable by electron tomography, but not traditional TEM. The defect is specific to the “forearm” of the ODA and is detected only at the proximal portion of the cilium where DNAH11 is located.

The transverse section electron tomography technique used in this study is advantageous in that it uses existing fixed embedded samples already stored for diagnosis by standard TEM, thereby avoiding additional sampling. A strength of the technique used in this study is the quantification of results and the use of the MTD volume as an internal control during quantification, to minimise bias introduced by image analysis, such as manual selection of image features and thresholding. Quantification showed no overlap in ODA volume measurements between healthy or PCD controls and DNAH11 subjects, confirming the clear-cut nature of the underlying defect at this imaging resolution. The reliability of the test results suggests that the technique has potential use in the diagnostic pathway for highly atypical PCD cases.

Using immunofluorescence and electron tomography we demonstrate that DNAH11 mutations do not result in loss of the entire ODA. We found that a partial heavy chain of the ODA head region (presumed to be the DNAH5 protein), along with at least part of the remaining ODA structure (NME8-positive), remains intact along the length of the proximal cilia region in patients with DNAH11 mutations. The localisation of these retained structures, coupled with the proximal nature of the DNAH11 loss, provides the likely explanation for the longstanding puzzle of apparently normal cilia structure in DNAH11 patients when using conventional TEM. Figure 5 shows a cartoon to demonstrate the predicted model of the ODA.

![Immunofluorescence showing a proximal axoneme location for DNAH11 in a healthy nasal epithelial cell (green), with DNAH5 running the whole length of the cilium (red). Inset: the top of two further ciliated cells, again showing the proximal location of DNAH11 (green) with acetylated tubulin (red, top image) running the length of the cilia and DNAH9 (red, bottom image) in the distal region. White arrows indicate the position of DNAH11 throughout. b) High-resolution immunofluorescence from patient #2 who carries a frameshift and premature truncation mutation in the gene. DNAH11 (green) is absent from the cilia. Whereas DNAH5 (main image), acetylated α-tubulin (top inset) and DNAH9 (bottom inset) are present throughout the length of the cilia (shown in red). c) An alternative antibody to DNAH11 (red) was used to validate results (Abcam ab126571) and to show presence of DNAH11 in the peripheral airways. Cilia are marked in green with acetylated tubulin. Ciliated epithelium was obtained from a lung resection. d) Cilia are marked by acetylated α-tubulin antibody (red); NME8, a protein predicted to be closely associated with the β-dynein heavy chain, is shown to be retained (green) in an individual with primary ciliary dyskinesia (PCD).]

Figure 3 a) Immunofluorescence showing a proximal axoneme location for DNAH11 in a healthy nasal epithelial cell (green), with DNAH5 running the whole length of the cilium (red). Inset: the top of two further ciliated cells, again showing the proximal location of DNAH11 (green) with acetylated tubulin (red, top image) running the length of the cilia and DNAH9 (red, bottom image) in the distal region. White arrows indicate the position of DNAH11 throughout. b) High-resolution immunofluorescence from patient #2 who carries a frameshift and premature truncation mutation in the gene. DNAH11 (green) is absent from the cilia. Whereas DNAH5 (main image), acetylated α-tubulin (top inset) and DNAH9 (bottom inset) are present throughout the length of the cilia (shown in red). c) An alternative antibody to DNAH11 (red) was used to validate results (Abcam ab126571) and to show presence of DNAH11 in the peripheral airways. Cilia are marked in green with acetylated tubulin. Ciliated epithelium was obtained from a lung resection. d) Cilia are marked by acetylated α-tubulin antibody (red); NME8, a protein predicted to be closely associated with the β-dynein heavy chain, is shown to be retained (green) in an individual with primary ciliary dyskinesia (PCD).
loss in DNAH11 cases. These data arising from 3D tomographic imaging advance our understanding of the cell biology of PCD disease and how it affects the cilia, such that the majority of structures remain completely undisturbed while a highly specific loss of only a portion of the ODA is sufficient to cause ineffective mucociliary clearance and its clinical consequences.

In this study we have shown that transverse section electron tomography can differentiate between DNAH11 and control cases without the need for more complex longitudinal section or cryo-electron tomography techniques. The proximal location of DNAH11 means appropriate selection of a proximal area for transverse electron tomography is crucial for this technique. In the present study the presence of microvilli was used as a marker for proximity to the cell surface and therefore the DNAH11 region; however, this method is imperfect, as occasional tip structures from bent cilia could reside in this region. The clear distinction between control and DNAH11 cases suggest this was not a major limitation in defining a DNAH11 defect in the present study. Other limiting factors of the electron tomography technique is that it is time-consuming, labour intensive and requires specialist training. It can take days to collect, reconstruct and average each tomogram, resulting in only a small number of cross-sections being assessed and precluding its use as a high-throughput diagnostic screening tool. However, high throughput was never our intention, and this technique could be reserved for specialist cases at a small number of.

**FIGURE 4 a)** Data from volume measurements from averaged transverse tomograms from the proximal portion of the axoneme from six healthy controls, seven DNAH11 mutation patients and six subjects with primary ciliary dyskinesia (PCD) due to other defects (HYDIN n=2, CCDC65 n=3 and DRC1 n=1). There is a significant difference between the DNAH11 mutation group and the two control groups. **: p<0.01 (Mann–Whitney U-test). **b)** Representative transverse section tomograms averaged, rendered and false-coloured, showing two different angles for each case: i) the tomographic average of a microtubular doublet (MTD) (blue), from a healthy control; ii) tomographic averages from DNAH11 mutation patient #1, who carries a premature truncation mutation and frameshift mutation; iii) tomographic averages from DNAH11 mutation patient #2, who carries a different premature truncation mutation and frameshift mutation. The predicted location of DNAH5 is coloured red; the green triangles have been added to the images on the right to indicate where DNAH11 is predicted to be missing. **c)** Tomograms from a healthy control as visualised in Chimera showing the bounding box method for quantification of i) the MTDs and ii) the outer dynein arm (ODA), enabling the calculation of the percentage volume of the ODA out of the total MTD volume (used in a)).
reference centres since specimens which have already been embedded can easily be transported between centres. Use of chemically embedded sections and the restricted tomographic tilt range (±65° rather than ±90°) are both factors that limit the full potential of tomographic resolution. The majority of the DNAH11 protein volume when imaging the ODAs in transverse section is in the depth (z-direction), which results in loss of resolution compared to image acquisition in the longitudinal plane. Use of longitudinal sections or cryo-electron tomography would overcome some of the resolution limitations and could thereby further elucidate the structural role of human DNAH11 [20]. However, we found longitudinal tomography technically challenging and were not able to collect a full dataset using this technique (figure 2b).

Cryo-electron tomography is not practical for diagnostic evaluation in clinic or for the review of existing, previously unsolved, clinical samples, due to the requirement for a cryo-electron microscope and the need for immediate preparation of samples following biopsy or culture [12].

Immunofluorescence can be used for confirmation of a DNAH11 defect. However, antibodies for DNAH11 are not widely available and others have shown that the protein remains present in cilia from individuals with PCD caused by pathogenic DNAH11 missense mutations since the staining is indistinguishable from controls [10]. One individual (subject #5) in the present study carries a homozygous missense mutation. Cilia from this subject demonstrate the same 25% tomographic volume loss as seen in the other six subjects carrying predicted null mutations (premature truncation and
frame-shift mutations). We speculate that the ability to detect protein loss arising from missense mutations by electron tomography is due to the superior resolution of the technique compared to immunofluorescence; however, this would need to be verified in further missense variants. It is possible that the difference in ODA volume could represent a conformational change in the dynein rather than a direct loss of the DNAH11 protein. This would provide another possible explanation for loss of volume in a missense mutation, where some protein is predicted to remain present. Interestingly, Dougherty et al. [10] and data in this study have shown that DNAH9 can enter the proximal area of the axoneme in the absence of DNAH11. Our study suggests that any such replacement protein still does not fully occupy the structural defect left by DNAH11 protein.

Therefore, this study provides proof of concept that electron tomography can be used to confirm a diagnosis of PCD, where traditional TEM appears normal. We suggest that electron tomography, due to the requirement for microscopy equipment and its time-consuming nature, could be used in specialist centres in cases where other tests for PCD have been inconclusive and the genetic testing if available rests upon variants of uncertain significance that require functional confirmation (e.g. unpublished missense mutations). The online supplementary material demonstrates an example case study where tomography was used to confirm a diagnosis in a patient with a missense variant in DNAH11.

In summary, we have shown a consistent ultrastructural defect in subjects that carry deleterious DNAH11 mutations once thought to have “normal ultrastructure”. We have shown that the tomography technique has good resolution and accuracy for the detection of PCD defects in DNAH11-deficient patients with apparently normal ultrastructure. Cilia electron microscopy has facilitated the major advances in PCD. Improved resolution and 3D visualisation of tomography has promise to provide important cell biological insights into the causes and variability of PCD along with an improved understanding of the significance of novel gene mutations.

Acknowledgements

We would like to thank the PCD families for their participation in this study and NHS England for support of the UK PCD specialised service. We would like to acknowledge the work of Michael Simpson (King’s College London, London, UK), Lucy Jenkins and Christopher Boustred (NE Thames Regional Molecular Genetics Service at Great Ormond Street Hospital for Children NHS Foundation Trust, London) for genetic analysis. We acknowledge Wellcome Trust award WT991310 to UK10K and are grateful to the UK10K consortium (investigators listed at www.uk10k.org/), in particular the Rare Diseases Group, for whole-exome sequencing of PCD patients. Authors are members of the BESTcilia FP7 and COST Action BEAT-PCD (BM1407).

References


