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The Evolving Spectrum of Ciliopathies and Respiratory Disease

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Abstract

Purpose of review—Research on the biology of cilia, complex hair-like cellular organelles, has greatly informed our understanding of its crucial role in respiratory health and the pathogenesis of Primary Ciliary Dyskinesia (PCD), including the genetics behind this condition. This review will summarize the current state of the art in the field highlighting its clinical implications.

Recent findings—The genetics of PCD have exploded over the past few years as knowledge acquired from model systems has permitted the identification of genes that are key components of the ciliary apparatus and its function. In addition clinical criteria and diagnostic tools have emerged that are permitting more clear identification of affected individuals.

Summary—The rate of progress in the field continues to accelerate through international collaborative efforts and standardization of methods. Although the genetics behind PCD are complex given the large number of genes associated with disease as well as the large number of possible mutations even at the individual gene level, this knowledge is rapidly translating in improved diagnostics and hopefully in the near future in the identification of potential therapeutics.

Keywords

Primary Ciliary Dyskinesia; cilia; ciliopathies; Nasal Nitric Oxide

Introduction

Primary ciliary dyskinesia (PCD) is a heterogeneous, predominantly autosomal recessive genetic disease that is the result of impaired ciliary function and defective mucociliary clearance in the respiratory tract. As a direct consequence of this defect, patients with this disorder are prone to serious respiratory infection and develop progressive obstruction and infection of the bronchial airways, as well as recurrent infections of the middle ear and paranasal sinuses. The first clinical description of PCD can be credited to the Russian physician A.K. Zivert, who in 1904 reported on a case of *situs inversus totalis* complicated by chronic sinusitis and bronchiectasis(1). It was not until 1933 that Manes Kartagener in his studies on the etiology of bronchiectasis, reported on a case series of subjects with similar manifestations and thus establishing what became known as the Kartagener's triad of *situs inversus totalis*, chronic sinusitis and suppurative bronchiectasis as a distinct clinical entity(2)(Figure 1). Although in the following decades a large number of cases accumulated making the association clearly recognized, as well as its recessive inheritance pattern, the

etiology for the syndrome remained elusive. It was not until 1976 that B. Afzelius(3) and H. Pedersen (4) working independently reported on axonemal ultrastructural abnormalities demonstrated by Electron Microscopy (EM) leading to lack of motility in the sperm tail (Afzelius) and respiratory cilia (Pedersen) as the basic defect in affected patients. Soon after, Afzelius followed this report with a recognition that individuals with defects in their ciliary structure could also present with *situs solitus*, thus expanding on the phenotype associated with the disease(5). Later it also became apparent that in some affected patients immobility in their cilia could not be demonstrated but rather a disordered and ineffective pattern of motion(6), which made the preferred term to describe affected patients as a distinct disease entity as Primary Ciliary Dyskinesia (PCD). It was not until 1999 that the first gene associated with ciliary dysfunction in humans was reported (a defect in the outer dynein arm component DNAI1)(7). Through the investigation of the human orthologs of genes involved in the assembly and function of the ciliary apparatus in protozoans (8) as well as the development of model organisms(9, 10), we are now witnessing an explosion of knowledge as to the genetic determinants of ciliary function and those genes associated with human disease(11).

Biology of Respiratory Cilia

Cilia are hair like complex organelles composed primarily of a backbone of microtubule arrays and multiple other proteins required for its structure, function and maintenance(12). Although its basic structure is evolutionarily well conserved across multiple species, in human cells 4 different types of cilia can be distinguished based on their basic microtubular structure and motility (13). Most cells contain a single non-motile cilium (monocilium or primary cilium), considered a sensory organelle, which has 9 pairs of microtubules (“9+0” arrangement) and absence of dynein arms. The second type can be found in the embryonic nodal cilia (key for determining laterality), which have a similar microtubular arrangement to the primary cilium but in contrast do have dynein conferring a rotational motion. Thirdly, highly specialized cells like the respiratory epithelium and the ependymal lining have a large number of motile cilia with 9 pairs of microtubules arranged around a central pair (“9+2” arrangement, Figure 2). The fourth type is found in the kinocilium of the hair cells in the inner ear, which also have a 9+2 microtubular arrangement, but are devoid of motion. All 4 types of cilia play key roles in the development and function of organ systems, so not surprisingly there is increased recognition of a multitude of complex conditions associated with unique defects in any of these. Then, beyond PCD, there is a growing number of conditions where defective ciliary function is being demonstrated to be the primary defect behind the phenotypes observed (14). As a result, there is a large number of disorders that fall under what has become known as the ciliopathy spectrum. As many of these do have associated defects in the motile cilia, they will also be associated with significant respiratory morbidity. For example, patients with X-linked retinitis pigmentosa from mutations in the RPGR gene have been reported with sensory hearing deficits and features of PCD(15, 16), an X-linked oral-facial-digital type I syndrome has been described from mutation in the OFD1 gene that had as features mental retardation, macrocephaly and ciliary dysfunction(17), and a reported patient with cranioectodermal dysplasia from mutation in

the WDR35 gene had recurrent respiratory infections with demonstrated defects in ciliary motility (18).

The crucial role that an intact ciliary apparatus has for effective mucociliary clearance in the respiratory tract is now well established (19). The airways are endowed with a number of innate mechanisms for protection against invasion by microorganisms. The mucosal surface constitutes the first line of defense against environmental offenders and by itself provides an effective barrier. In addition, the epithelium maintains and tightly regulates an array of defense mechanisms. These include an apical surface fluid layer and an apposed protective mucus barrier, and mixed in these are a number of peptides and small molecules with both anti-infective and immunodulatory activities. This barrier is kept in an almost continual motion towards the larynx by the activity of the apical ciliary brush. The coordinated motion of the cilia creates a smooth wave that permits the unidirectional flow of the mucus layer and its contents for effective clearance(20). This allows for the elimination of inhaled trapped particles, debris, and microorganisms. The function of the ciliary apparatus is finely regulated and is responsive to changes in the airway surface. The cilia themselves have a sensory ability through receptors on its surface, like the bitter taste receptors (T2Rs), which trigger signaling events to modulate ciliary beat (21). In vitro studies utilizing sophisticated imaging techniques have demonstrated an autoregulatory mechanism where ciliary beating is increased by a calcium-dependent pathway when a load of mucus causes a reduction in the periciliary liquid layer and ciliary brush height. This increase in ciliary beating in turn will result in the mobilization of the mucus mass and restoration of baseline conditions (22). To complement this finding, more recently it has been demonstrated that the transport of particles of different sizes by the cilia can also occur in the absence of mucus and without eliciting other cellular responses. However, if clearance fails and particles accumulate, then mucus release will result as well as increasing the ciliary beat frequency to effect particle clearance(23). The regulation of ciliary beat frequency itself is quite complex and the result of intracellular signaling through second messengers. These include cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), (24) as well as nitric oxide signaling (NO)(25). Additional insight has been provided by the finding that in the absence of the protein Anterior Gradient 3 (AGR3, a member of the Protein disulfide isomerase family) calcium-dependent regulation of Ciliary beat frequency in airway cells in response to ATP stimulation is defective (26). These findings demonstrate that defects other than those already reported in the biogenesis and structural components of the ciliary apparatus are also likely to be involved in defective ciliary function and could be associated with human disease.

Features of Primary Ciliary Dyskinesia

Despite the significant morbidity associated with PCD and its characteristic clinical features, it is notoriously challenging to diagnose by standard approaches and a high index of clinical suspicion is required. Given the severe defect in an important innate mechanism of defense in the lungs and its congenital nature, it is likely that pulmonary manifestations are present from early in life and progress during early childhood. However, delays in diagnosis despite typical clinical features are commonplace, with a reported mean age at diagnosis in a large European series of around 5 years of age(27). In this same study it was also apparent that

delays in diagnosis were more prominent in countries with lower levels of health care expenditures. This delay in diagnosis possibly allows for end organ irreversible damage to occur, in the lungs represented by the development of bronchiectasis. Longitudinal studies suggest that although there might be an advantage from early diagnosis (28, 29), evidence for significant lung disease can be found early in life(30) and its natural history is that of a progressive loss of pulmonary function as the patients age(31). This certainly stresses the importance of both an early and accurate diagnosis. However, there are recognized challenges in arriving at a conclusive diagnosis. It has already become apparent though the study of large cohorts of patients that the diagnostic test long held as the 'gold standard' based on the examination of airway epithelial cells by EM to evaluate ciliary structure can correctly identify only 50–60% of the patients. The use of EM as diagnostic test for PCD comes from the early descriptions of outer dynein defects found in affected patients(3, 4). However, the use of EM evaluation of respiratory mucosal biopsies as a diagnostic strategy has not been formally subjected to rigorous assessment as to its performance metrics. In part this is the result of the inherent difficulties in the procurement of adequate specimens, its correct processing, and as importantly its interpretation. In a multicenter study of PCD patients assessed through a network of centers with well validated standardized procedures it was noted that close to 90% of the specimens were of adequate quality for evaluation, but with a lower success rate for children (85%) and also better success rates seen only at the most experienced sites(32). An additional caveat to consider pertains to the frequent occurrence of secondary changes in cilia as a result of injury to the respiratory tract, and primarily in association with acute infection(33). Further, besides the sub-group of patients with defects in their outer dynein arms, EM has limitations given the spectrum of possible ciliary defects that can result in PCD. There are many defects that will be difficult if not impossible to demonstrate by EM exclusively, so a normal EM does not necessarily exclude the possibility of PCD. Some defects such as those introduced by mutations in the radial spoke protein RSPH1 produce very subtle defects that can only be demonstrated with highly sophisticated techniques such as cryo-electron tomography and thus not available on a clinical basis(34). Other defects such as mutations in the DNAH11 gene do not result in defects in the axonemal structure detectable by EM and produce subtle alterations in ciliary beat with a hyperkinetic pattern recognizable only by high-speed videomicroscopy (35). As an additional complimentary diagnostic tool, immunofluorescence (IF) directed at specific axonemal proteins has emerged as a valuable tool. To follow on the case of DNAH11, a recent report delineates a specific IF staining pattern for the normal DNAH11 distribution in the distal axoneme that clearly identified subjects with a defective gene (36). This points to the potential utility of IF as a confirmatory strategy for the presence of specific defects in suspected affected patients (37, 38).

A second strategy that has been frequently utilized to evaluate for the possibility of ciliary dysfunction involves the direct assessment of ciliary beating under light microscopy. Freshly obtained ciliated cells, typically from the mucosal surface of the nasal inferior turbinates as they are easily accessible, are prepared in a wet mount and the ciliary beating frequency (CBF) is assessed by a variety of methodologies. However, as already mentioned, in some instances the CBF could be assessed as normal and only an analysis of the beating pattern (CBP) can reveal the presence of abnormalities. This is certainly the case for defects in

radial spoke arms (rotational pattern) or DNAH11 (hyperkinetic pattern), where the subtlety of the defects can be easily overlooked. Cilia normally beat at a frequency that ranges from 10 to 15 Hz. This will entail for a full cycle of forward propulsion followed by backward recovery to be completed in 66 – 100 msec. Then only capturing the ciliary motion with the use of high-speed video recording at sampling rates in the order of 400 – 500 frames per second allows capturing the full cycle of motion with sufficient detail to be able to discern defects. High-speed video microscopy (HSVM) protocols for the evaluation of ciliary function have been reported and demonstrated of value as a true functional assay for the identification of defective ciliary function in support of a PCD diagnosis(39). Normative data and performance metrics have been formally assessed in support of the methodology(40). However, in addition to its being available only through highly specialized centers, acute infection has a substantial effect on the measurements(41, 42) so repeated testing to demonstrate repeatability of the defect in the individual patients is recommended(39). It is also recognized that the motility patterns identified in patients with specific defects can be quite diverse and are related not just to the gene affected but also the mutation identified(43, 44). Attempts at automating the interpretation through the use of computerized algorithms for both the analysis of CBF(45) and CBP (46, 47) have been reported and await validation through larger studies. However, these types of assays are at this time available only through highly specialized centers, where not only the procedures can be correctly performed but also its interpretation. As the performance metrics and standardization of HSVM are further developed, it is foreseeable that images could be acquired at regional programs for later analysis of the digitized images at centralized interpretive centers. The development of such a physiologic based biomarker will not only allow for better diagnostics but will also open the door to establish the methodology to more effectively screen for drugs that could potentially help restore normal function.

Genetics of Primary Ciliary Dyskinesia

Given the recognized limitations with the different microscopy methodologies available and the increasing genetic information associated with PCD, it is becoming quite apparent that genetic testing could play a prominent role in the diagnostic evaluation of patients suspected of PCD (48, 49). In fact, at this time there are 36 different genes that have been associated with ciliary dysfunction (Table 1).

This list is likely to grow as it is estimated that several hundred genes are involved in the correct assembly and function of the ciliary apparatus (12). Then not surprisingly, in a cohort of well characterized patients with PCD only 28% were found to carry mutations in the DNAH5 gene and at this time it is estimated genetic testing can probably identify at best 60–70% of the affected individuals, though this could potentially be augmented by the application of strategies such as whole-exome sequencing(78). Ongoing efforts to better define the ability of genetic panels to aid in the diagnosis of PCD include assessing the performance metrics of testing by applying next generation sequencing to a panel of 32 genes associated with PCD ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02389049) NCT02389049).

Nasal Nitric Oxide and Primary Ciliary Dyskinesia

A simpler tool that has proven of great value in the correct identification of affected individuals constitutes the measurement of nasal NO production. Ciliary function is known to be regulated by NO and epithelial cells produce NO through epithelial NO synthases (NOS) (79). In contrast to healthy controls, epithelial cells from PCD patients are known to be defective in their ability to induce NOS2 expression and produce NO under infectious challenge(79). In the nasal cavity large concentrations range of NO close to the ppm can be detected, with it predominantly arising primarily from the sinuses(80). Low concentrations of nasal NO have been for long recognized among patients with PCD (81) and ATS/ERS has provided recommendations for the measurement conditions(82). Recently, through a large collaborative effort in North America utilizing a standardized operating procedure a disease specific cut-off of 77 nL/min was identified. This lower limit of normal provides good confidence in the separation of affected patients from controls, including diseased controls. The only exception to this is among patients with CF who as a population do demonstrate a degree of overlap with the values seen in patients with PCD(83). A second group utilizing a breath hold maneuver for sampling has reported utilizing an arbitrarily defined lower cut-off for increased sensitivity, but also demonstrating increased sensitivity and specificity by combining the nNO results with other diagnostic procedures such as HSVM and EM analysis of cilia biopsies(84). However these studies included only children older than age 5 years old, in part due to the requirement to exhale through a mouthpiece resistor to maintain expiratory flow at a level around 50 mL/sec while nasal gas is sampled. Normative data for younger children and infants have not been established, though there is preliminary single center data that suggests the use of nasal NO determinations could be extrapolated to this patient population(85). With the multiple challenges and limitations encountered with the different available diagnostic methodologies there is an identified need to better define clear cut diagnostic criteria for PCD. Work conducted through the North American Genetic Disorders of Mucociliary Clearance Consortium (GDMCC) on well-defined and characterized cohorts of patients with a confirmed diagnosis of PCD have identified an early onset of respiratory morbidity as a cardinal feature for this condition(86). Further, unexplained neonatal respiratory distress has emerged as a strong criterion for clinical diagnosis (87). From this information and the classic phenotypic features a set of criteria can be defined for application in the clinical setting to screen patients for the possibility of PCD (Table 2). Then, the application of clinical criteria alone has excellent discriminatory power to identify subjects at risk that merit more detail evaluation for the possibility of PCD(88). These clinical criteria in combination with nasal NO measurements provides good confidence in excluding PCD as a possibility, and thus avoiding unnecessary additional testing as well as refocusing the diagnostic evaluation.

Conclusion

Rapid progress continues to be made in the understanding of the pathophysiology of PCD, in great part thanks to the translation of knowledge gained from basic studies in model systems. In addition, the identification of genes associated with PCD will likely continue to expand. Perhaps of most importance for the clinician, validated clinical criteria have emerged that permit the identification of patients with high likelihood of being affected.

With the combination of all these efforts PCD has certainly become a paradigm for genomic and translational medicine.

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Conflicts of interest

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Key Points

Translation of basic cilia biology from model systems has permitted the expansion of the genetics of PCD.

Although still a challenging diagnosis, increasing efforts at the validation of diagnostic tools is providing a clearer picture.

Product of large collaborative efforts, clinical criteria are now available that allow for the identification of affected subjects with good confidence.

nasal NO will be considered highly suggestive of PCD. For any age group, if nasal NO is part of criteria, CF must be confidently excluded. (Adapted from (60)).

Figure 1.

Kartagener's triad in a patient with PCD. A. *situs inversus totalis* as seen in a chest radiogram. B. Chronic pansinusitis demonstrated in a coronal view of a sinus CT. C. Diffuse bronchiectasis demonstrated in an axial cut of CT of the chest. D. Ciliary biopsy EM demonstrating absence of the outer dynein arms (120,000 $\times$ magnification).

Figure 2.
Structure of the normal respiratory cilia axoneme (9+2) in cross-section.

Table 1

Genes associated with PCD.

Gene name	Defect	Reference
<i>ARMC4</i>	ODA	(50)
<i>C21orf59</i>	ODA	(51)
<i>CCDC103</i>	ODA	(52)
<i>CCDC114</i>	ODA	(53)
<i>CCDC151</i>	ODA	(54)
<i>CCDC164 (DRC1)</i>	<i>Nexin-Dynein Regulatory Complex</i>	(55)
<i>CCDC39</i>	IDA	(56)
<i>CCDC40</i>	IDA	(57)
<i>CCDC65 (DRC2)</i>	<i>Nexin-Dynein Regulatory Complex</i>	(51)
<i>CCNO</i>	<i>Ciliogenesis</i>	(58)
<i>DNAAF1 (LRRC50)</i>	ODA + IDA	(59)
<i>DNAAF2 (KTU)</i>	ODA + IDA	(60)
<i>DNAAF3</i>	ODA + IDA	(61)
<i>DNAAF5 (HEATR2)</i>	ODA + IDA	(62)
<i>DNAH1</i>	IDA	(63)
<i>DNAH11</i>	Normal Structure	(35)
<i>DNAH5</i>	ODA	(64)
<i>DNAH8</i>	ODA	(65)
<i>DNAI1</i>	ODA	(7)
<i>DNAI2</i>	ODA	(66)
<i>DNAL1</i>	ODA	(67)
<i>DYX1C1 (DNAAF4)</i>	ODA + IDA	(68)
<i>GAS8 (DRC4)</i>	<i>Nexin-Dynein Regulatory Complex</i>	(69)
<i>HYDIN</i>	Central Apparatus	(70)
<i>LRRC6</i>	ODA + IDA	(71)
<i>MCIDAS</i>	Ciliogenesis	(58)
<i>NME8 (TXNDC3)</i>	ODA	(72)
<i>OFD1</i>	Ciliogenesis	(17)
<i>RPGR</i>	Transitional Zone	(16)
<i>RSPH1</i>	Radial Spoke Head	(73)
<i>RSPH3</i>	Radial Spoke Head	(74)
<i>RSPH4A</i>	Radial Spoke Head	(75)
<i>RSPH9</i>	Radial Spoke Head	(75)
<i>SPAG1</i>	ODA + IDA	(76)
<i>WDR35</i>	Intraciliary Transport	(18)
<i>ZMYND10</i>	ODA + IDA	(77)

ODA = Outer dynein arm, IDA = Inner Dynein Arm.

Table 2

Clinical criteria for the diagnosis of PCD. Upper panel presents the 4 clinical criteria and the lower panel age-dependent considerations. The combination of clinical criteria and

PCD Major Diagnostic Criteria:	
1. Unexplained neonatal respiratory distress (term or near term) with lobar atelectasis or collapse and/or need for respiratory support with CPAP and/or oxygen for > 24hours	
2. Laterality defect- situs inversus totalis, situs ambiguous or heterotaxy	
3. 3A. Daily, year-round wet cough starting in first year of life AND / OR	
3B. Bronchiectasis on chest CT	
4. 4A. Daily, year- round nasal congestion starting in first year of life AND / OR	
4B. Pansinusitis on sinus CT	
Age Dependent Diagnostic Considerations:	
Newborns (up to 1 month of age)	
<ul style="list-style-type: none"> Situs abnormality (situs inversus or heterotaxy) AND Unexplained neonatal respiratory distress in the first week of life with lobar atelectasis or collapse by imaging and/or need for respiratory support with CPAP or prolonged oxygen need (> 2days) AND / OR Diagnostic ciliary EM ultrastructural defects 	
Children under 2 years of age:	
<ul style="list-style-type: none"> 3 or more major criteria but including neonatal respiratory distress. <i>Nasal NO not included at this age.</i> 	
AND / OR	
<ul style="list-style-type: none"> Diagnostic ciliary EM 	
Children 2–5 years of age with chronic respiratory symptoms	
<ul style="list-style-type: none"> 2 or more major criteria (including neonatal respiratory distress); plus nasal NO during tidal breathing <50 nl/min on 2 occasions >1month apart OR 3 or more major criteria (including neonatal respiratory distress); plus nasal NO during tidal breathing <50 nl/min (on 1 occasion) AND / OR Diagnostic ciliary EM 	
Children 5–18 years of age with chronic respiratory symptoms	
<ul style="list-style-type: none"> 2 or more major criteria; plus, nasal NO during plateau <77 nl/min (breathing against resistance) AND / OR Diagnostic ciliary EM 	
Adults (18 years of age)	
<ul style="list-style-type: none"> Bronchiectasis; plus, at least 1 other major criteria and nasal NO during plateau <77 nl/min (breathing against resistance) 	
AND / OR	
<ul style="list-style-type: none"> Diagnostic ciliary EM 	
AND / OR	
<ul style="list-style-type: none"> Bronchiectasis; plus, laterality defect 	