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# Picking up speed: advances in the genetics of primary ciliary dyskinesia

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## Abstract

Abnormal ciliary axonemal structure and function are linked to the growing class of genetic disorders collectively known as ciliopathies, and our understanding of the complex genetics and functional phenotypes of these conditions has rapidly expanded. While progress in genetics and biology has uncovered numerous cilia-related syndromes, primary ciliary dyskinesia (PCD) remains the sole genetic disorder of motile cilia dysfunction. The first disease-causing mutation was described just thirteen years ago, and since that time the pace of gene discovery has quickened. These mutations separate into genes that encode axonemal motor proteins, structural and regulatory elements, and cytoplasmic proteins that are involved in assembly and preassembly of ciliary elements. These findings have yielded novel insights into the processes involved in ciliary assembly, structure, and function, which will allow us to better understand the clinical manifestations of primary ciliary dyskinesia. Moreover, advances in techniques for genetic screening and sequencing are improving diagnostic approaches. In this manuscript, we will describe the structure, function, and emerging genetics of respiratory cilia, review the genotype-phenotype relationships of motor ciliopathies, and explore the implications of recent discoveries for diagnostic testing for primary ciliary dyskinesia.

## INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare disease of children, and was the first human disease linked to cilia dysfunction. Nearly four decades have passed since the disease was linked to ultrastructural defects of the axoneme (1, 2). Following that time, and particularly in the last decade, there has been an explosion in knowledge related to cilia. Progress in cilia biology has been accompanied by the recognition that a large number of previously uncharacterized syndromes in children are the result of ciliary defects. This review will



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summarize major areas of progress in cilia-related disease, focusing on the genetics of PCD and areas for future investigation.

## CILIA STRUCTURE AND FUNCTION

Cilia are present on the surface of most cells, and the basic axonemal structure is evolutionarily conserved. Cilia have historically been segregated into motile or primary (or sensory) types, and based on patterns of microtubule structure, fall into three general classes: motile “9+2”, motile “9+0”, and non-motile “9+0” (FIGURE 1). Several lines of evidence suggest that this classification system may be overly simplistic as their molecular features and functions overlap (3, 4).

Cilia are anchored to the cytoplasm by a basal body, a specialized centriole located at the base that localizes the cilium in the extracellular space and organizes cilia assembly. Each cilium consists of hundreds of proteins arranged around a scaffold of α- and β-monomers of tubulin arranged into helical protofilaments in the recognized pattern of paired microtubules (FIGURE 2). The central fibrillar structure, or axoneme, is covered by a membrane continuous with the plasma membrane. Major strides have been made in understanding how the structural and functional elements of cilia are synthesized and assembled within the cytoplasm and moved to the basal body prior to transport into the cilium. A well-described process called intraflagellar transport (IFT) mediated by microtubule-associated motor proteins, kinesin and dynein, moves proteins cargos to and from the ciliary axoneme for the construction and maintenance of cilia (5). The mechanism by which proteins move from the cytoplasm to the apical domain of the ciliated epithelial cell for the proper construction of the cilium, and how this process is disrupted in disease still needs to be defined.

Motile cilia are highly conserved to provide cell locomotion, fluid movement, and sexual reproduction. In humans, they line the upper and lower surface of the respiratory tract (including the Eustachian tubes), ependymal cells in the brain ventricles and spinal canal, as well as the Fallopian tubes. The flagellum of male spermatazoa has a similar axonemal structure. Cilium beating is generated by motor proteins that bind the nine outer microtubule doublets and interact with the central pair (central apparatus). Information regarding the motor proteins was first deduced from electron microscopic (EM) analysis of these dynein motors in the biflagellate green algae, *Chlamydomonas reinhartii* (6). Ultrastructural appearance and the localization of the dynein proteins relative to the microtubules have led to the term “inner” and “outer” arms to describe the different sets of motors. Subsequent biochemical analysis in alga and other organisms have revealed that the arms are complex structures, consisting of several heavy, intermediate, and light dynein chains that attach to the A microtubule, and contain ATPases. Nexin links, which are components of the dynein regulatory complex, connect adjacent outer microtubular doublets, and with the radial spokes, control the sliding of cilia (7).

The result is a ciliary stroke and beating at approximately 8-20 Hz under normal conditions that contributes to a coordinated mucociliary wave moving with intra- and inter-cellular synchrony. The regulation of motile cilia movement can be influenced by changes in the ciliary microenvironment, such as the depth and viscosity of the apical surface fluid, redox

conditions and various infections and pollutants (including cigarette smoke) leading to an acquired ciliopathy (8-10). Thus, acquired or genetic disruption to the coordinated movement of motile cilia can result in ineffective mucociliary clearance and lung disease.

Distinct from motile cilia are primary (sensory) cilia, which are solitary structures present during interphase on most cell types. In mammals, sensory cilia are present on well-differentiated epithelia of sensory organs, biliary ductules, renal tubules, chondrocytes, and astrocytes. For years, these structures were considered vestigial remnants of little or no physiological significance (11). However, over the past fifteen years it has been shown that primary cilia are important signaling organelles and sense the extracellular environment. Cilia detect mechanical stimulation, chemosensation, and in specialized cases, changes in light, temperature, and gravity (12, 13). In addition, development, growth, and repair functions are mediated by primary cilia through surface receptors, including sonic hedgehog (SHH), epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor (PDGFR) (14, 15). Given this range of function, genetic defects in primary cilia have been associated with a growing number of clinically diverse pediatric conditions, collectively known as ciliopathies (16).

Motile cilia also have sensory functions, and syndromes caused by mutated proteins that overlap between motile and primary cilia are reported. For example, blindness due to mutations in the retinitis pigmentosa GTPase regulator gene (*RPGR*) is associated with motile cilia dysfunction (17). Further overlap in function is suggested by finding the expression of the polycystic kidney disease genes in motile cilia and an association with bronchiectasis in those individuals with cystic kidney disease (18, 19). Motile cilia on human respiratory epithelia possess several members of the family of bitter taste receptors, identical to those in the tongue and nose (20). These sensory properties likely allow the motile cilia to adjust their movement in response to changes in their immediate environment.

The nodal cilia are a third distinct class of cilia, but exist only transiently in the ventral node of the gastrula during embryonic development. Nodal cilia share a similar “9+0” microtubule arrangement as primary cilia, but contain dyneins to provide motility (21, 22). The leading hypothesis as to the function of the nodal cilia posits that the node includes two populations of cilia: motile and sensory (23, 24). Through this arrangement, the motile cilia spin and generate leftward flow of extracellular fluid across the nodal surface that is then detected by sensory cilia, and transformed to a biochemical signal that activates a cascade of transcription and growth factors to establish body sidedness (25). Defects within the nodal cilia pathways cause left-right laterality defects, such as *situs inversus totalis*, *situs ambiguus*, and heterotaxy associated with congenital heart disease, asplenia, and polysplenia (26, 27). These findings have led to searches for ciliary anomalies in children with congenital heart disease to uncover the genetic cause of these defects (28).

## DIAGNOSIS OF PRIMARY CILIARY DYSKINESIA: CHANGING STRATEGIES

Advances in cilia biology has been accompanied by rapid improvements in the diagnosis of the motile cilia dysfunction syndrome of PCD. In this regard, existing diagnostic studies, including transmission electron microscopy (EM) and cilia beat frequency, are expected to

give way to nasal nitric oxide (NO) measurements and genetic testing. Recognition of clinical manifestations, which include neonatal respiratory distress, laterality defects, persistent infantile rhinitis, and recurrent upper and lower respiratory tract infections, continues to be the most important indication for diagnostic testing. Ciliary dysfunction leads to impaired mucociliary clearance and chronic airway infections, which results in progressive airway obstruction, atelectasis, and bronchiectasis, even early in life. Other manifestations of PCD include infertility and rarely, perinatal hydrocephalus. For a broader review, the reader is referred to several recent reviews that summarize the different clinical aspects of PCD (17, 29).

The use of EM for the diagnosis of PCD dates to the original description of dynein arm defects in four subjects (1). Since then, ultrastructural defects have been the cornerstone for diagnosing PCD (29). Dynein arm defects account for 90 percent of cases with defined ultrastructural abnormalities. However, there are many limitations to the use and interpretation of EM. Ciliary defects can be acquired, and careful interpretation of the ultrastructural findings is necessary, since nonspecific changes may be seen related to exposure to environmental pollutants or infections. Chronic inflammation and infection common in patients with PCD often precludes collection of an adequate epithelial biopsy with abundant ciliated cells required for analysis. Even when this limitation is overcome, high quality tissue processing and ultrastructural analyses require sophisticated imaging equipment and expertise that are only available in few centers. Lastly, newly discovered disease-causing gene mutations cause functional defects not associated with a consistent ultrastructural defect (30). Thus the presence of normal axonemal ultrastructure does not exclude PCD, which underscores the limitation of using EM as a sole diagnostic criterion.

High-speed videomicroscopy has been advocated as a diagnostic test for PCD for measurement of cilia beat frequency and waveform analysis. The normal beat frequency of cilia obtained from healthy individuals ranges between 8-14 Hz, and varies with testing temperature and handling (31). Waveform analysis is an emerging method that may be reliable for diagnosing PCD (32), especially as more automated methods are introduced, but this approach currently lacks objective measures that can reliably be employed by clinicians. Ciliary motion in airway epithelial samples from PCD subjects can widely vary, from near immotility to subtle changes in beat pattern or frequency, all leading to ineffective mucociliary clearance. High quality videomicroscopy is available only at a limited number of research centers, and requires sophisticated software and expertise in analysis. This approach should be relegated to research only. Most importantly, inspection of motor cilia using standard light microscopy is *never* sufficient to support the diagnosis of PCD.

Perhaps the most important diagnostic advance for PCD is the use of nasal NO measurement as a screening test. Nasal NO level measurements are sensitive and specific for the diagnosis of PCD, and are currently part of the diagnostic criteria routinely employed by several centers in Europe and the United States (33, 34). Low levels are also observed in some individuals with cystic fibrosis, thus emphasizing the importance of excluding this diagnosis when considering PCD. The precise relationship between the motile cilia and nasal NO level is unclear, though the proximity of several regulatory enzymes to the ciliary basal bodies provides clues regarding their involvement in regulating cilia motility (35, 36).

Experimentally, NO can be detected in cells during normal cilia beating (37). Nitric oxide synthase-2 and 3 (NOS2 and NOS3) have been found to be markedly reduced in subjects with PCD, and related mechanisms of NO metabolism have been implicated (38, 39).

## GENETICS OF PRIMARY CILIARY DYSKINESIA: AN EMERGING CLASSIFICATION SYSTEM

In addition to the identification of cilia-related proteins, advances in techniques of genetic screening and sequencing are leading the way for the use of genetic testing as a diagnostic tool. PCD is usually inherited as an autosomal recessive trait, though rare cases of autosomal dominant and X-linked transmission have been reported (40, 41). PCD is highly heterogenic owing to the large number of proteins involved in cilia assembly, and a growing number of genes have been implicated in disease. These mutant genes encode proteins that are involved in axonemal motors, structure and regulation, or ciliary assembly and preassembly (TABLE 1). The rate of discovery of new PCD genes has accelerated during the past two years. It is estimated that known PCD-causing mutations account only for about 60% of known PCD cases, but considering the pace of new discoveries, it is reasonable to expect that this percentage will increase.

The identification of PCD-associated genes has relied on a combination of experimental models, proteomic analysis, and sequencing of candidate genes (17, 29, 42-44). The best-studied model of motile cilia is the alga, *C. reinhartii* (45, 46). Screening algae with defective motility or abnormal flagellar structure has been routinely used to study the function of orthologous ciliary proteins, some of which were linked to human disease (17, 29, 42-44, 47). The second major advance leading to PCD gene identification was the collection of ciliogenesis related transcriptomes and cilia proteomes that provided lists of cilia genes that could be linked to newly found disease mutations (48). More recently, massive parallel sequencing to analyze regions of interests in the genome has allowed more rapid identification of multiple new mutations in cohorts of PCD subjects without prior knowledge of candidate genes (49). Approaches that previously took months can now be completed in weeks or days. In addition, whole exome sequencing has the potential to unravel the genetic causes of rare diseases, and was recently successfully used to identify new candidate genes associated with PCD (50-52).

To date, mutations in 19 different genes have been linked to PCD (TABLE 1), and more candidates are being verified. Early searches for PCD-related mutations have focused on genes that encode proteins integral to axonemal structure and function; outer dynein arm: *DNAH5*, *DNAI1*, *DNAL1*, *DNAI2*, *TXNDC3* and *DNAH11*; inner dynein arm and axonemal organization: *CCDC39*, *CCDC40*, *CCDC164*; central apparatus and radial spokes: *RSPH9*, *RSPH4A*, and *HYDIN*. More recently, mutations in several genes coding for several cytoplasmic proteins not found in the axoneme have been linked to PCD. These proteins are presumed to have roles in cilia assembly or protein transport, and mutations lead to ultrastructural abnormalities: *HEATR2*, *DNAAF1*, *DNAAF2*, *DNAAF3*, *CCDC103*, *LRRC6*, and *CCDC114* (30, 42-44, 50-68). Little is known about most of these proteins, but their association with PCD has contributed to advances in our knowledge of cilia biogenesis.

Most PCD-causing mutations have been identified in components of the ciliary axoneme and mutations in two genes in particular, dynein axonemal intermediate chain 1 (*DNAI1*; MIM 604366) and dynein axonemal heavy chain 5 (*DNAH5*; MIM 603335) that encode components of the outer dynein arm, account for more than 30% of all cases (17, 69). *DNAI1* was the first disease-associated gene to be identified using a candidate gene approach, relying on screening algae for abnormal flagellar beat and outer dynein arm defects. *DNAI1* mutations were found in a large cohort at a prevalence of 9% of all identified PCD subjects (70). Mutations in *DNAH5*, another component of the outer dynein arm, were discovered using homozygosity mapping in large affected endogamous families (71), and later identified by sequencing in PCD subjects (53). Similarly, homozygosity mapping in consanguineous families has been used to identify mutations in other structural proteins, including the radial spoke head proteins 9 (*RSPH9*; MIM 612648) and 4A (*RSPH4A*; MIM 612647), both associated with absence of the central pair and motility defects (58, 72). Other examples of mutated axonemal proteins associated with PCD discovered using the candidate gene approach include intermediate dynein chain *DNAI2* (MIM 605483) (60), which comprise about 2% of PCD patients (55), and *TXNDC3* (MIM 607421) (56).

Mutations in the dynein axonemal heavy chain 11 (*DNAH11*; MIM 603339) gene, that encodes an outer dynein arm protein, present with an intriguing phenotype (30, 73). Unlike other PCD-causing mutations, it is not associated with an ultrastructural defect and cilia have normal (or more rapid) beat frequency. It is presumed that an abnormal waveform results in ineffective mucociliary clearance. Mutations in other genes, such as coiled-coil domain containing proteins *CCDC39* (MIM 613798) and *CCDC40* (MIM 613799), produce inconsistent ultrastructural abnormalities characterized by disordered microtubules in some but not all cilia, which underscores the clinical observation that current diagnostic testing will miss some PCD cases.

Several non-structural cilia-associated proteins have been found to be mutant in PCD individuals and result in absent outer and inner dynein arms. These mutations involve proteins that are considered to function in cilia assembly or “preassembly” pathways. Identification of the involvement of these proteins has spurred research into the mechanisms of cilia biogenesis. To understand the role of these mutations, cilia biogenesis can be conceptually divided into the following processes: intra-axonemal transport, transfer into the cilium, and cytoplasmic preassembly.

As an organelle, the cilium projects into the environment and is uniquely isolated from the cell. Transfer of proteins from the cytoplasm into the cilium is limited by the periciliary diffusion barrier, which separates the ciliary membrane from the cell membrane (74, 75). Direct access to the cilium is guarded by a ciliary pore complex, a structure analogous to nuclear pores (76, 77). Located at the base of the cilium, the complex surrounds the basal body and its associated proteins. It is believed that proteins targeted to the cilium from the Golgi gain access by employing a ciliary localizing sequence (CLS), similar to the nuclear localizing sequence (NLS), as was described for KIF17 (78). Once through the ciliary pore, proteins move to a second regulatory region called the transition zone in the proximal part of the ciliary axoneme. Providing transport through this compartment and into cilia are

numerous well-known IFT proteins, which move essential protein cargos anterograde and retrograde along the length of the cilium (5). Precisely how specific proteins are coded for precise delivery and assembly within the cilium and how the transition zone functions as a gate is not yet known.

Several gene mutations in preassembly proteins have been found to cause PCD. The first PCD-associated preassembly protein found was DNAAF2 (MIM 613190) (43), which was identified in mutant *Oryzias latipes* and later in PCD subjects who had complete absence of outer dynein arms. Localized within the apical region of the cell cytoplasm, DNAAF2 belongs to the proteins interacting with Hsp90 (PIH) family and interacts with DNAI2 and the chaperone heat shock protein HSP70 to facilitate assembly or dynein complex transport into the cilia (43, 79). Similar to DNAAF2, MOT48 is a PIH protein localized in the cell body that was identified in the *C. reinhartii* ida10 strain that display motility defects, and is associated with preassembly of both outer dynein arms and a subset of inner dynein arms, likely needed for the stability of dynein heavy chain components. Mutations in the related proteins DNAAF1 (LRRC50; MIM 612517) (42, 56) and DNAAF3 (MIM 614566) (44) caused outer dynein arm defects in subjects with PCD. Evidence for assembly roles of these proteins is substantiated in PCD-mutant cells where components of the inner dynein arm were found to accumulate in the cytoplasm of mutated cells and fail to move into the cilium. These findings indicate the existence of a multistep cytoplasmic assembly pathway.

Another preassembly protein with mutations causative of PCD is HEATR2 (MIM 614864), which was recently implicated in dynein arm assembly (50). Similar to other preassembly factors, HEATR2 is expressed in the cytoplasm of ciliated cells. However, HEATR2 is diffusely expressed in the cytoplasm rather than in the apical region, which suggests it either functions at different stages of dynein assembly or is part of a chaperone complex that facilitates transfer of different dynein complexes along an “assembly line.” This role was supported by the finding that HEATR2 mutations are associated with mislocalization of inner arm proteins. Other proteins with HEAT-containing repeats are implicated in ciliary and nuclear import (78), suggesting that HEATR2 can potentially have similar mechanisms.

Mutations in *LRRC6* (MIM 244400) also lead to PCD (66, 67). A member of a protein family with diverse functions, including splicing factors and nuclear transport (80), LRRC6 is found in the cytoplasm (67) and co-localizes with basal body markers. Mutations in LRRC6 were shown to down-regulate expression of other dynein arm proteins and indicate an additional regulatory role. Thus, dynein arm preassembly is intricately regulated by both positive and negative feedback mechanisms.

## AREAS FOR FUTURE STUDY IN PRIMARY CILIARY DYSKINESIA AND CONCLUSIONS

As described previously, newer techniques hold promise for discovery of additional PCD-associated mutations and genetic screening for PCD. Massive parallel sequencing has been used to analyze regions of interests and in the absence of candidates, whole exome sequencing have been used to successfully identify new candidate genes associated with PCD (50-52). These advances, together with the rapid identification of genes associated with

PCD seen over the last two years, have the potential to revolutionize the diagnostic testing and lead to earlier identification and treatment of affected children. There is every expectation that soon we will be able to identify the genetic cause for PCD in most suspected cases, which would potentially allow massive genetic screening of all PCD-associated genes in suspected individuals. The use of emerging technologies such as DNA microchips may facilitate the commercialization of such tests. It is envisioned that these approaches will be used in combination with clinical symptoms and nasal NO levels to diagnose patients with PCD.

The lack of an extensive mapping of mutations in PCD has hampered our ability to define a genotype-phenotype relationship. Moreover, the heterogeneity of disease and compound allelic mutations further complicate pinning specific phenotypes on unique mutations. The creation of specialized clinics in pediatric academic centers for the diagnosis and management of affected individuals with PCD in the US and Europe will aid in the collection of genetic and clinical data. In parallel, analysis of the protein affected by specific gene mutations using biochemical and physiologic approaches will be an essential component of this work.

Despite progress in the genetics and diagnosis of PCD and related cilia disease, we currently lack effective or well-tested therapies. Instead, current treatments for PCD are directed at symptoms, largely extrapolated from experience with other conditions associated with bronchiectasis. Ultimately, it is our hope that the investigation of the mechanisms of cilia assembly and function, together with careful genetic and biochemical assessment, will provide better therapeutic options for individuals with PCD.

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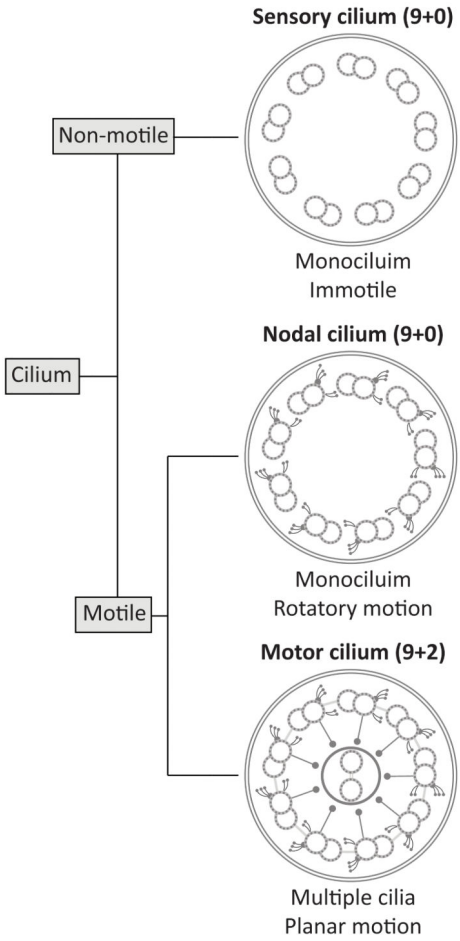


**Figure 1.**
General classification of cilia.

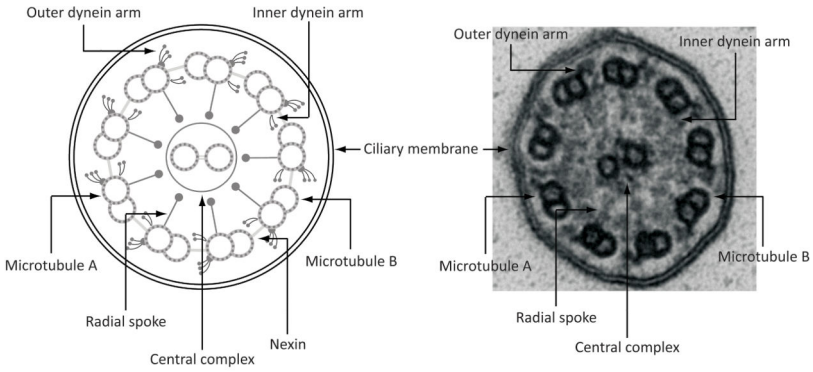


**Figure 2.**

Ultrastructural features of the cilia. Schematic diagram (left) and transmission electron photomicrograph (right) of a normal motor cilium in cross-section, which shows the structural elements of the ciliary axoneme.

## Table 1

Genes mutated in primary ciliary dyskinesia.

| Gene | Axonemal component | Ciliary ultrastructural defects | OMIM | Reference |
|---|---|---|---|---|
| *DNAH5* | ODA-HC | ODA defects | 603335 | (58) |
| *DNAH11* | ODA-HC | Normal | 603339 | (34, 66) |
| *DNAI1* | ODA IC | ODA defects | 604366 | (59, 65) |
| *DNAI2* | ODA IC | ODA defects | 605483 | (60) |
| *DNAL1* | ODA-LC | ODA defects | 610062 | (62) |
| *TXNDC3* | ODA LC/IC | Partial ODA defects | 607421 | (61) |
| *RSPH4A* | RSH | CP defects | 612647 | (63) |
| *RSPH9* | RSH | CP defects or normal | 612648 | (63) |
| *CCDC39* | DRC | Microtubule disorganization | 613798 | (64, 67) |
| *CCDC40* | DRC | Microtubule disorganization | 613799 | (64, 68) |
| *CCDC164* | DRC | DRC links defects | TBD | (73) |
| *CCDC103* | ODA docking | ODA defects | 614677 | (69) |
| *CCDC114* | ODA docking | ODA defects | 615038 | (56, 57) |
| *HYDIN* | Central pair | CP defects | 610812 | (70) |
| *DNAAF1* (LRRC50) | Cytoplasmic | ODA+IDA defects | 613190 | (47) |
| *DNAAF2* | Cytoplasmic | ODA+IDA defects | 612517 | (48) |
| *DNAAF3* | Cytoplasmic | ODA+IDA defects | 614566 | (49) |
| *HEATR2* | Cytoplasmic | ODA+IDA defects | 614864 | (55) |
| *LRRC6* | Cytoplasmic | ODA+IDA defects | 614930 | (71, 72) |

ODA: outer-dynein arm; IDA: inner-dynein arm; RSH: radial spoke; CP: central pair; DRC: dynein regulatory complex; HC: heavy chain; IC: Intermediate chain; LC: light chain; TBD: to be determined.