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CLINICAL & BASIC RESEARCH

Cilia Ultrastructure Associated with Primary Ciliary Dyskinesia in Omani Patients

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Abstract: ***Objectives:*** Primary ciliary dyskinesia (PCD) is a disorder affecting the structure and function of the motile cilia of the respiratory system. Transmission electron microscopy is one method that can be used to examine ciliary ultrastructure in airway biopsies. Although the role of ultrastructural findings in PCD has been described in the literature, this role has not been well-studied in the Middle East or, specifically, Oman. This study aimed to describe ultrastructural features in Omani patients with high suspicion of PCD. ***Methods:*** This retrospective cross-sectional study included 129 adequate airway biopsies obtained from Omani patients attending pulmonary clinics at Sultan Qaboos University Hospital and the Royal Hospital, Muscat, Oman, from 2010 to 2020 who were suspected of having PCD. ***Results:*** Ciliary ultrastructural abnormalities in the current study population were outer dynein arm (ODA) associated with inner dynein arm (IDA) defects (8%), microtubular disorganisation associated with IDA defect (5%) and isolated ODA defect (2%). Most of the biopsies showed normal ultrastructure (82%). ***Conclusion:*** In Omani patients suspected to have PCD, normal ultrastructure was the most common feature.

Keywords: Cilia; Primary Ciliary Dyskinesia; Biopsy; Transmission Electron Microscopy; Ultrastructure; Oman.

Advances in Knowledge

- *Transmission electron microscopy (TEM) is a feasible diagnostic tool for primary ciliary dyskinesia (PCD).*
- *Normal ciliary ultrastructure features are a common finding when using TEM.*
- *Pepsinogen levels are also directly correlated with the risk of rebleeding; higher levels were associated with an increased risk of rebleeding.*

Application to Patient Care

- *A normal ciliary ultrastructure finding does not exclude PCD in Omani patients.*
- *Other tests need to be considered, including genetic testing, if a ciliary ultrastructure finding is normal but symptoms persist.*

Primary ciliary dyskinesia (PCD) is a hereditary disorder affecting the structure and/or function of motile cilia.[1,2] PCD is particularly challenging to manage and research, and diagnosis is typically delayed due to shared clinical features with other diseases including cystic fibrosis, immunodeficiency, chronic pulmonary aspiration, asthma and recurrent respiratory viral infection.[2–4]

The symptoms of PCD are initially observed in organs in which cilia motility is essential for normal function and manifest in organs outside the respiratory tract as well as in sinuses and the lungs.[3] In the respiratory system, PCD-related mucociliary clearance impairment can cause chronic wet cough, recurrent respiratory tract infections, bronchitis and bronchiectasis.[4–7] The effects may vary among patients but what is common is that they never fully resolve, despite administering systemic antibiotics.[4] Outside the respiratory system, PCD patients may suffer from fertility issues and hearing difficulties due to glue ear, and approximately 45–50% have *situs inversus*.[5,7,8] PCD patients may also have inborn heart defects due to *situs ambiguus*.[6]

The official American Thoracic Society (ATS) clinical practice guidelines for the diagnosis of PCD recommend testing for PCD if two clinical PCD phenotypes are present.[4] The recommended testing methodologies are the examination of ciliary ultrastructure using TEM, genetic testing, nasal nitric oxide (nNO) measurement in children five years of age or older and high-speed video microscopy (HSVM).[3,7,8] Although HSVM is useful for accessing ciliary beat frequency and its pattern and length, such testing is limited to specialised PCD centres.[4]

Ultrastructural studies of ciliary axonemes using TEM remains one of the most widely used and reliable diagnostic methods for PCD.[9] However, using this diagnostic process is challenging, because it may not be easy to obtain an adequate sample with a sufficient number of cilia that are technically acceptable for interpretation.[3,10] Nevertheless, using TEM to identify a consistent ultrastructural abnormality

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within the ciliary axoneme helps expedite disease management, as it indicates a definite diagnosis.[11] Ciliary ultrastructural features, including the location of the central pair complex, the availability of the dynein arms, orientation of peripheral microtubules and epithelial cells abnormalities are definitive clues that lead to a PCD diagnosis.[5,10,12]

International guidelines for reporting PCD using TEM were established to regulate and direct the diagnostic efforts.[10] According to these guidelines, ciliary ultrastructure can be classified as normal or as having class 1 or class 2 defects.[10] Normal ultrastructure is defined as the presence of the well-known 9 + 2 axonemal structure with a clear identification of the outer dynein arms (ODA), inner dynein arms (IDA) and the central microtubules in the middle of the axoneme [Figure 1]. Class 1 defects are considered hallmark defects (i.e. diagnostic), while class 2 defects may possibly be used to indicate a diagnosis of PCD if the findings are consistent across multiple samples.[10] In this case, and if clinical symptoms are persistent, it is required to confirm the diagnosis using other modes of testing, for example, HSVM or genetic testing.[10]

Class 1 or hallmark defects can include isolated loss of ODA, or combined ODA and IDA absence from >50% of cross-sections. However, when it is <50% (i.e. 20–50%), it is referred to as a class 2 defect. In addition, microtubular disorganisation combined with IDA defects is considered a class 1 defect, while microtubular disorganisation when IDA is present is referred to as class 2 defect.[10] Additionally, the central complex defect and the mislocalisation of basal bodies with few or no cilia are also considered class 2 defects.[10]

PCD is no longer considered a mild disease and further research is needed to expedite PCD management in order to prevent complications from the disease. Therefore, this study aimed to determine the most common ciliary ultrastructural defects in Omani PCD patients and to use those results to assist in patient management.

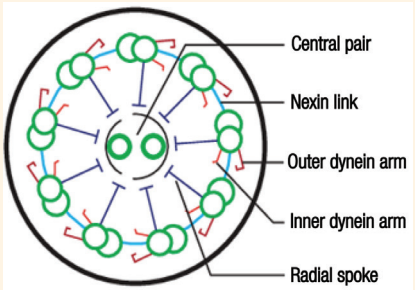

Figure 1: A schematic diagram of the cross-section of a motile cilium illustrating the normal 9 + 2 microtubular structure. The nine fused doublet microtubules with outer and inner dynein arms are arranged in the outer periphery surrounding a pair of singlet microtubules in the middle of the ciliary axoneme. The central pair is surrounded by a central sheath and radial spokes are radiating between them and the outer doublets. *From: Ishikawa H, Marshall WF. Intraflagellar Transport and Ciliary Dynamics. Cold Spring Harb Perspect Biol 2017; 9:a021998. https://doi.org/10.1101/cshperspect.a021998.*

Methods

This retrospective cross-sectional study included all airway biopsies sent to the Electron Microscopy Unit (EMU) at Sultan Qaboos University (SQU) from 2010 to 2020. This sample included biopsies from all Omani patients who were highly suspected of having PCD, based on clinical phenotypes and symptoms. Specimens were taken from patients ranging between one month and 70 years of age. These patients had presented to pulmonary clinics at SQU Hospital (SQUH) and the Royal Hospital (RH) with at least two out of four ATS-defined PCD symptoms. These symptoms included recurrent chest infections, wet, productive cough, the presence of laterality defects and neonatal respiratory distress.[4] In addition, two adequate normal control samples were obtained from two healthy adult candidates.

Samples were considered adequate if screening 50 cross cilia were possible using the TEM. Samples of adequate airway biopsies from highly suspected PCD Omani patients were all included, if they met the inclusion criteria. They were included if the patients presented with at least two of the following symptoms: recurrent chest infections with no response to antibiotics or laterality defects or respiratory distress during early infancy or year-round wet cough. After reviewing the patients' clinical charts, all inadequate samples were excluded as were samples from patients diagnosed with conditions other than PCD.

Samples had been collected using the following steps: nasal airway biopsies were taken in outpatient clinics. The clinicians obtained the specimens by scraping the nasal inferior turbinate using either a brush or rhino-pro curette. Specimens were received in Karnovsky's fixative and then transferred into a sodium cacodylate buffer and kept at 4°C. Specimens were then fixed in Osmium tetroxide, washed in distilled water and dehydrated using a series of graded acetone. The dehydrated specimens were infiltrated in a mixture of acetone and araldite resin, embedded in freshly prepared pure araldite resin and polymerised at 60°C overnight. The control specimens were processed following the same protocol.

The blocks containing ciliated cells were cut using a diamond knife and thin sections were placed on copper grids. Sections were stained using supersaturated Uranyl acetate and Reynolds' lead

Table 1: Distribution of cilia ultrastructure findings detected by transmission electron microscopy from 2010 to 2020 among Omani patients (N = 129)

Finding	n (%)
Ultrastructural defect	
Class 1	
ODA and IDA defects	10 (8)
Microtubular disorganisation with IDA defect	7 (5)
ODA defect	2 (2)
Class 2	
Central complex defect	3 (2)
Microtubular disorganisation with IDA present	1 (1)
Normal ultrastructure	106 (82)

ODA = outer dynein arm; IDA = inner dynein arm.

citrate. In total, 50 cross cilia from each sample were screened at high magnification using a JEOL JEM-1230 TEM at 80 KV (JEOL Ltd., Tokyo, Japan). Images were captured using a Gatan MSC SI003 1 digital camera system (Gatan Inc., Pleasanton, California, USA) and analysed.

Medical ethics approval was obtained to include all airway biopsies for ciliary ultrastructural examination from 2010 to 2020 from SQU's EMU. The research was approved by the Medical Research Ethics Committee (MREC), College of Medicine and Health Sciences at Sultan Qaboos University (MREC #2089), and the Scientific Research Committee (SRC) at the Royal Hospital, Ministry of Health, Sultanate of Oman (SRC #23/2020).

Results

A total of 421 airway biopsies were received and processed during the study period, out of which 129 biopsies (31%) were adequate. From the adequate samples, 114 were from individuals between one month and 18 years old, and only 15 were from patients above 18 years old. These samples were taken from patients from different regions of the country.

Image analysis was done following the ATS international guidelines for reporting PCD using TEM.[10] Out of the 129 adequate samples, 23 (18%) showed alterations in the ciliary ultrastructure [Table 1]. The absences of ODA and IDA were the most frequently observed abnormalities in the studied group (n = 10, 8%) [Figure 2], followed by the microtubular disorganisation associated with IDA defect (n = 6, 5%). Both of these abnormalities are considered class 1

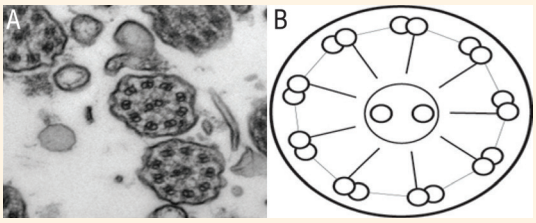

Figure 2: A: An electron micrograph at ×100000 magnification of a class 1 defect showing the absence of both outer dynein arm (ODA) and inner dynein arm (IDA) in the 9 + 2 ultrastructure of the ciliary axoneme from an adequate patient's sample. **B:** A schematic diagram of the cross-section of a motile cilium illustrating the absence of both ODA and IDA.

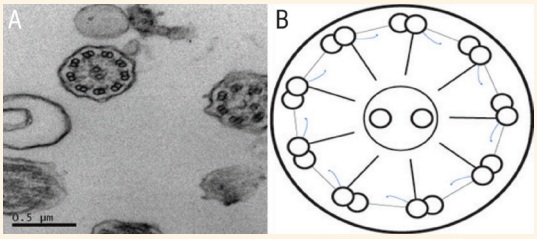

Figure 3: A: An electron micrograph at ×150000 magnification showing a class 1 defect where the outer dynein arm (ODA) are missing from the ciliary axoneme, while the inner dynein arm can be seen. **B:** A schematic diagram of the cross-section of a motile cilium illustrating the absence of ODA.

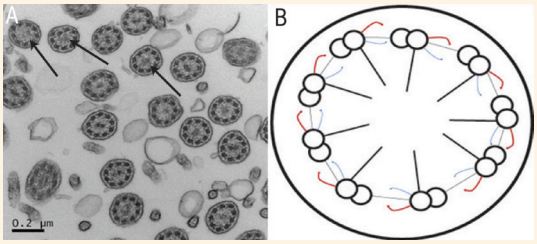

Figure 4: A: Electron micrograph at ×80000 magnification of a class 2 defect. The majority of the cilia showed the absence of a central complex (black arrows). However, outer dynein arm and inner dynein arm can be identified in those cilia. **B:** A schematic diagram of the cross-section of a motile cilium illustrating the absence of the central complex.

defects. The least common class 1 ultrastructural defect was the isolated absence of ODA (n = 3, 2%) [Figure 3]. Additionally, some samples (n = 3, 2%) showed central complex defects [Figure 4] and one sample (n = 1, 1%) showed microtubular disorganisation without IDA defect. These types of defects are classified as class 2 defects and require another mode of testing (e.g. testing for genetic mutations) to confirm PCD diagnosis.

Most of the sample (n = 106, 82%) showed normal ultrastructure of the ciliary axoneme [Figure

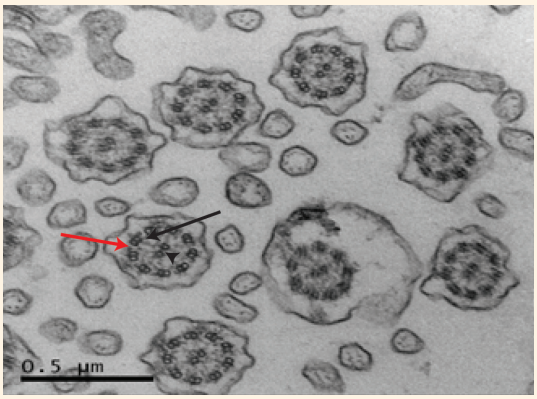

Figure 5: Electron micrograph at ×120000 magnification showing normal 9 + 2 ultrastructure of the ciliary axoneme from an adequate patient's sample. The outer dynein arm (red arrow), inner dynein arm (black arrow) and central complex (black arrow head) can be clearly identified.

5]. On review, it was found that all of these individuals had fulfilled the ATS clinical criteria for testing; PCD was highly suspected due to their presentation with at least two out of the four PCD clinical phenotypes. Furthermore, 65 patients (50%) from this group had a negative sweat chloride test and 57 (44%) had a negative workup for immunodeficiency. However, 37 patients (29%) were not tested for immunodeficiency or sweat chloride for clinical reasons.

Discussion

Ciliary ultrastructure analysis requires special expertise. Analysts are required to be knowledgeable of normal versus abnormal ciliary structures and TEM availability.[13] It is important that healthcare institutions overcome these challenges, however, because this type of analysis is essential to the process of diagnosing PCD.[8,9] TEM is feasible in approximately 70% of PCD patients, but TEM alone is not sufficient to establish a reliable diagnosis.[14] In the current study, most of the studied samples showed normal ultrastructure under TEM (n = 106, 82%). Other researchers have reported similar findings.[7,13,14] For example, Papon *et al.*'s study found that more than half of their PCD-positive sample showed normal ultrastructure.[14]

Other researchers' findings, and those of the current study, suggest the potential role of gene mutations in causing normal ciliary ultrastructure.[7,11,15–17] *DNAH11* gene mutations, for example, have been found to cause PCD but are associated with normal ciliary ultrastructure.[16] These mutations affect the structural proteins, and subsequently the function of ODA, but the structure still appears normal through a TEM.[16] The *HYDIN* autosomal recessive gene mutation is also associated with a normal ultrastructure.[18] This gene is involved in the production of proteins for the central pair complex.[18]

Due to high rates of consanguinity in Oman, it is expected that certain PCD-associated genes are predominant in the Omani population. If the most common PCD-associated gene mutations in this region are associated with normal ciliary ultrastructure, then this may explain the current study's results; however, genetic testing is needed to confirm this theory. In Omani cases of suspected PCD, it is recommended to re-evaluate clinical phenotypes in individuals who show repetitive normal ciliary ultrastructure. If symptoms of PCD persist, then other diagnostic investigations are highly recommended to confirm PCD. A similar recommendation should be followed if the ultrastructural features suggest class 2 defects. Unlike class 1 defects, a final diagnosis of class 2 defects requires confirmation of disease by applying a further test, for example, genetic testing.[10]

Defects of ODA and IDA and microtubular disorganisation combined with IDA defects were among the ciliary ultrastructural abnormalities reported in the current study – both of which are considered class 1 defects and confirm PCD.[10] In the current study, ODA associated with an IDA defect were reported in 10 patients (8%) and microtubular disorganisation associated with an IDA defect was reported in seven patients (5%). On the other hand, an isolated ODA defect was reported in only 2% (n = 3) of the studied group. In these cases, PCD diagnosis was confirmed using TEM and while testing for gene mutations causing these abnormalities was an option, it was not a priority.[10]

The high inadequacy rate of the samples submitted for TEM analysis was a challenge for the current study. However, inadequacy might also indicate a specific cause of PCD. It is now well-known that if multiple specimens from the same patient all show a low percentage of or no cilia in the epithelial cells, then it may indicate specific PCD gene mutations.[18] This type of finding indicates that something is not right in the ciliogenesis process. In such cases, re-evaluating the clinical presentation is highly recommended. If symptoms persist with no other explanation, then genetic testing for PCD to explore certain gene mutations, such as in the protein coding *CCNO* or *MCIDAS* genes may be considered.[18] Future research should examine possible genetic mutations in Omani PCD patients and correlate them with the clinical and ultrastructural phenotypes of patients.

Conclusion

Based on the findings of this study, it is recommended to perform ciliary ultrastructural studies for PCD patients in Oman. If class 1 defects are identified, early PCD management might limit or even prevent lung damage due to disease complications. In this case, genetic testing is optional and may not be necessary, unless required for family planning. The percentage of cases diagnosed using TEM was not high, but TEM cannot exclude PCD upon normal ultrastructure findings nor when multiple specimen inadequacy is observed within the same patient. At this point, a combination of tests are required to confirm PCD including TEM, genetic testing, nNO and HSVM.

AUTHORS' CONTRIBUTION

KAA carried out the electron microscopy laboratory work related to this research and prepared some of the samples, then screened them, captured the images and analysed the results of the research under the supervision of the team. TB was the primary supervisor for this research and reviewed the ultrastructure of the cilia and helped in the results analysis. MAR authorised the final reports of the ultrastructure for the patients included in this research. AAA and HAK were the co-supervisors for this research and participated in the analysis of results. HAK was also one of the clinicians who participated in the analysis of the clinical features for the patients included in this research. NAS and KAS were the clinicians who reviewed the clinical features and participated in the setup of this research. All authors approved the final version of this manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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