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Inner dynein arm defects causing Primary Ciliary Dyskinesia: Repeat testing required

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ABSTRACT

Background: Primary ciliary dyskinesia (PCD) results in chronic nasal symptoms and chest disease leading to bronchiectasis. We noted a number of patients referred for diagnostic testing whose initial results suggested PCD due to an inner dynein arm or radial spoke defect but on retesting no abnormality was found.

Methods: An audit of all patients referred for PCD diagnostic testing over a three year period whose initial electron microscopy (EM) and beat pattern analysis suggested an inner dynein arm or radial spoke defect.

Results: Twenty-one patients referred for diagnostic testing for PCD suspected of an inner dynein arm defect and six suspected of a radial spoke defect on initial EM and beat pattern analysis had repeat testing performed. On repeat testing five patients initially suspected of an inner dynein arm defect and one with a radial spoke defect had normal electron microscopy and beat pattern leading to the initial diagnosis being questioned.

Conclusion: Patients suspected of PCD due to an inner dynein arm defect or radial spoke defect should have the diagnosis reassessed if based on only one diagnostic sample.

Keywords:

Bronchiectasis; Cilia; Inner dynein arm defect; Primary ciliary dyskinesia: PCD; Radial spoke defect

INTRODUCTION

Primary ciliary dyskinesia is a congenital disorder with a prevalence of between 1:2,500 to 1:30,000 live births [1-3]. It causes impaired mucociliary clearance due to motionless or dyskinetically beating cilia leading to chronic upper and significant lower respiratory tract disease [4-6]. Specialist diagnostic testing is required to make the diagnosis and the diagnosis of PCD can be difficult [7].

PCD is associated with a number of different ultrastructural defects of the ciliary axoneme that result in defective ciliary function [4] and specific ultrastructural defects have been shown to produce characteristic ciliary beat patterns [8]. Currently two diagnostic techniques: the analysis of ciliary beat pattern [9] and frequency and transmission electron microscopic analysis are recommended as the principal diagnostic tests for patients suspected of PCD [4].

However, assessment of ciliated epithelial samples can be difficult, particularly if there is underlying epithelial damage. Even in cilia from healthy ciliated epithelium, electron microscopic analysis can problematic, particularly for the analysis of inner dynein arms because of their low contrast on electron microscopy [10].

Because of concerns with regards to the possibility of false positive diagnoses, we repeated diagnostic testing of cases where a positive diagnosis of PCD was suggested from analysis of ciliary function and electron microscopy on the initial brush biopsy. We noted that occasionally when an inner dynein arm defect was suspected on electron microscopy and functional analysis, and a subsequent sample was taken, ciliary function and electron microscopy were normal, excluding the diagnosis. This was also noted in a patient who was suspected of having a radial spoke defect on their initial sample. A radial spoke defect is a combination of an inner dynein arm defect where there is also disarrangement of the peripheral and central microtubules of the ciliary axoneme. In contrast, patients whose initial sample suggested PCD due to either an outer dynein arm defect, a combined inner and outer dynein arm defect, or a transposition defect, repeat testing confirmed the diagnosis in each case. In this paper we audit the patients referred to our PCD diagnostic centre over the last three years where an inner dynein arm defect or radial spoke defect

was suspected on examination of the first epithelial brush biopsy to determine the proportion of cases where the diagnosis was excluded on subsequent biopsies.

METHODS

The audit consisted of patients referred to one of the National Diagnostic Centres for PCD (Leicester) in the UK over a three year period where a diagnosis of an inner dynein arm defect or a radial spoke defect was strongly suspected from analysis of the initial biopsy.

An inner dynein arm defect was defined as a complete absence of all inner dynein arms or where all inner dynein arms were present universally as very short stub-like projections as opposed to the fully developed arm. In a radial spoke defect, again there is an absence of inner dynein arms associated with disarrangement of the microtubules within the ciliary axoneme allowing the displacement one of the outer doublets inwards towards the central pair.

Ciliated samples were obtained by brushing the nasal epithelium with a 2 millimetre cytology brush (Olympus Keymed Ltd, UK). When the nasal brush biopsy was taken, all patients had reported that they had not had a symptomatic acute upper respiratory tract infection for at least six weeks.

Ciliary beat frequency and ciliary beat pattern analysis were performed as described previously using a digital high-speed video camera (Kodak Motioncorder Analyser 1000: Eastman Kodak, Rochester, NY) [8, 11] or the Motion Pro X4 digital high-speed video camera (Lake Image Systems, USA).

Ciliary ultrastructure assessment

Samples were processed for transmission electron microscopy for ultrastructural analysis of the ciliary axoneme [12, 13]. In summary, EM sections were examined in a methodical grid square search. The health and condition of cells was noted at low power (x 5,000). Cells were then observed under medium power (x 66,000) to assess for the quality of the cross-sections. Sections that had been cut in the best alignment were suitable for microtubular and dynein arm assessment. A high power examination (x 250,000) of the cilia from each

cell was assessed for the presence and absence of dynein arms and for microtubular structure and arrangement. Data was recorded on all of the cross-sections seen. Whenever possible, at least 10 individual cells were studied. The percentage of cilia with dynein arm microtubular defects was recorded [12].

Ciliary function assessment was blind to the results of EM analysis, and vice versa as is the standard practice in our referral centre.

NO measurement

Nasal Nitric Oxide n[NO]: n[NO] was measured using a chemiluminescence analyser (Sievers 280i, Analytix, UK) with a transnasal flow rate of 2 litres per minute following the ATS guidelines [14]. n[NO] was measured in patients attending the Leicester clinic who and had patent airflow across the nasopharynx and, due to difficulty of the restricted exhaled breath manoeuvre, were over five years old [14].

Air liquid interface cell culture

We have described this methodology in detail previously [15]. Briefly, nasal brush biopsy samples were grown on Collagen (0.1%, Vitrogen, Netherlands) coated tissue culture trays (12 well) in Bronchial Epithelial Growth Media (BEGM, Lonza, USA) for 2-7 days. The basal cells were seeded on a collagen coated 12mm diameter transwell clear insert (Costar, Corning, USA) under BEGM for 2 days. The basal cell monolayer was fed on the basolateral side only with ALI-media (50% BEGM and 50% Hi-glucose minimal essential medium containing 100nM retinoic acid). When cilia were observed on the cultures of difficult patients (with a high dyskinesia score on the original brush biopsy) or suspected PCD patients, they were physically removed from the transwell insert by gentle scraping with a spatula and washing with 1ml of HEPES (20mM) buffered medium 199 containing penicillin (50µg/ml), streptomycin (50µg/ml) and Fungizone (1µg/ml). The recovered ciliated epithelium was then dissociated by gentle pipetting. 100µl of the cell suspension was placed in a microscope chamber slide and ciliary beat frequency and pattern assessed as described above. The remaining 900µl was fixed in glutaraldehyde for TEM analysis of

the axoneme structure. Cell culture is done in all samples that are suspected of having PCD [15].

RESULTS

Over a three year period 724 patients were referred for diagnostic testing for PCD. Twenty one of the patients referred were suspected of PCD due to an inner dynein arm defect and 6 due to a radial spoke defect on initial sampling. Repeat diagnostic sampling of these patients, on one further occasion, did not confirm the diagnosis in six cases (22%). The confirmed radial spoke and inner dynein arm cases are described below followed by a more detailed description of the cases where a repeat biopsy gave normal results, excluding the diagnosis of PCD.

We had previously noted that although some patients with suspected inner dynein arm defects and radial spoke defects showed no abnormalities on retesting this was not the case for other common phenotypes of PCD. Because of this not all of the patients with other defects were retested during the study period reported in this paper. However, twenty seven patients diagnosed with other PCD phenotypes (18 with an outer or outer plus inner dynein arm defect out of a total of 52 patients diagnosed with these defects: 9 out of 9 patients diagnosed with a of transposition defect) had repeat biopsies that in each case confirmed the original diagnosis.

Confirmed radial spoke defects: Six cases of a radial spoke defect were diagnosed, five in children (ages 1 month to 9 years) and one in a 60 year old. All children had daily respiratory symptoms from the first year of life and nasal symptoms from early infancy. Although all were born after 36 weeks gestation, three had been admitted to a special care baby unit following delivery because of respiratory distress. One patient had glue ear causing hearing difficulties and two patients under one year of age were awaiting hearing assessment. Two patients had situs inversus. One of the patients with situs inversus also had transposition of the great arteries and a ventricular septal defect. The mean ciliary beat frequency was 8.6 (range 3-13) Hz on the first biopsy and 8.3 (range 5-12.5) Hz on the second biopsy. Nasal nitric oxide was low at 48, 59 and 5 parts per billion, in the three patients old enough to be tested. Electron microscopy revealed an absence of inner dynein

arms and disarrangement of the peripheral and central microtubular pairs, typical of a radial spoke defect, on both biopsies.

Confirmed inner dynein arm defects: Fifteen cases of an inner dynein arm defect were diagnosed in fourteen children (ages 1 to 15 years) and one in a 30 year old. All had daily respiratory symptoms from the first year of life and nasal symptoms from early infancy. Although all were born after 36 weeks gestation, six had been admitted to a special care baby unit following delivery because of breathing difficulties. Ten patients had glue ear causing hearing difficulties and five had had grommets inserted. Six patients had situs inversus. One patient also had a ventricular septal defect and polysplenia. The mean ciliary beat frequency was 10.4 (range 4-17) Hz on the first biopsy and 9.9 (range 5-16) Hz on the second biopsy. Nasal nitric oxide was measured in 11 patients and in all but one patient (NO =142 ppB) was below 50 ppB. Electron microscopy revealed an absence of inner dynein arms on both biopsies. Cell culture to a ciliated phenotype was possible in eight cases confirming an absence of inner dynein arms and a stiff beat pattern of all cilia observed.

Diagnosis of PCD unlikely on retesting: In six cases the initial sample suggested an inner dynein arm defect (n=5) or a radial spoke defect (n=1) on both beat pattern analysis and transmission electron microscopy. In four of the initial samples the epithelium obtained was recorded as being unhealthy. The details of these patients are outlined in Tables 1 and 2.

DISCUSSION

In 27 patients out of 724 patients referred for diagnostic testing for PCD over a three year period, the initial epithelial brush biopsy suggested a possible diagnosis of an inner dynein arm defect or a radial spoke defect. In all cases the biopsy was repeated and in six cases (22%), five with suspected inner dynein arm defect and one with a radial spoke defect, the diagnosis was excluded considered unlikely by the presence of a normal ciliary beat pattern and a normal ciliary ultrastructure on electron microscopy. These results strongly suggest that any patient suspected on initial testing of an inner dynein arm defect or an inner dynein arm defect associated with a radial spoke defect should have their diagnosis confirmed by repeat testing. In PCD due to an outer

dynein arm defect, a combined inner and outer dynein arm defect or transposition defect repeat biopsies have proved identical to the initial sample on each occasion.

The reasons for the change in the initial beat pattern and EM phenotype are unclear. In four of the initial samples, the epithelium obtained was recorded as being unhealthy, however with enough ciliated edges to allow analysis to proceed. However, in two cases the epithelium appeared healthy. It is known that damage to the epithelium, for example following a viral upper respiratory tract infection can affect ciliary function and electron microscopic appearance of cilia [13, 16-18]. Although we do not biopsy patients who have reported a symptomatic upper respiratory tract infection within the previous six weeks, we know that a proportion of people with a viral infection may show significant damage to the ciliated respiratory epithelium without being aware of any symptoms [13].

Four of the initial samples had been couriered from peripheral units and examined within eight hours of the initial brushing. It was noted that the epithelium appeared unhealthy in these samples but sufficient ciliated edges were seen to allow analysis. We have previously observed that couriered samples may have a higher incidence of secondary ciliary dyskinesia [19] and it is possible that this may have contributed to our initial findings in two cases. However, two of the patients who were siblings and who were seen together at the diagnostic centre on both occasions, were suspected of an inner dynein arm on the initial sample. The respiratory epithelium obtained on both occasions appeared healthy.

Cell culture to a ciliated phenotype was possible in eight cases with isolated inner dynein arm defects and in one case with a radial spoke defect. In all but one case culture confirmed the initial suspicion of the underlying ciliary defect. In one case, where an inner dynein arm defect was suspected on examination of the initial biopsy, ciliary structure and function of the cultured epithelium was normal. Cell culture, however, is a highly specialised technique and our success rate of culturing nasal ciliated epithelium from patients suspected of PCD using at an air liquid interface method is only 54% [15]. If cell culture results were successful from all of the initial biopsies this would have proved helpful. Unfortunately cell culture was only successful on one of the five patients suspected of an inner dynein arm defect on their initial biopsy. When this patient's ciliated culture was examined the ciliary beat pattern was found to be normal and inner dynein arms were clearly seen. This again suggests a secondary problem in the initial sample.

The clinical presentation of patients with PCD is well described in adults [6] and in children [9]. From the clinical history diagnostic testing was strongly indicated in five of the six patients whose repeat testing was normal. These patients had a history of a chronic wet sounding or productive

cough from infancy and chronic nasal symptoms. The other patients had similar respiratory problems but only intermittent nasal symptoms. It is of interest, however, that none of these patients had situs inversus and none required admission to a special care baby unit because of respiratory distress following birth. In the group where the diagnosis was confirmed on retesting, situs inversus was observed in 33% and 43% had been admitted to a special care baby unit because of respiratory distress after birth.

Various studies have shown that levels of exhaled nitric oxide, particularly nasal nitric oxide, are very low in patients with PCD [6, 20, 21]. None of the patients where the diagnosis of PCD was excluded on retesting had their nasal nitric oxide measured when their initial sample was taken. One patient was too young and in one case there was a technical failure relating to the measurement. The remaining four samples were couriered from peripheral units where measurement of nasal nitric oxide is not available. When measured in three of the patients during repeat diagnostic testing their nasal nitric oxide was within the normal range, again suggesting the initial suspicion of PCD was incorrect [5]. However, a few patients with PCD have been found to have nitric oxide levels within the normal range [20]. Indeed one of our patients with findings consistent of an inner dynein arm defect on initial and subsequent sampling had a nasal nitric oxide within the normal range.

We have not repeated the nasal brushing of the six patients where the diagnosis of PCD was initially suspected and later considered unlikely to determine if dynein arms were again impossible to see and ciliary function suggested an inner dynein arm defect. Therefore, we were not able to comment on whether the inner dynein arms could be temporarily knocked down in certain situations, for example following a viral infection. These patients all had chronic respiratory symptoms and we do not know if the inner dynein arm defect maybe intermittent and reappear representing an unusual ciliary phenotype.

It is acknowledged that inner dynein arms are more difficult to observe due to the decreased repeats along the ciliary axoneme compared to the outer dynein arms and Escudier and colleagues [10] have suggested the use of computer assisted analysis to help detect inner dynein arm abnormalities. However, such analysis is not widely used. Evaluation of this technique in other centres and the development of other methods to enhance visualisation of inner dynein arms are required.

As previously noted the use of ciliary beat frequency alone to screen for biopsies requiring further assessment by electron microscopy may result in diagnoses being missed. This is true for a

proportion of the patients in this study where the beat frequency was above 11 Hz, a cut off previously suggested above which electron microscopy was not indicated [22].

In summary, the results of this audit suggest that patients in whom primary ciliary dyskinesia has been diagnosed or is suspected due to an inner dynein arm defect alone, or an inner dynein arm defect combined with a radial spoke defect, should be retested to avoid the possibility of a false-positive diagnosis. We currently repeat diagnostic testing of patients who were suspected of an inner dynein arm defect or radial spoke defect and those who have an unusual phenotype of PCD and those whose initial biopsy shows significant secondary damage making interpretation difficult.

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Table 1: Clinical details of patients whose initial epithelial brush biopsies suggested an isolated inner dynein arm defect or an inner dynein arm defect associated with a radial spoke defect.

Patient	Suspected diagnosis on initial sample	Age	Neonatal Respiratory Distress	Nasal Symptoms	Cough	Ear Problems	Situs Inversu
1	Inner dynein arm defect	3	Nil	Rhinorrhea from birth	Continuous wet cough from birth	Glue ear and grommets	Nil
2	Inner dynein arm defect	15	Nil	Rhinorrhea first year	Continuous wet cough from first year	Nil	Nil
3	Inner dynein arm defect	6	Nil	Rhinorrhea from birth	Intermittent wet cough	Glue ear (right nasal polyp)	Nil
4	Radial spoke defect	7	Nil	Intermittent rhinorrea	Continuous wet cough from first year	Nil	Nil
5	Inner dynein arm defect	3	Nil	Rhinorrhea from birth	Continuous wet cough from birth	Nil	Nil
6	Inner dynein arm defect	2	Nil	Rhinorrhea from birth	Continuous wet cough from birth	Nil	Nil

Table 2: Analysis of ciliary beat frequency, beat pattern and transmission electron microscopy of patients whose initial epithelial brush biopsies suggested an isolated inner dynein arm defect or an inner dynein arm defect associated with a radial spoke defect.

Patient	Suspected defect on initial sample	Condition of initial epithelial sample	Ciliary Beat Frequency (Confidence Interval) Hz			Ciliary Beat Pattern			
			Sample 1	Sample 2	Cell Culture Sample	Sample 1	Sample 2	Cell Culture Sample	Sar
1	Inner dynein arm defect	Unhealthy	10.7 (10.4-11.1)	12.9 (12.7-13.2)	12.6 (12.3-12.7)	Stiff 100%	69% normal		96% inner arms
2	Inner dynein arm defect	Unhealthy	4.6 (4.1-5.1)	13.4 (13.0-13.8)		Stiff 100%	100% normal		92% no in dyne (159/
3	Inner dynein arm defect	Unhealthy	7.6 (7.0-8.2)	11.2 (10.9-11.6)	10.4 (9.8-10.8)	Stiff 100%	51% normal	65% normal	96% no in dyne (160/
4	Radial spoke defect	Unhealthy	10.6 (10.2-11.1)	12.7 (12.4-12.9)		Stiff 100%	57% normal		96% no in dyne (232/
5	Inner dynein arm defect	Healthy	11.0 (10.7-11.3)	12.5 (12.0-13.0)	9.8 (9.4-10.2)	Stiff 100%	63% normal	71% normal	97% no in dyne (240/
6	Inner dynein arm defect	Healthy	9.4 (9.0-9.9)	12.7 (12.5-12.9)		Stiff 100%	69% normal		96% no in dyne (192/